Reproductive decisions of the hermaphroditic tapeworm *Schistocephalus solidus*

Dissertation

zur Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Christian-Albrechts-Universität

zu Kiel

vorgelegt von

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Kiel 2002

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Korreferent:

Tag der mündlichen Prüfung:

Zum Druck genehmigt:

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SUMMARY

Most helminths are large, endoparasitic worms. They have a global distribution and infect both vertebrate and invertebrate hosts. The helminth studied in this thesis is the simultaneous hermaphroditic tapeworm *Schistocephalus solidus*. As most tapeworms, *S. solidus* parasitizes several hosts during its life from larva to adult worm. This worm reproduces in the gut of fish eating birds e.g. kingfisher or heron. The eggs fall into the water with the birds faeces where they hatch and eventually will be eaten by a copepod. When sticklebacks feed on infected copepods, the worm is transmitted to the fish host. Birds, the final hosts, feed on infected fish. The worm reproduces in the gut of the bird by self- or cross-fertilization and eggs will eventually reach water, which closes the life cycle of *S. solidus*

In this thesis I studied the reproductive biology of *S. solidus* in the context of mating system evolution, genetic variability and gamete exchange. I used microsatellite markers to determine if progeny had been reproduced by self- or cross-fertilization. Therefore, I developed a protocol that allowed DNA extraction and amplification from small amounts of tissue such as a single freshly hatched worm larva.

The key questions and their answers were the following:

(I) Does worm size influence the proportion of selfed and outcrossed progeny of worm pairs? - Worms produced more outbred offspring not only with increasing total weight of the pair, but also with decreasing weight difference between the two paired worms. This suggests that this parasite species reproduces by mixed-mating (i.e. reproduction through both, selfing and outcrossing) and reproductive conflict may prevent worm pairs from achieving an optimal proportion of outcrossing to selfing.

(II) Does the potential fitness gain from self-fertilized progeny influence the outcrossing probability of worms? - Worms whose selfed offspring had a high hatching rate produced fewer outcrossed offspring than worms whose selfed offspring had a lower hatching rate. The rate of outcrossing was furthermore influenced by the individual heterozygosity of the offered partner. Worms produced a higher proportion of outcrossed offspring with more heterozygous partners. This suggests that worms can adaptively adjust their outcrossing rate depending both on their fitness as selfers and on the genetic quality of the offered partner.

(III) How do parasites and their stickleback hosts differ in genetic variability? - Parasites showed a significant heterozygote deficiency, reflecting the reproductive mode, i.e. mixed-mating of this species. In uninfected fish individual heterozygosity was higher in fish with better condition. Heterozygote advantage did however, not reduce the susceptibility to infection with *S. solidus*.

(IV) Does *S. solidus* exchange sperm already as larvae in the second intermediate host? - Offspring from singly breeding worms that originated from a doubly infected stickleback were all self-fertilized. It is therefore very unlikely that *S. solidus* engaged in gamete exchange in its fish host.

(V) Are there ideal animal model systems for the study of mixed-mating system evolution? - In an essay I discussed and consider several potentially suitable endoparasite species to test predictions from mixed-mating theory under controlled conditions in the lab. Theory on mixed-mating system is not unified and often contradictory, therefore, empirical evidence from new model systems is a necessity for a deeper understanding of the evolution of mixed-mating systems.

In conclusion, *S. solidus* reproduces through a mixture of selfing and outcrossing (i.e. mixed-mating) when mating in pairs. The usually low densities in their final host force worms sometimes to self-fertilize as last resort. Selfed offspring of worm lineages which have always selfed a certain proportion of their eggs (despite the availability of a partner), may produce fitter progeny than do offspring from worms that have always outcrossed when both have to self in a year of low worm density. The investment in selfing and outcrossing was influenced by mating partner size as well as by partner heterozygosity. Moreover, worms adaptively adjusted their outcrossing rate depending on whether their selfed progeny had a high or a low fitness in terms of hatching rate. This gives them a selective advantage compared to individuals that would outcross and self at random. Genetic variability in a natural population of *S. solidus* shows a heterozygote deficiency which is in line with the mode of reproduction found in the lab namely mixed-mating. The *S. solidus* system is to date one of the best established model systems to study mixed-mating in animals in the lab as well as in the field. Future experiments exploring this species are expected to discover further fascinating aspects that led to the evolution of a mixed-mating system.

ZUSAMMENFASSUNG

Eingeweidewürmer wie Nematoden, Kratzer oder Bandwürmer sind weltweit verbreitet. Die meisten von ihnen parasitieren während ihres Lebenszyklus mehrere Wirte, darunter sowohl Wirbeltiere als auch Wirbellose. In dieser Arbeit habe ich den Bandwurm *Schistocephalus solidus* untersucht. Der Lebenszyklus dieses Wurmes umfasst zwei Zwischenwirte und einen Endwirt. Der Wurm pflanzt sich im Darm eines Wasservogels durch Selbst- oder Fremdbefruchtung fort. Die Eier fallen mit dem Vogelkot ins Wasser, wo nach einer Weile die Larven schlüpfen. Diese Larven werden von Copepoden, kleinen Krebstieren, gefressen und fangen im Copepoden an zu wachsen. Stichlinge infizieren sich mit dem Wurm, indem sie parasitierte Copepoden fressen. Durch Wasservögel, die infizierte Stichlinge gefressen haben, gelangt der Wurm in den Endwirt und pflanzt sich dort fort. Der Zyklus beginnt wieder von neuem.

In der Arbeit habe ich die Fortpflanzungsbiologie von *S. solidus* untersucht. Im speziellen bearbeitete ich Fragen zum Fortpflanzungssystem, zur genetischen Variabilität und zum Gametenaustausch. Mit Hilfe von Mikrosatelliten-Markern konnte ich bestimmen, ob Nachkommen durch Selbst- oder Fremdbefruchtung produziert worden waren. Um die Analysen an einzelnen, frisch geschlüpften Larven machen zu können, habe ich ein Verfahren entwickelt, das mir erlaubte, mit nur sehr kleinen DNA-Mengen zu arbeiten.

Ich habe folgende Themenkreise untersucht:

(I) Beeinflusst die Wurmgrösse den Fortpflanzungsmodus (Selbst- oder Fremdbefruchtung) von Wurmpaaren? – Die Auskreuzungsrate stieg mit dem mittleren Gewicht des Wurmpaares an, und sie sank mit zunehmendem Gewichtsunterschied zwischen Würmern eines Paars. Die Würmer haben sich also trotz des vorhandenen Fortpflanzungspartners auch selbstbefruchtet. Das deutet darauf hin, dass sich *S. solidus* durch Selbst- und Fremdbefruchtung ("mixed-mating" System) fortpflanzt.

(II) Hat der Fitnessvorteil von durch Selbstbefruchtung entstandenen Nachkommen einen Einfluss auf die Wahrscheinlichkeit, dass sich ein Wurm auskreuzt? – Würmer, deren selbstbefruchtete Nachkommen hohe Schlupfraten aufwiesen, haben weniger ausgekreuzte Nachkommen produziert als Würmer, deren selbstbefruchtete Nachkommen niedrige Schlupfraten hatten. Zusätzlich war die Auskreuzungsrate von der individuellen Heterozygotie des angebotenen Partners abhängig. Die Auskreuzungsrate war höher mit Partnern mit einer hohen individuellen Heterozygotie. Das deutet darauf hin, dass *S. solidus* Individuen ihre Auskreuzungsrate abhängig von ihrer Fitness als Selbstbefruchter und der genetischen Qualität des Fortpflanzungspartners anpassen können.

(III) Wie unterscheidet sich der Parasit vom Wirt hinsichtlich der genetischen Variabilität? – Die Parasiten wiesen ein deutliches Heterozygotendefizit auf, was die Fortpflanzung dieser Art durch Selbst- und Fremdbefruchtung widerspiegelt. Die Kondition von nicht-infizierten Fischen korrelierte positiv mit ihrer individuellen Heteorzygotie. Dieser Zusammenhang trat jedoch nicht bei Fischen auf, die mit *S. solidus* infiziert waren.

(IV) Tauschen *S. solidus* Individuen auch schon im letzten Zwischenwirt Spermien aus? – Allein brütende Würmer, die mit einem zweiten Wurm den Fisch parasitiert hatten, produzierten nur selbstbefruchtete Nachkommen. Es ist deshalb ziemlich unwahrscheinlich, dass *S. solidus* Individuen schon im Fisch Spermien austauschen.

(V) Gibt es besonders geeignete Modellorganismen für die Erforschung von Fortpflanzungssystemen mit möglicher Selbst- und Fremdbefruchtung? – Die theoretischen Grundlagen für die Evolution solcher Fortpflanzungssysteme sind uneinheitlich. Empirische Untersuchungen an neuen Modellsystemen sind notwendig. Ich diskutiere mehrere Arten von parasitischen Würmern, die sich für diese Art von Fragestellungen eignen könnten.

Aus der Arbeit ergeben sich folgende Schlussfolgerungen:

S. solidus Individuen pflanzen sich fort, indem sie sich sowohl auskreuzen als auch selbstbefruchten. Einzelne Würmer sind dann gezwungen, sich durch reine Selbstbefruchtung fortzupflanzen, wenn sich kein anderer Wurm in dem selben Endwirt befindet. Nachkommen, welche wieder durch Selbstbefruchtung enstanden sind, können, wenn sie sich selbstbefruchten müssen, fittere Nachkommen erzeugen, als Nachkommen von Wurmlinien, die nicht an die Selbstbefruchtung angepasst sind. Die Investition in selbst-befruchtete bzw. ausgezüchtete Nachkommen war vom Gewicht und von der individuellen Heterozygotie des Fortpflanzungspartners abhängig. Ausserdem haben *S. solidus* Individuen ihre Tendenz auszukreuzen davon abhängig gemacht, ob ihre selbstbefruchteten Nachkommen gut oder schlecht schlüpften. Die Würmer haben so einen Fitnessvorteil gegenüber Individuen, die zufällig auskreuzen oder selbstbefruchten. Auch Messungen an einer natürlichen *S. solidus* Population weisen ein Heterzygotendefizit auf, was ebenfalls auf Fortpflanzung durch ein Gemisch von Selbstbefruchtung und Fremdbefruchtung hindeutet. Das *S. solidus* Modellsystem ist sehr weit entwickelt und deshalb für weiterführende Untersuchungen zur Evolution von Fortpflanzungssystemen ausgesprochen geeignet.

1 - We are living in a "Wormy world"

In his classic essay on human helminth infections the parasitologist Norman Stoll was struck by the great number of infectious species and concluded that we are living in a "Wormy World" (Stoll 1947). The term helminth is used for species of the following four phyla: platyhelminthes (flatworms), nematoda (roundworms), nematomorpha (hairworms) and acanthocephala (spiny-head worms). All helminths are parasitic with the exception of the members of the turbellarians which are almost entirely free living (Smyth 1994).

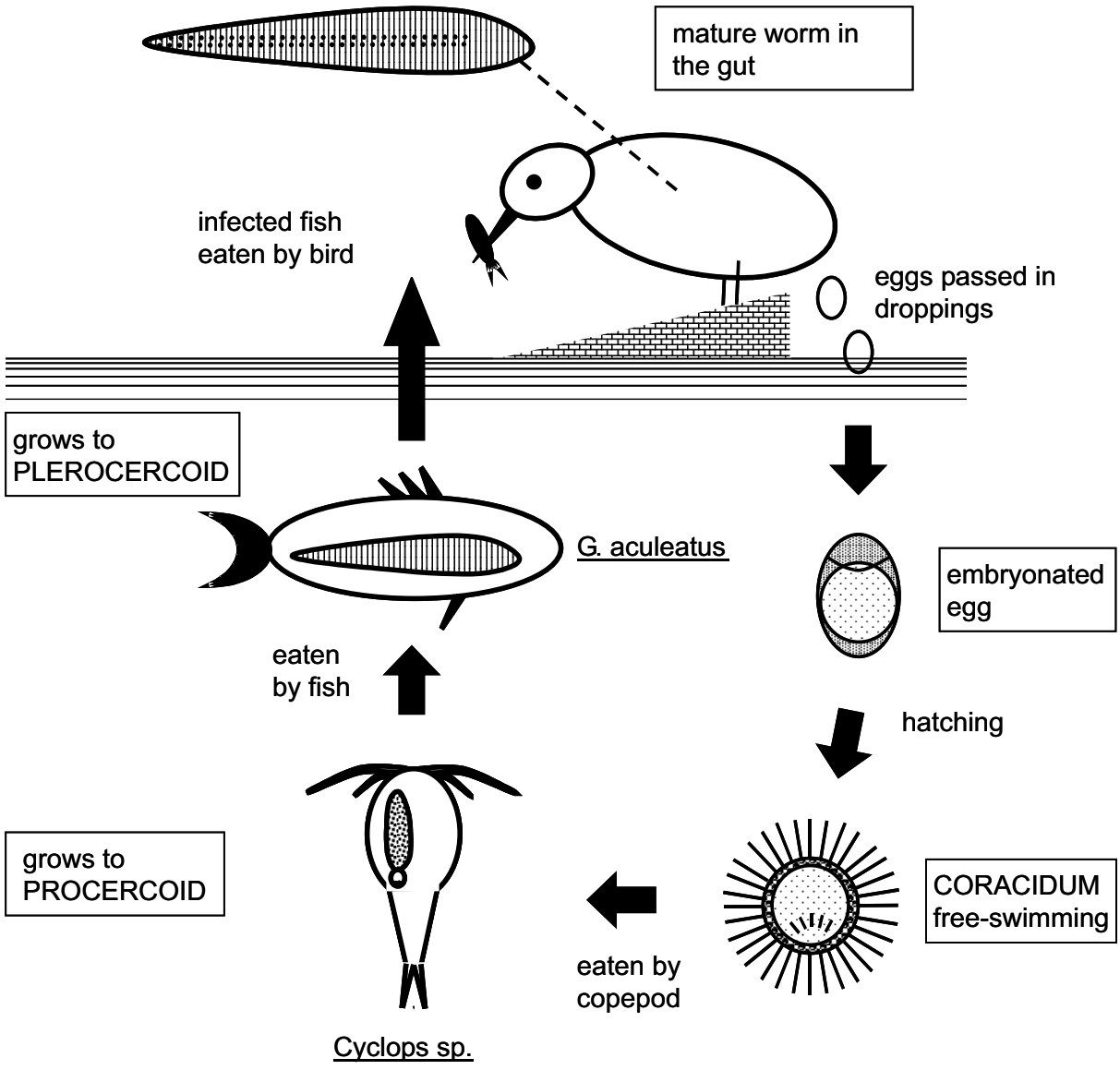
In the following paragraph I will explain the life cycle of the protagonist helminth of this thesis *Schistocephalus solidus* in more detail. Since this species is not infecting humans, it serves as a safe and experimentally accessible model system to study helminths under a wide range of evolutionary and ecological questions. This species parasitizes two intermediate hosts (a copepod and the stickleback) and one final host (a fish eating bird).

The adult worm is non specific in its choice of final host and a wide range of bird hosts have been reported (for example the kingfisher, the grey heron or the cormorant) . When infected fish are eaten by birds, the worms mature rapidly in the birds gut and start to release eggs via the birds faeces. For obvious ecological reasons, faeces from fish eating birds have a good chance to end up in water. The embryo is unsegmented when laid and is surrounded by yolk cells. In the water the embryo develops in one to two weeks depending on water temperature, into a ciliated larvae (the coracidium) with small hooks. The coracidium larva hatches and swims actively. It contains few food reserves and dies if not eaten by a copepod within a few hours after hatching.

An adult *Schistocephalus solidus* freshly dissected from a stickleback.

Infected copepod. The worm larvae is in the tail of the copepod.

Inside the stomach of the copepod, the coracidium loses its outer epitelium and the enclosed larva penetrates the gut wall with the aid of the hooks. The larva bores its way rapidly through the intestine into the body cavity of the copepod, to become a procercoid larva. A procercoid reaches an infective stage when the hooks become separated from the body into a constricted



The life - cycle of *Schistocephalus solidus.*

posterior region called the cercomer.

When a cyclops containing a procecoid in the infective stage is eaten by a stickleback, the larva penetrates the gut wall of the fish to develop in the body cavity into the final larval stage, the plerocercoid. The distribution of *S. solidus* in sticklebacks is overdispersed with most infected fish containing only one worm. An infection with this parasite is causing severe fitness reduction to the fish. The gonads develop less well in infected fish compared to uninfected ones. Moreover, fish harbouring infective plerocercoids (about 50 mg in weight) swim closer to potential predators than uninfected fish.

Stickleback in a late stage of infection.

The kingfisher (*Alcedo atthis*) one of the possible final hosts of S. solidus. (Photo by M. Delpho)

Spermatogenesis and oogenesis of the worm take place in the final host, triggered by a rise of temperature from about 15°C in the fish to 40°C in the bird. Within the short time period of reproduction which is terminated within a few days these hermaphroditic worms can copulate several times by self- and/or cross-fertilization. The final host is not harmed by the worms as worms cease to take up energy during reproduction. Low densities in the final host may sometimes force worms to self-fertilize all their eggs as last resort.

The *S. solidus* life cycle described in the previous paragraph may seem quite simple to understand, but it is complex in its details and the integrity is in fact a compilation of results from a huge number of studies on this one species (reviewed in Smyth (1994)). Research on *S. solidus* has already more than 200 years of history. By feeding infected sticklebacks to ducks, and, obtaining the adult worms on autopsy several days later, the Danish biologist Peter Abildgaard demonstrated the transmission of a parasite from one host to another (Abildgaard 1790). This was the first demonstration of a helminth life cycle.

The revelation of helminth life cycles was a major step into the management of human helminth infections. At least 340 different helminth species are infecting humans. In 1998 UNICEF estimated that of 5753 million humans around 4457 million were infected by

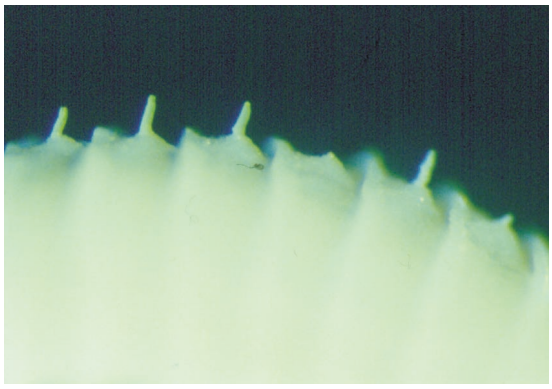
helminths (UNICEF 1998). However, only an estimated 15 % of these infected people will eventually become ill through a helminth induced disease.

New research suggests that humans passed tapeworms about 10'000 years ago onto domesticated cows and pigs, and not the other way round as was thought for the last 50 years (Hoberg *et al*. 2001). It is now believed that ancestors of modern humans caught tapeworms already over two million years ago, from the game they hunted on the plains of Africa. So, the human-tapeworm partnership is a longstanding and successful one - for the tapeworm at least.

Being the biggest among the most well known of the parasites, tapeworms have a certain mythology build around them. A famous legend has been passed on since the 1920's is "The Hollywood Tapeworm Diet", whereby starlets and supermodels are intentionally infecting themselves with large tapeworms in an effort to loose weight. Since then, a number of famous women, from opera singer Maria Callas to supermodel Claudia Schiffer, are alleged to have tried this revolting eating plan. As for the dietary benefits of tapeworms, the large parasites like the beef tapeworm (*Taenia saginata*) and *Ascaris* certainly do compete for us for nutrients. However, like any method of starvation, they tend to rob us of micronutrients (like vitamins) before they get to the stuff we don't really need. To make matters worse, one of the symptoms of having a large lump of tapeworm protein in one's gut is an immune response. This leads to a collection of fluid in the abdomen resulting in a pot belly - not exactly what dieters are looking for!

2 - Some basics about hermaphrodites

Hermaphroditism, the occurrence of male and female sexual organs in one individual, is widespread in plants and is found in two-thirds of the c. 35 animal phyla, including entire groups such as the platyhelminthes, pulmonate gastropods or ascidians (Ghiselin 1969). Mating systems in these animals display bewildering variety, including amazingly complex courtship behaviours. Animals can either be sequential hermaphrodites or simultaneous hermaphrodites. Sequential hermaphrodites exhibit their two sex functions separated in time (e.g. some serranid fish), while simultaneous hermaphrodites exhibit both sex functions at the same time (e.g. the helminth *S. solidus* used in this work). Some hermaphrodites are self-compatible, which means that they have the possibility to self-fertilize their eggs, while other are self-incompatible.



Errected cirri of the internal fertilizer *S. solidus*. Each segment possesses male and female reproductive organs. (Photo by L. Schärer)

What led to the evolution of hermaphroditism? Self-compatible hermaphrodites have a 50 % advantage compared to dioecious organisms, because they can not only reproduce by fertilizing other eggs, but also by self-fertilizing their own eggs (Fisher 1941). Moreover, they are not dependent on a mating partner for reproduction. Therefore hermaphrodites have an advantage when living in low densities. If hermaphrodites search for a mating partner each conspecific is a possible mating partner, and not only every second as in dioecious species. In such species the cost of finding a mating partner is only half compared to the costs in dioecious species.

However, the costs of maintaining two sex functions within an individual are probably high and hermaphroditism only evolves if keeping two sexes provides considerable advantages such as the ones mentioned above. Moreover, the advantage of reproduction through self-fertilization is lowered by inbreeding depression (Charlesworth and Charlesworth 1987). Inbred offspring usually have lower fitness than outcrossed offspring, because deleterious recessive alleles become homozygous and are thus expressed in inbred offspring. Amazingly, hermaphroditic animals are often reproducing by a mixture of selfing and outcrossing (i.e. mixed-mating) (Thornhill 1993). Animals have therefore much to offer to an understanding of the evolution of hermaphroditism and the consequences for the mating system. Exploring the wide variety of breeding systems of hermaphrodites is in my opinion

one of the most fascinating topics in evolutionary biology to date.

The word hermaphrodite has its origin in greek mythology. Hermaphroditus was the son of Hermes and Aphrodite and was a creature with both male and female reproductive organs. Interestingly, mythology says that Hermaphroditus was actually a protandric hermaphrodite. He was born as a boy and stayed male until he met the nymph Salmacis (female), who fell immediately in deep love with the young and beautiful man. However, he was not interested at all and rejected her. Shortly after Hermaphroditus took a bath in a nymph water pond. Under water Salmacis grabbed Hermaphroditus and prayed so ardent to the gods not to let them be separated ever again that the gods had pity with her and unified the two bodies in one. Therefore, the name Hermaphroditus does in principle not point at the two sexes of the creature, but primarily at the fact that his parents were Hermes and Aphrodite.

Worms stored in alcohol for conservation after the experiment. (Photo by A. Hämmerli)

3 - Distinguishing selfed and outcrossed offspring using microsatellite markers

As a consequence of simultaneous hermaphroditism two mating *S. solidus* individuals can produce three different kinds of offspring: self-fertilized offspring by either one of the worms, or offspring resulting from cross-fertilization between the two worms. For experimenters collecting eggs of a worm pair the problem was that there was no way of measuring the proportion of eggs belonging to each of these three ways of reproduction. An elegant method to solve this problem is to use genetic markers with sufficient resolution to distinguish individuals. Microsatellite markers are perfectly suited for such purposes because they usually show high levels of polymorphism. Only recently, the first five microsatellite markers for *S. solidus* have been characterized (Binz *et al*. 2000). These are also the first microsatellite markers for a cestode species. This finding provided a powerful tool for experiments with *S. solidus*. It now became possible to distinguish offspring resulting from self-fertilization from offspring resulting from cross-fertilization using genetic markers. In this work I characterized three additional primers for *S. solidus*, which allowed me to look also at population genetic parameters with high enough resolution.

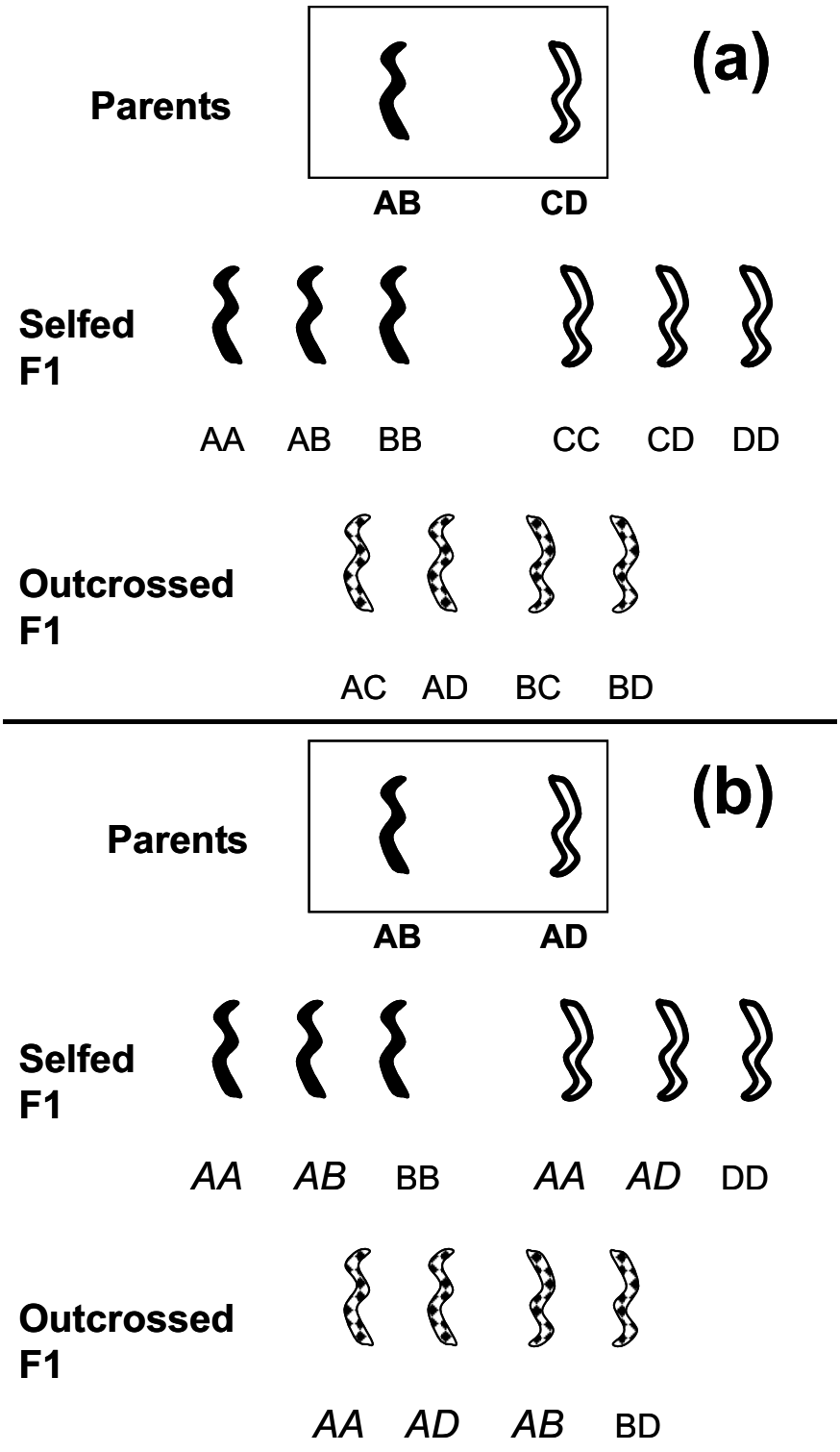
Microsatellites are tandemly repeated DNA sequences whose unit of repetition is between one and five base pairs long (Jarne and Lagoda 1996). They are found in a wide variety of eukaryotes and also in the chloroplastic region of plants. Microsatellites have been shown to have mutation rates of up to 10^{-2} events per locus per replication, which is much higher than the average 10^{-9} commonly found for point mutations on other DNA stretches. This largely explains their high variability. Microsatellites are highly polymorphic in natural populations, with average expected heterozygosity well above 50 % in general, peaking virtually at 100 %. Microsatellites are also codominant and inherited in a mendelian fashion, hence heterozygote individuals can be distinguished from homozygotes ones. Alleles at a given locus are identified following specific PCR (polymerase chain reaction) amplification by their relative electrophoretic migration, by comparison with a ladder of known size. In order to do so one needs primers that bind to the conserved flanking regions of a microsatellite. Primers are short DNA sequences which have the property to bind to other specified DNA sequences. Because DNA is double stranded there need to be a forward and a reverse primer, one of which is usually stained with a fluorescent colour molecule that can later be identified.

How can these primers be used to estimate selfing and outcrossing rates of worms? The first step was to develop an extraction method for eggs and coracidia (worm larvae). The primers had only been tested on extracted tissue of adult worms. However, in eggs and

coracidia (diameter of c. 50 μm) the amount of DNA was much smaller. There was no extraction method giving acceptable result for eggs. For coracidia, the simplest and least costly method - the Chelex © extraction (Walsh *et al*. 1991) - worked well, when PCR conditions were adapted to low DNA amounts. Therefore, I decided to estimate the selfing and outcrossing rates of worms based on hatched eggs i.e. coracidia.

When eggs were hatching coracidia were singly caught with a pipette and each transferred to a small tube containing the Chelex © solution. To amplify the microsatellite profile of each sample, DNA extract together with a cocktail of several substances including the microsatellite primers is processed in a polymerase chain reaction (PCR). The PCR reaction is the temperature cycling through the following biochemical steps: The double stranded DNA is split in single strands - cooling down - the microsatellite primers bind to the single stranded DNA - heating up - a new double stranded DNA is built (using polymerase and nucleotides) - heating up - the double stranded DNA is split again in single strands, and the cycle starts again. This amplification protocol results in an exponential accumulation of the microsatellite gene sequence. Different sized sequences have different running speeds through an electronic field. This property is used to detect the different sized alleles in each sample. In this work, PCR products were run on an optical automated sequencer machine. The fragments pass a laser, with smaller fragments passing earlier than larger ones. The laser detects the fluorescent dye of the primers. These signals are then transformed into a virtual image on the computer screen. Finally the bands on the gel image can be translated for each sample into alleles of different length. An internal size standard facilitates the determination of the exact size of each allele.

The alleles are then scored for their size individually for each sample. Samples processed through all these steps have received their microsatellite profile with two either identical (homozygote) or different (heterozygote) alleles for each analysed primer (up to eight primers in this work). To find out whether offspring resulted from self-fertilization or from cross-fertilization, we could now compare the microsatellite profiles of the offspring with that of the possible parents. First we had to find a microsatellite locus where the parents did not share any allele of the same length. If the parents shared one allele, seven out of ten possible genetic combinations in the offspring could not be assigned unequivocally to selfing or outcrossing. Only if parents didn't share any allele for a specific locus all offspring could be identified and assigned to either mode of reproduction. Apart from parentage analysis microsatellites can also be used to estimate the level of inbreeding in a population and the level of genetic differentiation between populations (as was done in Chapter III).



(a) Parents do not share an allele at that locus. All offspring can be assigned to outcrossing or selfing.

(b) Parents share allele A. Seven possible genetic combinations (letters in italics) cannot unequivocally be assigned to either selfing or outcrossing.

4 - Thesis outline

This thesis is organized in five chapters. The technical details of each study can be found in the specific chapters. Each chapter is organized in summary, introduction, methods, results and discussion, except chapter five which is written in the form of an essay. This outline is a very general introduction to the concepts of the research questions, explaining for each study why it was performed, what the question was and how it was tested.

The different aspects investigated were:

(I) Effect of worm size within a pair on selfing and outcrossing rates

(II) Effect of potential fitness of selfed offspring on outcrossing probability of worms

(III) Genetic variability of the parasite compared to the fish host

(IV) Test of premature sperm transfer of worms in the intermediate host

(V) Endoparasites as suitable model systems for studying mating-system evolution

(I) In the first chapter I discuss an experiment where I paired worms of different sizes and let them reproduce in an *in vitro* system. The motivation was to test whether female quality of worms (i.e. bigger worms are of higher female quality because they produce more eggs) had an influence on outcrossing rates of worm pairs. When worms reproduce in pairs, they have the opportunity to engage in both self- and cross-fertilization. The costs and benefits of self- vs. cross-fertilization are important factors determining the potential benefits that can be achieved by either mode of reproduction. Moreover, simultaneous hermaphrodites are expected to be in a conflict over the preferred sexual role (The Hermaphrodite's Dilemma), which may also influence a cooperative solution between two mating partners. In an *in vitro* experiment consisting of worm pairs varying in mean weight and weight difference within worms of a pair, I measured the total reproductive output and the proportion of selfed offspring of each worm using microsatellite markers.

(II) In the second chapter the aim was to investigate the effect of a worms' potential fitness through self-fertilization on its outcrossing probability. The motivation for this study was the observed worms' reproduction through selfing and outcrossing even in the presence of a mating partner in the experiment described in chapter one. The reason why I expect that fitness gained through self-fertilization can influence the outcrossing rate of a *S. solitus* individual is briefly explained in the following paragraph.

Low densities in their final host may force worms sometimes to self-fertilize as last resort. Selfed offspring of worm lineages which have always selfed a certain proportion of

their eggs (despite the availability of a partner), may produce fitter progeny than do offspring from worms that have always outcrossed when both have to self in a year of low worm density. Moreover, an individual which can adaptively adjust its outcrossing rate depending on the fitness gain it would achieve through selfing would have a selective advantage compared to individuals that outcross and self at random. In an experiment using an *in vitro* system I tested for adaptive outcrossing in worms where I first had measured the hatching success of selfed offspring.

(III) In the third chapter I looked at the genetic variability of a natural *S. solidus* population and their intermediate hosts, the sticklebacks. The starting point was that few studies have investigated the genetic structure of both hosts and parasites at the population and the individual level. Resistance of hosts towards parasites has been shown to be a function of individual inbreeding (Coltman *et al*. 1999). Similarly the virulence of parasites might be related to individual inbreeding. As a proxy for individual inbreeding, individual heterozygosity of hosts and parasites was measured. To increase the solution of this measure I developed and characterized three new microsatellites for *S. solidus*, which are also described in this chapter.

(IV) In the fourth chapter I tested the hypothesis that *S. solidus* is capable of premature gamete exchange as a plerocercoid in the last intermediate stickleback host. The existence of such a reproductive mode is suggested by the highly advanced gonadal development in the plerocercoid and the large fitness gain of outcrossing. To test this, offspring from singly breeding worms that originated from doubly infected sticklebacks were tested for paternity. If the hypothesis is correct some of these offspring should be outcrossed and share alleles with both the breeding worm and the second cestode in the coinfection.

(V) The fifth chapter contains an essay on experimental testing of hypothesis generated from the theory on mixed-mating systems (i.e. reproduction through both selfing and outcrossing). This has been and still is a vivid field of research mainly in plant biology. In this essay I propose the species rich group of endoparasitic helminths as ideal model systems for the investigation of mixed-mating systems in animals. Theory on mixed-mating systems is not unified and often contradictory, therefore, empirical evidence from new model systems is a necessity for a deeper understanding of the evolution of mixed-mating systems.

Experimental set-up. Worms are enclosed between two nylon layers. Eggs (dark shadows) fall on the bottom of the bottles.

Simultaneous hermaphrodites reproducing in pairs self-fertilize some of their eggs: an experimental test of predictions of mixed-mating and Hermaphrodite's Dilemma theory.

SUMMARY

Theory predicts (1) that mixed-mating systems (i.e. reproduction through both selfing and outcrossing) should usually not evolve and (2) that reproducing simultaneous hermaphrodites should be in a conflict over the preferred sexual role (The Hermaphrodite's Dilemma). In an *in vitro* system with the endoparasitic cestode *Schistocephalus solidus*, a simultaneous hermaphrodite, we tested predictions of both the mixed-mating and the Hermaphrodite's Dilemma theory. Using microsatellite markers, we measured the proportion of selfed offspring and the total reproductive output of each worm within pairs varying in mean weight and weight difference. Worms produced more outbred offspring not only with increasing total weight of the pair, but also with decreasing weight difference between the two paired worms. These results suggest: (1) that this parasite species reproduces by mixed-mating, which may be maintained by stochastic density fluctuations in the definitive host and hence unpredictability of self reproduction, (2) reproductive conflict may prevent worm pairs from achieving an optimal intermediate selfing rate.

INTRODUCTION

A large group of simultaneous hermaphrodites are the endoparasitic platyhelminthes (i.e. cestodes, trematodes, monogeneans), many of which are able to reproduce by both self- and cross-fertilization (Williams and McVicar 1968; Nollen 1983). Due to stochastic environmental fluctuations, parasitic helminths are at risk to end up without a mating partner in the final host (Smyth 1976) and selfing may thus be maintained in these species mainly because it offers reproductive assurance (Baker 1959; Lloyd 1980). Even though selfing may provide advantages such as assurance of reproduction, preservation of highly fit genotypes, reduced energy allocation to sperm production and less costly mating behaviour (Jarne and Charlesworth 1993; Thornhill 1993), detrimental inbreeding effects may act as a major selective force against self-fertilization when cross-fertilization is possible (Lande and Schemske 1985; Charlesworth and Charlesworth 1987). Moreover, low heterozygosity may reduce a parasite's competitive ability in the coevolutionary arms race with its host (Gemmill *et al*. 1997; Wedekind 1999). Nevertheless, worms from some strains of echinostomes (Trematoda) from different geographical regions both self- and cross-fertilize when mating partners are available (Nollen 1975; Trouvé *et al*. 1996; Nollen 1997). This suggests that a mixed-mating system, i.e. reproduction through both selfing and outcrossing, may be evolutionarily stable in these species.

Mixed-mating systems are a challenge for evolutionary biology. Fisher showed in 1941 (Fisher 1941) that a genotype reproducing by selfing has an advantage of 50% when it is introduced at low frequency into an outcrossing population. However, the low fitness of inbred progeny selects directly against self-fertilization. Since then, the evolution of mixed-mating systems has been modelled several times under different assumptions, yet there is no consensus about the predicted evolutionarily stable strategies. Most models on mating system evolution predict that a mixture of selfed and outcrossed progeny should not exist. Lande (1985) and Charlesworth *et al.* (1990) showed in quantitative genetic models that the balance between the cost of outcrossing and inbreeding depression should result in either complete selfing or complete outcrossing. With this quantitative genetic approach it was possible to include the important influence of the "selfer gene" on the rest of the genome. On the other hand, evolutionarily stable mixed-mating systems are predicted only for the following special cases: (1) when both self-fertilization and biparental inbreeding occur simultaneously (Uyenoyama 1986), and (2) when selfed progeny are less well dispersed than outbred progeny (Holsinger 1986). More recently Cheptou and Dieckmann (2002) could show that deterministic or stochastic environmental effects can also lead to evolutionarily stable mixed-mating systems. Their models

assume finite, explicitly fluctuating populations, which is in contrast to the traditional approaches. Endoparasitic simultaneous hermaphrodites may be suitable to detect mixed-mating systems, that are predicted by this model, because stochastic density fluctuations in the definitive host are influencing the probability for outcrossing. So far it has proved very hard to test any of these hypotheses (Charlesworth and Charlesworth 1998; Cheptou and Dieckmann 2002) and hence the evolution of selfing rates in natural populations is still far from being understood.

Our study animal, the simultaneous hermaphroditic cestode *Schistocephalus solidus*, has a life cycle that includes two intermediate hosts (a cyclopoid copepod and the three-spined stickleback (*Gasterosteus aculeatus*)) and one final host (any fish eating bird). This worm can reproduce by both self- and cross-fertilization. Previous studies uncovered several disadvantages of selfing compared to outcrossing in this species. *S. solidus* larvae that result from self-fertilization are hatching at a low rate (about 5 to 10% of the eggs hatch); this rate is 80% lower than that of eggs from worm pairs (about 50% of the eggs hatch) (Wedekind *et al*. 1998; Christen *et al*. 2002). Moreover, genetic homogeneity among *S. solidus* offspring decreases the probability of infection of the first intermediate host (Wedekind and Rüetschi 2000). In competition with outcrossed offspring selfed *S. solidus* infect the first intermediate host with a lower probability, and, when successful, have reduced growth, i.e. a reduced virulence, compared to outcrossed competitors (Christen *et al*. 2002). All disadvantages combined, inbreeding depression seems quite strong in this species and we therefore expect worms to always outcross when given the opportunity.

However, due to strong fluctuations in prevalence between years in this species, (e.g. Pennycuick 1971b) worms may have to self when they happen to be alone in the definitive host. Inbreeding depression is expected to be strongest for the first selfed generation and is thereafter expected to decrease during consecutive selfing events, due to purging of deleterious mutations with large homozygous effects on fitness (Charlesworth and Charlesworth 1998). Selfed offspring of worm lineages which have always selved a certain proportion of their eggs (despite the availability of a partner), may produce fitter offspring than do offspring from worms that have always outcrossed when both have to self in a low prevalence year. Depending on the frequency of such inescapable selving events, we may expect *S. solidus* individuals to reproduce by both selfing and outcrossing when mating partners are available. We could even speculate that the amount of eggs that individuals self depends on how inbred, i.e. how purged they are themselves. This latter mechanism may be a bit far fetched, because it assumes a specific kind of genomic imprinting in order to allow individuals to measure their inbreeding history. Even if this mechanism does not exist, we expect a certain proportion of

selfing, when the worms reproduce in pairs, selected by the pending risk of low prevalence in the next season because of stochastic elements in life cycle of this species so that most worms would then be forced to self.

If *S. solidus* is a simultanous hermaphrodite because it has to self when its prevalence is low, a further complex problem is inevitable: it is the conflict that arises when two simultaneous hermaphrodites meet for reproduction. Such mating encounters will conform to a conditional, non-zero sum game, which has been called the Hermaphrodite's Dilemma (Leonard 1990). It describes the conflict between mating partners over which individual of a pair will play the preferred role, i.e. sperm donor or sperm acceptor, in any mating encounter (Charnov 1979; Leonard 1990; Dugatkin and Reeve 1998; Michiels 1998). The model predicts that the cooperative solution to the dilemma will be a mating system based on reciprocity with potential cheating in the preferred sexual role. Reciprocity can be achieved through either egg or sperm trading depending on the preferred sexual role and has been found in several taxa (egg trading: Fischer 1984; Petersen 1995; Sella *et al*. 1997; Sella and Lorenzi 2000; sperm trading: Leonard and Lukowiak 1984; Vreys and Michiels 1998; Michiels and Bakovski 2000. Cheating yields a higher fitness pay-off than reciprocating, because defecting ("cheating") worms would not only fertilize their mating partners eggs, but also self-fertilize their own eggs. This prevents the cheated worm from acting as a sperm donor, and thus deprives it from the fitness gain through male function.

Cooperation between two mating partners may be especially difficult to achieve if they differ in quality (i.e. if asymmetric reciprocation is required). However, asymmetries in partner quality may be the rule under natural conditions. Two worms that meet for reproduction are unlikely to have the same size. Bigger worms of *S. solidus* are expected to be of higher female quality than small worms, because female fecundity is strongly positively correlated with body size (Wedekind *et al*. 1998; Schärer and Wedekind 1999). Moreover sex allocation is size-dependent: larger worms are more biased towards female allocation (Schärer *et al*. 2001). The higher female quality and attractiveness of big worms compared to that of small ones was confirmed in a mate choice experiment, where small worms preferred high fecundity, potential sperm acceptors (big worms) (Lüscher and Wedekind 2002). We therefore expect mating partners that differ in size to solve this conflict non-cooperatively more often than partners of similar size

In the present study we investigate experimentally whether and how individuals of *S. solidus* adjust their reproductive mode and output (selfing rate, egg number, egg size, hatching rate) when reproducing in pairs that vary in mean weight and weight difference within pairs. We manipulated mean weight of pairs to investigate if these pairs differing in

female fecundity are investing partly in selfing, despite the offered mating partner and the considerable inbreeding depression that is known for this species. We further manipulated weight difference of worms in pairs to create situations with major and minor potential reproductive conflict. Here, for the first time a potentially mixed-mating system as well as a Hermaphrodite's Dilemma is studied in detail in a hermaphroditic endoparasite. This became possible because of the recent rapid development of efficient molecular techniques, which allow an unambiguous assignment of individual offspring (selfed and outcrossed) to either parent worm. To perform this experiment under controlled conditions we replaced the bird's gut environment by an *in vitro* system which has been established by Smyth (1954) and was modified by Wedekind (1997) and Schärer and Wedekind (1999).

MATERIAL AND METHODS

Study organism

The life cycle of *S. solidus* includes two intermediate hosts. Its definitive host, i.e. any fish eating bird, is infected by consuming infected sticklebacks (*Gasterosteus aculeatus*), which themselves have been parasitised by consuming infected copepods. Spermatogenesis and oogenesis take place only in the definitive host, triggered by a rise of temperature from about 15°C in the fish to 40°C in the bird, ruling out the possibility of sperm transfer in the fish (Smyth 1946). *S. solidus*' entire reproduction, i.e. sperm and egg production, mating, and egg release, occurs in the bird's gut shortly after infection (Clarke 1954; Schärer and Wedekind 1999). Within the short time period of reproduction which is terminated within a few days worms can copulate several times (self- and/or cross-fertilization) (McCaig and Hopkins 1963; Tierney and Crompton 1992). It has only recently been shown that *S. solidus* reacts to situations with high sperm competition by transferring more sperm (Schärer and Wedekind 2001). After several days in the gut the worms die. The eggs are released into the water with the bird's faeces. After some weeks the free-living *S. solidus* larva (coracidium) hatches. The coracidium needs to be ingested by a cyclopoid copepod which, after the worm larva has become infective to the next intermediate host, is a preferred prey of sticklebacks (Wedekind and Milinski 1996). The reported prevalence of *S. solidus* in sticklebacks can be high, sometimes up to 100% (Dick 1816; Smyth 1947; Hopkins and Smyth 1951). The distribution of *S. solidus* in sticklebacks is overdispersed with most infected fish containing

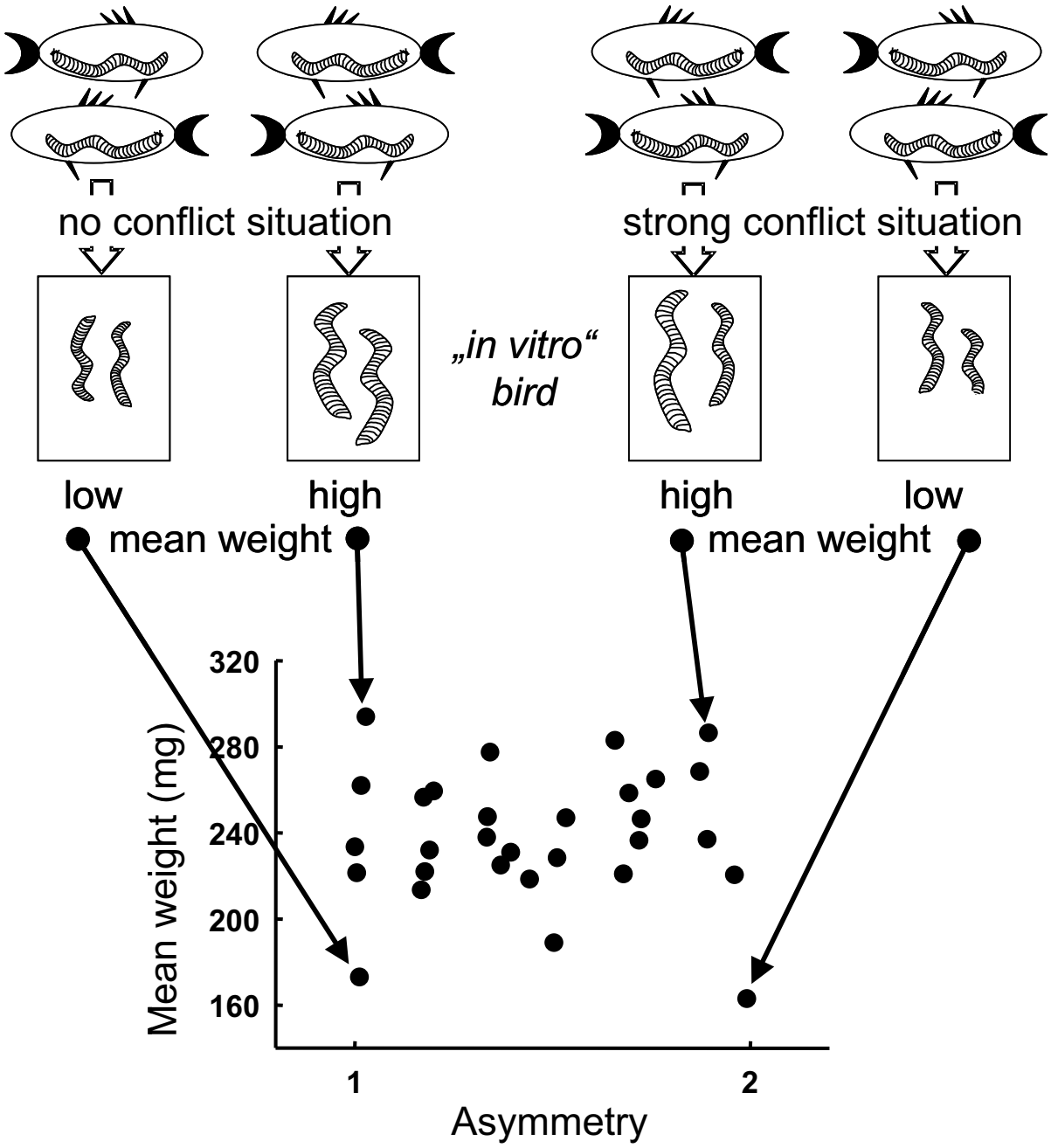


Fig. I. 1: Experimental design. Worms were obtained from singly infected fish. To create situations of minor or major reproductive conflict (Asymmetry variable), worms of different sizes were paired. The data points in this figure show the actual distribution of mean weights and weight difference of experimental pairs used in this study. See text for further details.

only one worm (Smyth 1994; A. Lüscher unpublished data). In birds, up to around 300 adult worms per individual host have been observed (Nybelin 1919; Vik 1954), but these numbers probably represent extreme cases.

Experimental procedure

Infected sticklebacks were caught during September and October 2000 in a brackish water bay near Neustadt (Schleswig-Holstein), Germany. Shoaling sticklebacks were dip-netted from the shore within a range of about 1 km. The fish were kept in 194 l tanks for six weeks before dissection. To obtain the worms, fish were killed by a cerebrospinal cut and the worms were removed aseptically from the stickleback. Only worms from singly infected fish were used for the experiment. The worms were weighed on a balance to the nearest mg and kept in culture medium at room temperature for a maximum of twelve hours before the start of the experiment. The culture medium consisted of sterilized Minimum Essential Medium with Earle's salts, L-glutamine, 25 mM HEPES buffer (Sigma) and additives (per litre of medium: 1 µl Antibiotic/Antimycotic solution (Sigma), 6.5 g D-Glucose), and had been titrated with NaOH to a pH of 7.5.

In order to create situations with minor or major reproductive conflict, worms of different sizes were paired (Fig. I. 1). This asymmetry in weight within pairs was calculated as the weight of the heavier worm divided by the weight of the lighter worm. Asymmetry in weight yielded a variable that is distributed uniformly between 1 and 2 (between no reproductive conflict and major reproductive conflict respectively) (see Graph in Fig. I. 1). We also controlled for mean weight of worm pairs, which ranged from 170 mg to 300 mg. In total we formed 29 worm pairs. To be able to analyse asymmetry and mean weight independently of each other, it was essential that there was no correlation between these two variables ($r = 0.018$, $F_{1,27} = 0.009$, $p = 0.93$) (Fig. I. 1).

Each worm pair was then transferred to a nylon mesh bag (25 x 75 mm). A mesh size of 200 µm was chosen so that eggs could easily pass the net whereas worms could not. The opening of the bag was closed by melting the ends of the nylon layer with a lighter. Each bag was attached to the lid of a culture bottle and then hung into 250 ml of culture medium. After closing the lids the bottles were placed in a 40°C water bath (SW 23, Julabo Labortechnik, Seelbach, Germany) and shaken continuously at 70 rpm.

Egg samples were taken five times during the experiment. The first sampling was done 38 hours after placing the worm bottles into the water bath and from then on every 24 hours. In total the worms were allowed to reproduce for 134 hours, which is the time the worms need to complete 95%

of their egg production (Schärer and Wedekind 1999). At each sampling time, eggs were collected from the bottom of the old bottles, and the lids with the worms were placed on new bottles with fresh, preheated medium. After the eggs had settled in the bottle about 95 % of the Medium was replaced by tap water. This procedure was repeated four times before transferring the eggs to a 15 ml tube and storing them at 4°C.

Number and size distribution of eggs were determined using a CASY, Cell Counter (Schärfe), which gives a particle volume distribution with 1024 size channels and the number of particles counted. We used a 150 μm diameter orifice tube with the sampling range set between 35 μm and 70 μm (egg diameter is expected to range from 45 to 55 μm (Wedekind *et al*. 1998)). About 50'000 eggs of each worm pair (10'000 per daily sample) were counted and measured.

After collection eggs were kept at darkness and 4 °C. Four weeks later about 90'000 eggs of each worm pair were transferred to Petri dishes for hatching. It was necessary to standardize the number of eggs of pairs to be able to exclude density in Petri dishes as a confounding variable acting on hatching success. After two weeks at 20 °C and darkness the eggs were moved to 15L/9D at 20 °C which induced hatching. Hatching rates of the clutches were determined 8 weeks after exposure of the eggs to light. Only empty eggs with open or removed operculum were considered as hatched (Swiderski 1994).

DNA extraction followed the Chelex © extraction method (Walsh *et al*. 1991). 1 mm^3 of worm tissue was mixed in a 50 μl 5 % Chelex © suspension and stored at –20 °C. 1μl of Proteinase K solution (Roche) was added to the sample in Chelex © suspension and incubated at 55 °C for 15 min and at 100°C for 15 min. Then the samples were placed on ice, spun down for 30 seconds at 13000 rpm, and stored at –20°C. PCR reactions were set up in 10 μl volumes, each containing 1μl of extract, 1x Promega reaction buffer, 1.5 mM MgCl2, 250 mM of each dNTP, 0.5 units Promega Taq Polymerase, 1 μl of 1 % Bovine serum albumin and 0.5 mM of one of four pairs of locus-specific fluorescent-labelled forward primer and non labelled reverse primer. Details on microsatellite characterization are given in (Binz *et al*. 2000). PCR cycling parameters were the following: 1 min at 94 °C, 27 cycles of 94 °C for 1 min, 1 min at 57 °C and 1 min at 72 °C, followed by a final step of 72 °C for 7 min. Fluorescent PCR fragments were separated and visualized on a ABI PRISM, 3100 Genetic Analyzer using the POP4™ Polymer (Applera Corporation). Nearly identical methods were used to amplify microsatellite fragments from coracidia (larvae). From each worm pair 90 coracidia were analysed. Each coracidium was singly mixed in 30 μl 5 % Chelex © suspension and the incubation step at 55 °C lasted 30 min. For amplification of coracidia the cycles in the PCR reaction were increased to 40.

To be able to determine for each larva if it resulted outcrossing, sefing by the bigger worm or selfing by the smaller worm of the pair, individuals in a pair were required not to share identical alleles on at least one of the four microsatellite loci. For 17 worm pairs we found such a locus that met this

requirement. The remaining pairs either shared alleles at all four loci (nine pairs) or microsatellite amplification with the used primers failed (three cases).

Statistical analysis

The data were analysed with the JMP 4.0.4. statistics package (Sall and Lehman 1996). Because of its binomial distribution the response variable 'percentage of outcrossed eggs' was arcsine transformed. Multiple regression and repeated measures ANOVA were used after data plots had indicated that the assumptions of such statistics were not violated (Sokal and Rohlf 1995).

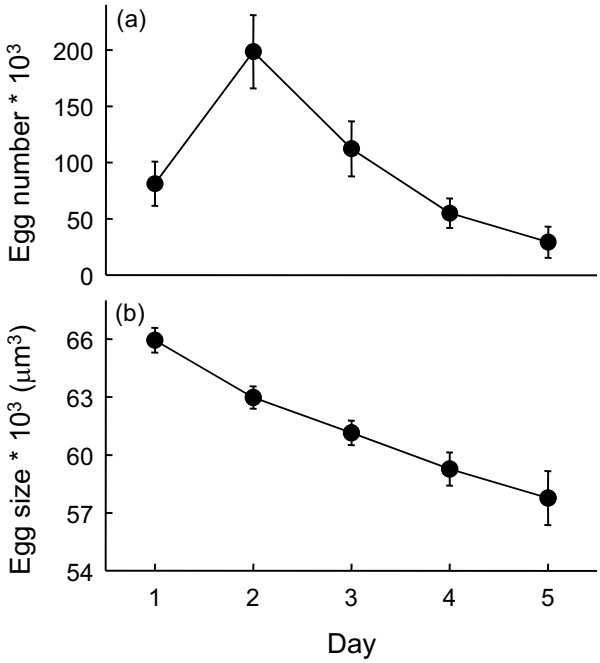


Fig. I. 2: (a) Mean (± s.d.) numbers of eggs produced by worm pairs during the five days of observation. (b) Mean size of eggs (± s. e.) produced by worm pairs during the five days of observation.

RESULTS

Egg production (number and size) of worm pairs

Worm pairs reached peak egg production on day 2 in the *in vitro* system, thereafter the egg output steadily decreased (Fig. I. 2a; ANOVA with repeated measures using Huynh-Feldt correction: $F_{3,87} = 381.57$, $p < 0.0001$). On day 1 the worm pairs produced the biggest eggs, thereafter the size decreased steadily until day 5 (Fig. I. 2b; $F_{2,53} = 27.76$, $p < 0.0001$). Worm weight had a significant influence on egg production, i.e. large worm pairs produced more eggs than small worm pairs (Multiple regression with weight of pairs and asymmetry in weight as independent variables, effect of weight: $n = 29$, $t = 7.14$, $p < 0.0001$). The effect of asymmetry in weight within worm pairs on total egg number was not significant, but there was a significant interaction between weight and asymmetry of worm pairs (effect of asymmetry: $n = 29$, $t = 0.99$, $p = 0.33$; interaction: $n = 29$, $t = 3.09$, $p = 0.005$; for details see Table I. 1).

Heavier worm pairs produced bigger eggs than smaller worm pairs (Multiple regression with weight of pairs and asymmetry in weight as independent variables, effect of weight:

Table I. 1. Multiple regression table with egg number as dependent and asymmetry and mean weight of pairs as independent variables (n = 29).

	Estimate	Std Error	t ratio	Prob >= t	Std beta
Asymmetry	45.96	46.48	0.99	0.33	0.11
Mean weight	3.72	0.52	7.14	< 0.0001	0.81
Interaction	4.73	1.53	3.09	0.0048	0.35

n = 29, t = 2.2, p = 0.037; for details see table I. 2). Asymmetry of worm weights of a pair had no significant effect on mean egg size (effect of asymmetry: n = 29, t = 0.65, p = 0.52), nor was there a significant interaction (n = 29, t = 0.17, p = 0.87).

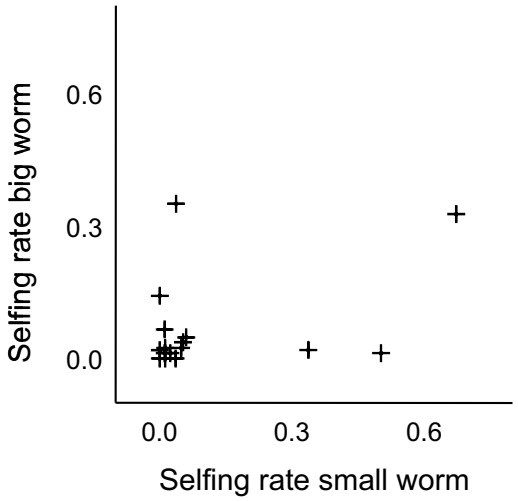


Fig. I. 3: Selfing rate of the small worm plotted against the selfing rate of the big worm. Note that 1 - (selfed hatched eggs small worm + selfed hatched eggs big worm) equals the outcrossing rates of pairs.

The intensity of reproductive conflict influences outcrossing and selfing rates

The selfing rate of the small worm is compared with that of the big worm in Figure I. 3. Only one worm pair (of the high asymmetry group) only selfed, all the remaining pairs produced both selfed and outcrossed offspring. Weight of worms had a significant influence on outcrossing probability: outcrossing rates increased with weight of worm pairs (Multiple regression with weight and asymmetry as independent variables: effect of weight: $F_{1,13}$ = 7.25, p = 0.018; Fig. I. 4a; for details see Table I. 3). Worm pairs that experienced a high level of conflict (big size difference within pairs) produced fewer outcrossed eggs than worm pairs at a low level of conflict (effect of asymmetry: $F_{1,13}$ = 4.65, p = 0.05; Fig. I. 4b).

Hatching success of the offspring

Hatching success was not significantly influenced by either weight of pairs or conflict intensity (all effects and interactions: F always < 1.58, p always > 0.23). Surprisingly there was no

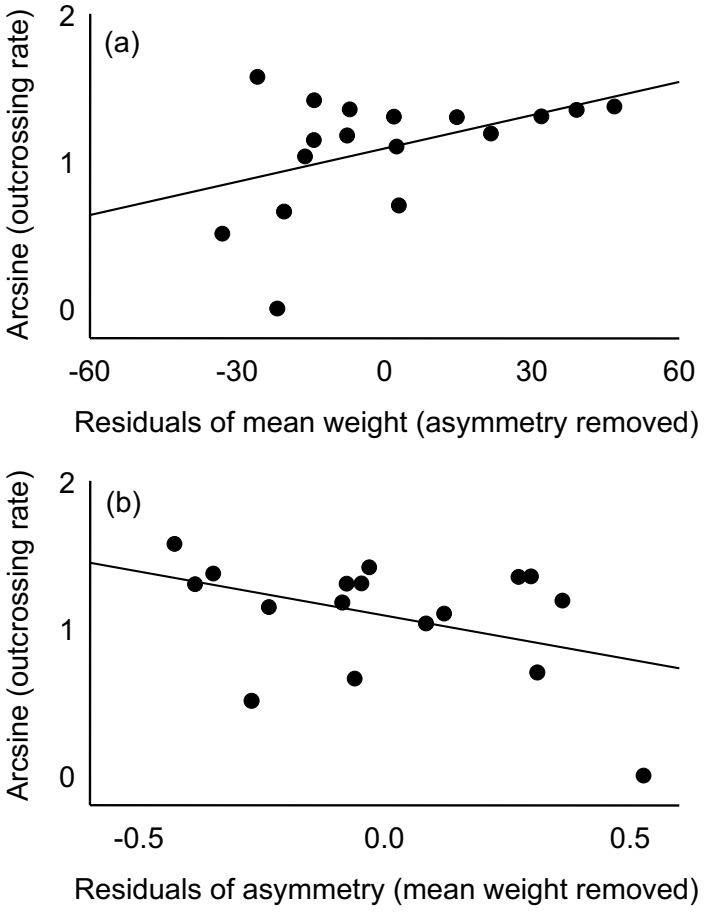


Fig. I. 4: Partial residual plot of mean weight of pairs and asymmetry in weight on outcrossing rates of pairs. For details on the multiple regression see Table 3. (a) Residual plot of the effect of mean weight (asymmetry removed) on outcrossing rate. (b) Residual plot of the effect of asymmetry (mean weight removed) on outcrossing rate.

significant correlation between outcrossing rate and hatching rate (n = 17, r = 0.24, p = 0.34), even though the proportion of hatched eggs seemed to increase with outcrossing rate (n = 17, r = 0.42 , p = 0.08).

DISCUSSION

All worms in pairs of *S. solidus* engaged in some self-fertilization, even though they had the opportunity to outcross. This is surprising, because several studies demonstrated various disadvantages of selfed offspring from singleton worms compared to offspring from pairs of worms (such as a reduced hatching rate, lower transmission probability and slower growth when competing for the same resources (same copepod) (Wedekind 1997; Christen *et al*. 2002). However, due to strong fluctuations in prevalence, individ-

Table I. 2. Multiple regression table with mean egg size as dependent and asymmetry and mean weight of pairs as independent variables (n = 29).

	Estimate	Std Error	t ratio	Prob >= t	Std beta
Asymmetry	1428.20	2196.07	0.65	0.52	0.12
Mean weight	54.23	24.61	2.20	0.037	0.41
Interaction	12.10	72.40	0.17	0.87	0.03

Table I. 3. Multiple regression table with outcrossing rate (arcsine transformed) as dependent and asymmetry and mean weight of pairs as independent variables (n = 17).

	Estimate	Std Error	t ratio	Prob >= t	Std beta
Asymmetry	-0.59	0.27	-2.16	0.05	-0.42
Mean weight	0.01	0.003	2.69	0.02	0.54
Interaction	0.02	0.01	1.76	0.10	0.35

uals of *S. solidus* may occasionally end up without a partner in the final host (Pennycuick 1971b). In such a situation their assurance of reproduction is to self-fertilize their eggs. For a species that normally fully outcrosses a sudden event of the most extreme kind of inbreeding , i.e. self-fertilization, is expected to cause a massive decrease in fitness due to deleterious mutations with homozygous effects on fitness (Charlesworth and Charlesworth 1987; Charlesworth and Charlesworth 1998). On the other hand, inbreeding depression is expected to decrease with every generation of successful self-fertilization due to purging of deleterious mutations. Hence, in a fully outcrossing population, offspring of a worm that invests both in outcrossing and in selfing may have a higher fitness when almost all individuals have to reproduce by selfing due to low density. The evolutionary stability of intermediate selfing rates for such a scenario has been confirmed recently in a model by Cheptou and Dieckmann (2002). They have demonstrated that intermediate selfing rates in simultaneous hermaphrodites can arise through both cyclical and stochastic density fluctuations.

In a population with a mixed-mating system, the fitness that individuals gain through selfing may vary depending on how purged they are (see Introduction). The large variability of hatching rates of eggs that were produced by singles of *S. solidus* may be a consequence of their history of inbreeding. The hatching rate of singletons' eggs varies between 3% and 30 % (Christen *et al*. 2002; A. Lüscher unpublished data). Moreover, we did not find a significant correlation between outcrossing rate and hatching rate of eggs from worm pairs in this study, which suggests that the average cost of selfing is lower when individuals can decide (within pairs) which proportion of their eggs they self-fertilize. Therefore, to maximise their fitness, worms may not only self- and cross-fertilize their eggs at random or at a fixed rate, but invest in either mode of reproduction depending on their fitness as selfers. We would expect that more inbred worms are better adapted to selfing (due to purging of deleterious mutations) and hence self a higher proportion of their eggs compared to less inbred worms.

We found that outcrossing rate increased significantly with mean weight of pairs. Do inbred worms grow less in their fish host than outbred worms? If this is the case, we should find small worms not only in small fish, but also in big fish, because worm size would not only be predicted by fish size, but also by the worms' inbreeding history. This, however, is not what we found: the size of the worms used in this experiment strongly correlated with the size of the fish they had been removed from: $n = 59$, $r = 0.74$, $F = 68.87$, $p < 0.0001$. Yet, there is 26% of variation that remains unexplained which leaves an opportunity for, e.g. genetics. Individual heterozygosity is expected to decrease the more inbred an individual is (Haldane 1949; Cockerham and Weir 1968). Hence, small pairs may not have outcrossed less than bigger pairs, because they were smaller, but because they were more inbred and therefore more adapted to reproduction through self-fertilization. We found, however, no significant correlation between mean weight of pairs and mean heterozygosity ($n = 17$, $r = 0.26$, $F = 1.18$, $p = 0.29$). Considering the small sample size, weight being a function of individual heterozygosity should not yet be excluded and will provide an interesting framework for further studies. Yet, the evolution of adaptive investment in selfing depending on inbreeding history may be hard to imagine, because this requires individuals to predict their potential fitness gain as selfers.

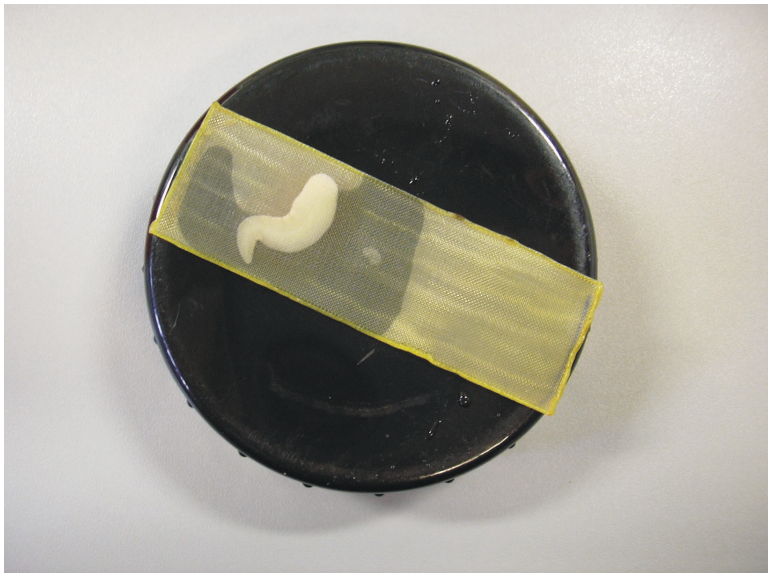
Outcrossing rate increased not only with mean weight of pairs, but also with similarity in weight difference. In situations of strong reproductive conflict (big weight difference within pairs), worm pairs produced fewer outcrossed offspring than in situations where cooperative solutions are more probable (small weight difference) (Fig. I. 4b). It is important to notice that there was no correlation between heterozygosity and weight difference within worm pairs, which could have blurred the influence of asymmetry on outcrossing rate ($r = 0.013$, $n = 17$, $F = 0.003$, $p = 0.96$). Interestingly, there were only five worm pairs out of seventeen, where one of the worms of a pair selfed much more than the other (Fig. I. 3). In all other combinations both worms of a pair were almost only outcrossing or in one case selfing a substantial proportion of their eggs. The worms which selfed much more than their partners increased their fitness, because they passed sperm not only through outcrossing but also through selfing. Every selfed egg of the defector is a lost fitness gain for the cheated worm. Indeed, this does not correspond to a cooperative solution to the Hermaphrodite's dilemma, which predicts reciprocity, i.e. gamete trading in equilibrium. In a pair consisting of two differently sized worms, we would therefore expect that the big worm is allowed to fertilize all eggs of the small one, while the small worm is at least allowed to fertilize an equal number of eggs of the big worm. This would leave the big worm with a certain proportion of selfed eggs, and the small one with only outcrossed eggs. This may be consistent with the three worm pairs where the

bigger worm selfed some of its eggs and the smaller worm fully outcrossed. It is, however, not consistent with the two pairs where the smaller worm selfed a considerable amount of it's own eggs and fertilized all of it's partners eggs. In terms of the Hermaphrodite's Dilemma model, those two worms were cheating on their partners, because they did not reciprocate the opportunity to the mating partner to reproduce as a sperm donor.

In this experiment we found mutually exclusive experimental evidence for predictions of both mixed-mating system theory and Hermaphrodite Dilemma theory. Both model are necessary to understand the reason why individual *S. solidus* in pairs self-fertilize some of their eggs. The *S. solidus* system is very promising for future studies, because it allows experimental manipulation of mating situations under controlled conditions and assignment of outcrossing and selfing rates of worms using fast molecular methods.

ACKNOWLEDGEMENTS

We thank Thomas Binz for the introduction to microsatellite analysis, and David Baird and Solveig Schjørring for statistical advice. August Hämmerli and Derk Wachsmuth generously provided Genstat and Matlab program codes. We thank Wolf-Rüdiger Wulff and Sybille Liedtke for lab assistance and several people from the Department of Evolutionary Ecology at the MPI Plön generously helped with catching and dissecting sticklebacks.



Contracted *S. solidus* individual enclosed between two nylon layers.

Potential fitness from selfing and heterozygosity of mating partner determine outcrossing rate in a simultaneous hermaphrodite

SUMMARY

Traditionally, inbreeding depression is thought to prevent the evolution of stable mixed-mating systems (i.e. reproduction through both selfing and outcrossing) in both hermaphrodite plants and animals through selection for high outcrossing rates. Recently, however, it was shown theoretically that environmentally induced fluctuations in population density can lead to mixed-mating systems, which are evolutionarily stable. Fluctuating densities render the environment unpredictable e.g. in terms of finding a mating partner. One large group of species for which this may be particularly relevant are the hermaphroditic endoparasitic worms (helminths). The usually low densities in their final host force them sometimes to self-fertilize as last resort. Selfed offspring of worm lineages which have always selfed a certain proportion of their eggs (despite the availability of a partner), may produce fitter progeny than do offspring from worms that have always outcrossed when both have to self in a year of low worm density. Moreover, an individual which can adaptively adjust its outcrossing rate depending on the fitness gain it would achieve through selfing would have a selective advantage compared to individuals that outcross and self at random. We tested this prediction experimentally in the cestode *Schistocephalus solidus* using an *in vitro* system. Focal worms whose selfed offspring had a high hatching rate produced fewer outcrossed offspring than worms whose selfed offspring had a lower hatching rate. The rate of outcrossing was furthermore influenced by the individual heterozygosity of the offered partner: focal worms produced a higher proportion of outcrossed offspring with more heterozygous partners. This suggests that worms can indeed adaptively adjust their outcrossing rate depending both on their fitness as selfers and on the genetic quality of the offered partner.

INTRODUCTION

Fisher (1941) showed that a selfer gene which is introduced into an outcrossing population at low frequency will have a 50% fitness advantage and will therefore spread rapidly. This would soon lead to complete selfing if it were not counteracted by inbreeding depression. Most models on mating system evolution predict either one of two extremes to be evolutionarily stable: complete selfing or complete outcrossing (Lande and Schemske 1985; Charlesworth *et al*. 1990). Which of the two is predicted depends mainly on whether inbred progeny are assumed to have a disadvantage through inbreeding depression of more or less than 50 % compared to outcrossed progeny. In contrast to these predictions however, data from natural populations show that many hermaphroditic plants and animals are reproducing through a mixture of selfing and outcrossing (mixed-mating) (Waller 1986; Jarne and Charlesworth 1993; Charlesworth and Charlesworth 1998). This is why the conditions under which mixed-mating could also be evolutionarily stable has come into focus of evolutionary biologists in the past decades.

Traditionally, evolutionarily stable mixed-mating systems are predicted only for the following special cases: (1) when both self-fertilization and biparental inbreeding occur simultaneously (Uyenoyama 1986), and (2) when selfed progeny are less well dispersed than outbred progeny (Holsinger 1986). Yet, these models still leave a diversity of mixed-mating systems unexplained, because many systems do not meet the rather specialized assumptions. Recently, however, Cheptou and Dieckmann (2002) showed that environmental fluctuations can lead to mixed-mating systems, which are evolutionarily stable under a wide range of conditions. Their models assume populations with fluctuating densities and this is in contrast to the traditional approaches. Fluctuating densities render the environment unpredictable in terms of, e.g. finding a mating partner. One large group of species for which this may be particularly realistic are the endoparasitic worms (helminths). The usually low densities in their final host (Shaw *et al*. 1998) force them sometimes to self-fertilize as last resort, which may be reflected in the high proportion of hermaprodites in this group of species. This is why the endoparasitic helminths are particularly suitable organisms to study inbreeding depression and mixed-mating systems in the new context of environmental unpredictability.

Within the large group of endoparasitic helminths (i.e. cestodes, trematodes), most are able to reproduce both, by self- and cross-fertilization (Williams and McVicar 1968; Nollen 1983; Trouvé *et al*. 1996). Environmental stochasticity increases the risk to end up in their final host without a mating partner (Pennycuick 1971b; Shaw *et al*. 1998). In these species, selfing maybe maintained simply because (1) it assures reproduction in an unpredictable

environment (Baker 1959; Lloyd 1980), (2) it may preserve highly fit genotypes, (3) reduces energy allocation to sperm production and (4) mating is less costly (Jarne and Charlesworth 1993; Thornhill 1993). Despite these advantages however, inbreeding depression may act as a major selective force against self-fertilization when cross-fertilization is possible (Lande and Schemske 1985; Charlesworth and Charlesworth 1987).

Our study animal is the simultaneous hermaphroditic cestode *Schistocephalus solidus*. Its life cycle includes two intermediate hosts (a cyclopoid copepod and the three-spined stickleback (*Gasterosteus aculeatus*)) and one final host (any fish eating bird). Reproduction by cross- or self-fertilization takes place in the gut of the final host. Several studies have shown that *S. solidus* suffers inbreeding depression upon selfing: lowered hatching rate by 80% (Wedekind *et al*. 1998; Christen *et al*. 2002), reduced infection rate of the first intermediate host (Wedekind and Rüetschi 2000) and reduced virulence compared to outcrossed competitors (Christen *et al*. 2002; Christen and Milinski subm). According to these findings we would expect that worms will always outcross when they have a partner. Results from a recent experiment are clearly at variance with this expectation. Worms reproducing in pairs invested in both, self- and cross-fertilization, despite strong inbreeding depression (Lüscher and Milinski subm.). How can we explain the findings? Mixed-mating in this species seems to be evolutionarily stable, but the conditions for stability are largely unknown.

A possible explanation for this observation is that mixed-mating may be evolutionarily maintained in this species due to the strong fluctuations of worm density in final hosts, as predicted by the model of Cheptou and Dieckmann (2002). Because of the short time (a few days) during which the whole reproduction takes place (McCaig and Hopkins 1963; Tierney and Crompton 1992), and the strong fluctuations in prevalence between years in this species, e.g. Pennycuick (1971b), worms have to self when they happen to be alone in the definitive host. Selfed offspring of worm lineages which have always selfed a certain proportion of their eggs (despite the availability of a partner), may produce fitter progeny than do offspring from worms that have always outcrossed when both have to self in a year of low worm density. Inbreeding depression is expected to be strongest for the first selfed generation and is thereafter expected to decrease during consecutive selfing events, due to purging of deleterious mutations with large homozygous effects on fitness (Charlesworth and Charlesworth 1998). Outcrossing rates of individuals can be determined in three ways: (1) there is a fixed population mean, (2) individuals outcross a random amount of their eggs or (3) individuals could adaptively adjust their outcrossing rate depending on their fitness to be gained through selfing. It is unlikely that outcrossing rate is fixed in a population, because outcrossing rate is highly variable between pairs of *S. solidus* (Lüscher and Milinski subm.). We therefore aimed

to test in this study if the variability in outcrossing rate could be explained by the reproductive success that worms achieve through selfing. An individual which could adaptively adjust its outcrossing rate depending on the fitness gain it would achieve through selfing, would have a strong selective advantage compared to individuals who outcross and self at random. If an individual's fitness gain through selfing is high, it should invest less in outcrossing than an individual whose selfed offspring have a low fitness. For this mechanism to work, a specific kind of memory, e.g. genomic imprinting is assumed in order to allow individuals to take their inbreeding history into account.

The aim of the present study was to test (a) whether and how individuals of *S. solidus* adjust their outcrossing rate depending on their fitness through self-fertilization and (b) whether the time span a worm had been forced to self had an influence on its outcrossing rate or egg production. An individual may increase its outcrossing rate, if it had to self-fertilize already a large proportion of its eggs or it may delay its egg production while selfing, to be able to increase it, when there is a possibility for outcrossing. We further manipulated the weight difference of worms in a pair, to test whether the difference in female quality (larger worms produce more eggs (Wedekind *et al*. 1998; Schärer and Wedekind 1999)) had an influence on the outcrossing rate. A previous experiment showed that worms produce more outbred offspring with decreasing weight difference between two paired worms (Lüscher and Milinski subm.). In the present study we tested if we find the same result in worm pairs where one of the two worms had already reproduced alone by selfing before the pairs were formed.

To test these hypotheses we performed an experiment under controlled conditions in which we replaced the bird's gut environment by an *in vitro* system (Smyth 1954; Wedekind 1997; Schärer and Wedekind 1999).

METHODS

Study Organism

The life cycle of *S. solidus* includes two intermediate hosts. Its definitive host, i.e. any fish eating bird, is infected by consuming infected sticklebacks (*Gasterosteus aculeatus*), which themselves have been parasitised by eating infected copepods. Spermatogenesis and oogenesis take place only in the definitive host, triggered by a rise of temperature from about 15°C in the fish to 40°C in the bird, ruling out the possibility of sperm transfer in the fish (Smyth

1946). *S. solidus*' entire reproduction, i.e. sperm and egg production, mating, and egg release, occurs in the gut of birds shortly after infection (Clarke 1954; Schärer and Wedekind 1999). Within the short time period of reproduction which is terminated within a few days worms can copulate several times (self- and/or cross-fertilization) (McCaig and Hopkins 1963; Tierney and Crompton 1992). It has only recently been shown that *S. solidus* reacts to situations with high sperm competition by transferring more sperm (Schärer and Wedekind 2001). After several days in the gut the worms die. The eggs are released into the water with the bird's faeces. After some weeks the free-living *S. solidus* larva (coracidium) hatches. The coracidium needs to be ingested by a cyclopoid copepod which, after the worm larva has become infective to the next intermediate host, is a preferred prey of sticklebacks (Wedekind and Milinski 1996). The reported prevalence of *S. solidus* in sticklebacks is often high, sometimes up to 100% (Dick 1816; Smyth 1947; Hopkins and Smyth 1951). The distribution of *S. solidus* in sticklebacks is overdispersed with most infected fish containing only one worm (Smyth 1994; A. Lüscher unpublished data). In birds, up to around 300 adult worms per individual host have been observed (Nybelin 1919; Vik 1954), however, these numbers probably represent extreme cases.

Experimental Procedure

Infected sticklebacks were caught during September and October 2001 in a brackish water bay near Neustadt (Schleswig-Holstein), Germany. Shoaling sticklebacks were dip-netted from the shore within a range of about 1 km. The fish were kept in 194 l tanks for eight weeks before dissection. To obtain the worms, fish were killed by a cerebrospinal cut and the worms were removed aseptically from the stickleback. Only worms from singly infected fish were used for the experiment. The worms were weighed on a balance to the nearest mg and kept in culture medium at room temperature for a maximum of twelve hours before the start of the experiment. The culture medium consisted of sterilized Minimum Essential Medium with Earle's salts, L-glutamine, 25 mM HEPES buffer (Sigma) and additives (per litre of medium: 1 ml Antibiotic/Antimycotic solution (Sigma), 6.5 g D-Glucose), and had been titrated with NaOH to a pH of 7.5.

Forty focal worms were transferred singly to nylon mesh bags (25 x 75 mm). A mesh size of 200 μm was chosen so that eggs could easily pass the net whereas worms could not. The opening of the bags was closed by melting the ends of the nylon layer with a lighter. Each bag was attached to the lid of a culture bottle and then hung into 250 ml of culture medium. After closing the lids the bottles were placed in a 40°C water bath (SW 23, Julabo

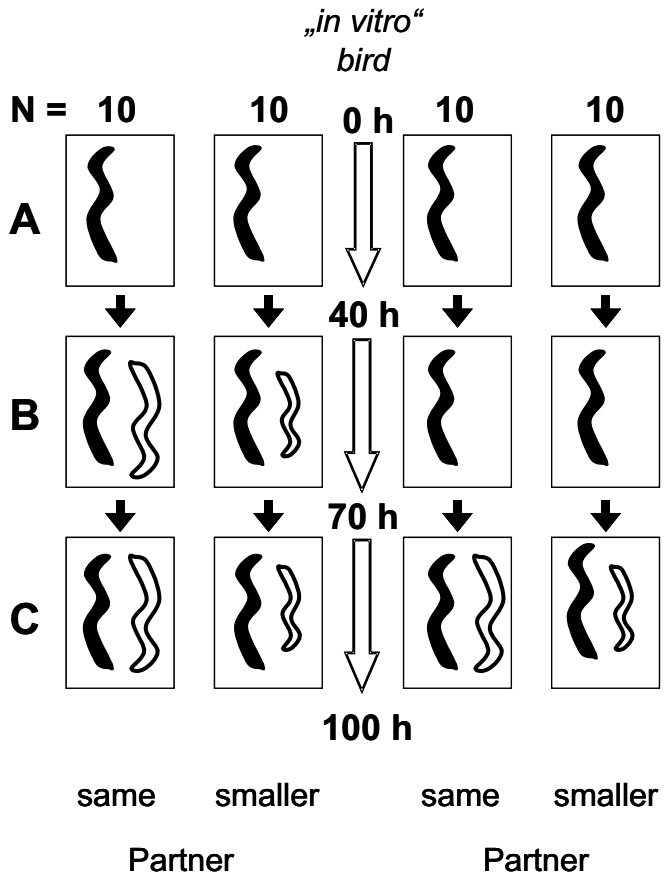


Fig. II. 1: Experimental design. To one half of the focal worms (filled worms in figure) a partner worm (translucent worms in figure) was added after the focal worm had reproduced alone for 40 hours (left panel in figure). To the other half of the focal worms a partner worm was added after 70 hours of reproducing alone (right panel in figure). In half of the pairs the partner worm had half the weight of the focal worm ("smaller") and in the other half the partner worm had the same weight as the focal worm ("same"). The experiment was ended after focal worms had reproduced for 100 hours. We measured egg number, egg size, hatching rate, outcrossing and selfing rates at three points in time: from 0 to 40 hours (in the text referred to as "sampling A"), from 40 to 70 hours (referred to as "sampling B") and from 70 to 100 hours (referred to as "sampling C"). See text for further details.

Labortechnik, Seelbach, Germany) and shaken continuously at 70 rpm. Egg samples were taken three times during the experiment. The first sampling was done 40 hours after placing the worm bottles into the water bath (Fig. II. 1. this will further on be referred to as "sampling A") , the second one after 70 hours (this will further on be referred to as " sampling B") and the last one after 100 hours (this will further on be referred to as " sampling C"). In total the worms were allowed to reproduce for 100 hours, which is the time the worms need to complete about 90% of their egg production (Schärer and Wedekind 1999).

The experiment consisted of four treatment groups each starting with ten focal worms (Fig. II. 1, filled worms). In half of the samples (n = 20) a second worm from a freshly dissected fish was inserted into the nylon bag after focal worms had reproduced alone for 40 hours (left

panel in Fig. II. 1). In ten pairs the second worm (Fig. II. 1, translucent worms) had the same size as the focal worm (in Fig. 1. II. referred to as "same") and in the other ten pairs the second worm had half the weight of the first worm (in Fig. II. 1. referred to as "smaller"). In the other half of the samples (n = 20) a second worm from a freshly dissected fish was inserted into the nylon bag after focal worms had reproduced alone for 70 hours (right panel in Fig. II. 1). Again, in ten pairs the second worm had the same size as the focal worm (= "same" in Fig II. 1), while in the other ten pairs the second worm weighed only half the weight of the focal worm (= "smaller" in Fig. II. 1). This resulted in a completely balanced design. We controlled for mean weight of worm pairs, which ranged from 241 mg to 368 mg (mean = 293 mg, s.d. = 36.98 mg). There was no difference in worm weight between the four treatment groups (n = 40, $F_{3, 36}$ = 0.0039, p = 0.9997).

At each of the three sampling times, eggs were collected from the bottom of the old bottles, and the lids with the worms were placed on new bottles with fresh, preheated medium. After the eggs had settled in the bottle about 95 % of the medium was replaced by tap water. This procedure was repeated four times before transferring the eggs to a 15 ml tube and storing them at 4°C.

Number and size distribution of eggs were determined using a CASY, Cell Counter (Schärfe), which gives a particle volume distribution with 1024 size channels and the number of particles counted. We used a 150 µm diameter orifice tube with the sampling range set between 35 µm and 70 µm (egg diameter is expected to range from 45 to 55 µm (Wedekind *et al*. 1998)). About 60'000 eggs of each worm pair (20'000 per sample) were counted and measured.

After collection eggs were kept at darkness and 4 °C. Four weeks later about 45'000 eggs of each worm pair were transferred to Petri dishes. It was necessary to standardize the number of eggs of pairs to be able to exclude density in Petri dishes as a confounding variable acting on hatching success. After two weeks at 20 °C and darkness the eggs were moved to 15L/9D at 20 °C which induced hatching. Hatching rates of the clutches were determined eight weeks after exposure of the eggs to light. Only empty eggs with open or removed operculum were considered as hatched (Swiderski 1994).

DNA extraction followed the Chelex © extraction method (Walsh *et al*. 1991). 1 mm^3 of worm tissue was mixed in a 50 µl 5 % Chelex © suspension and stored at –20 °C. 1µl of Proteinase K solution (Roche) was added to the sample in Chelex © suspension and incubated at 55 °C for 15 min and at 100°C for 15 min. Then the samples were placed on ice, spun down for 30 seconds at 13000 rpm, and stored at –20°C. PCR reactions were set up in 10 µl volumes, each containing 1µl of extract, 1x Promega reaction buffer, 1.5 mM MgCl2, 250

mM of each dNTP, 0.5 units Promega Taq Polymerase, 1 µl of 1 % Bovine serum albumin and 0.5 mM of one of eight pairs of locus-specific fluorescent-labelled forward primer and non labelled reverse primer. Details on microsatellite characterization are given in Binz *et al*. (2000). PCR cycling parameters were the following: 1 min at 94 °C, 27 cycles of 94 °C for 1 min, 1 min at 57 °C and 1 min at 72 °C, followed by a final step of 72 °C for 7 min. Fluorescent PCR fragments were separated and visualized on a ABI PRISM, 3100 Genetic Analyzer using the POP4™ Polymer (Applera Corporation). Nearly identical methods were used to amplify microsatellite fragments from coracidia (larvae). From each worm pair 30 coracidia were analysed per sampling time. Each coracidium was singly mixed in 30µl 5 % Chelex © suspension instead of 50 µl and the incubation step at 55 °C lasted 30 min instead of 15 min. For amplification of coracidia the cycles in the PCR reaction were increased to 40.

To be able to determine for each larva if it resulted from outcrossing, selfing by the bigger worm or selfing by the smaller worm of the pair, individuals in a pair were required not to share identical alleles on at least one of the microsatellite loci. For 35 worm pairs we found such a locus that met this requirement. The remaining pairs either shared alleles at all four loci (two pairs) or microsatellite amplification with the used primers failed (three cases).

Statistical Analysis

The data were analysed with the JMP 4.0.4. statistics package (Sall and Lehman 1996). Because of their binomial distribution the response variable 'percentage of outcrossed eggs' was arcsine transformed. Multiple regression and repeated measures ANOVA were used after data plots had indicated that the assumptions of such statistics were not violated (Sokal and Rohlf 1995).

RESULTS

Egg Production (Number and Size)

At sampling A all the worms were reproducing as singles. At sampling B a second worm was added to half of the worms reproducing as singles, and at sampling C a second worm was added to each of the remaining singly reproducing worms (for experimental design see Fig. II. 1). The pattern of egg production that resulted from this sequential design is shown in Fig. II. 2. Egg production depended on whether worms reproduced alone or in pairs (Fig. II. 2).

	F	NumDF	DenDF	Prob>F
Between subjects				
Insertion	28.21	1	36	< 0.0001
Size	0.13	1	36	0.72
Insertion x Size	2.01	1	36	0.17
Within subjects				
Time	70.75	1.98	71.41	< 0.0001
Time x Insertion	43.16	1.98	71.41	< 0.0001
Time x Size	7.01	1.98	71.41	0.002
Time x Insertion x Size	12.35	1.98	71.41	< 0.0001

Table II. 1: Two-way ANOVA on egg number; with repeated measurements and time point of insertion (40 h or 70 h) and size of the second worm (same size as focal worm or half the size) as fixed factors using Huynh-Feldt correction (n = 40). See also Fig. II. 2.

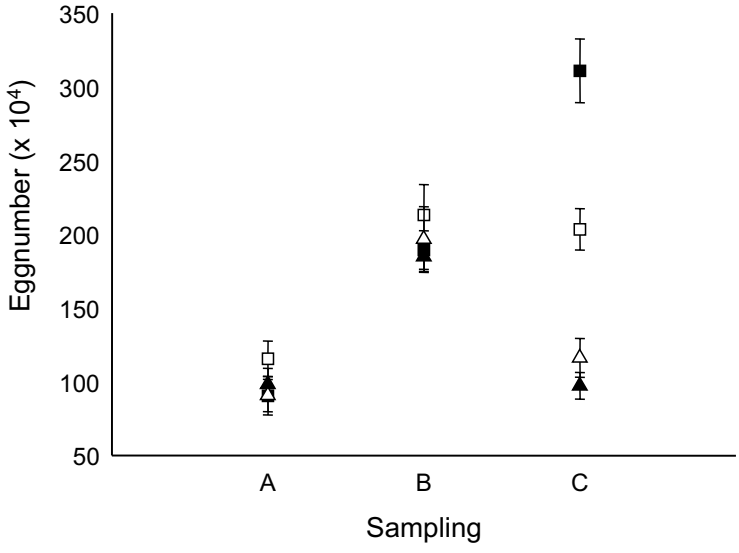


Fig. II. 2. Mean (± s.e.) numbers of eggs produced by worms during the 100 hours of observation (divided up into the three sampling times). Filled squares = a partner worm of the same size was added to the focal worm 40 hours after the start of the experiment (n = 10); open squares = a partner worm half the size was added to the focal worm 40 hours after the start of the experiment (n = 10); filled triangles = a partner worm of the same size was added to the focal worm 70 hours after the start of the experiment (n = 10); open triangles = a partner worm half the size was added to the focal worm 70 hours after the start of the experiment (n = 10). For full model see Table II. 1.

Only at sampling C a difference in egg production is visible in pairs where the second worm had been added at sampling B (two-way ANOVA with repeated measurements, using Huynh-Feldt correction; interaction of insertion of second worm X time: n = 40, $F_{1.9,\ 71.4} = 70.75$, $p < 0.001$; see Table II. 1 for full model). This is because the peak of egg production of the focal worms occurs at sampling B, while the egg production of the worms added at sampling B is very low at this point and only increases at sampling C. Moreover, the influence of the size of the added mating partner (added at sampling B) on egg

Table II. 2: Two-way ANOVA on egg size with repeated measurements and time point of insertion (40 h or 70 h) and size of the second worm (same size as focal worm or half the size) as fixed factors using Huynh-Feldt correction (n = 40).

	F	NumDF	DenDF	Prob>F
Between subjects				
Insertion	0.29	1	36	0.59
Size	0.39	1	36	0.54
Insertion x Size	1.56	1	36	0.22
Within subjects				
Time	16.69	1.55	56.02	< 0.0001
Time x Insertion	2.10	1.55	56.02	0.14
Time x Size	0.48	1.55	56.02	0.58
Time x Insertion x Size	0.65	1.55	56.02	0.49

production is visible at sampling C: unequally sized pairs produced less eggs than equally sized pairs (two-way ANOVA with repeated measurements, using Huynh-Feldt correction; interaction of size of the added worm X time: n = 40, $F_{1.9, 71.4} = 7.01$, p = 0.002; for full model see Table II. 1). This was expected, since focal worms in each of these two treatment groups were size matched, therefore the total weight of unequal sized pairs was smaller than that of similar sized pairs.

No difference is found at sampling C in worm pairs, where the second worm was added only at sampling C (Fig. II. 2). This can be explained by the low number of eggs the added worm contributes to the egg production of the pair in its first 30 hours of reproduction.

To test whether egg production of pairs is additive, i.e. is corresponding to the amount of eggs each of the two worms in a pair would produce alone, we compared the observed values from this study with expected values generated from another data set (Lüscher and Milinski subm.). We used those data, because in this previous experiment worms of similar sizes were reproducing in pairs during the whole 100 hours and the *in vitro* system was exactly the same. The difficulty in this calculation is that the reproductive cycles of the two worms in a pair were shifted, because the second worm was added only after the focal worm had already reproduced alone for either 40 or 70 hours. We therefore had to calculate the percentage of eggs a single worm (either focal or added worm) within a pair was expected to produce during each of the three sampling times. From there the percentage of eggs expected to be produced by pairs of worms at sampling time B and C could be calculated. These

expected percentages were then compared with the observed values. The observed values did not differ significantly from the expected values in either of the two treatment groups: when the second worm was added after the focal worm had reproduced alone for 40 hours (n = 20, d.f. = 2, $\chi 2$ = 0.688, p > 0.5) and when the second worm was added after the focal worm had reproduced for 70 hours (n = 20, d.f. = 2, $\chi 2$ = 0.117, p > 0.9). The same procedure was used to compare the values of equal and unequal sized pairs in the two treatment groups. Also there the observed values did not significantly differ from the expected values (n=10, d.f. = 2, $\chi 2$ always > 0.284, p always > 0.5). This suggests that egg production was additive and not influenced by our treatment.

Worms produced the biggest eggs in the first 40 hours of reproduction, thereafter egg size steadily decreased (two-way ANOVA with repeated measures; effect of time: n = 40, $F_{1.6, 56.0}$ = 16.69, p < 0.001; see Table II. 2 for full model). Neither the time point of insertion of the second worm nor its size or interactions between the variables had a significant influence on egg size (two-way ANOVA with repeated measures: F always < 2.1, p always > 0.14; see Table II. 2 for full model).

Table II. 3. ANOVA on selfing rate with two repeats: the focal and the stimulus worm of a pair and the two measurements in time, and one fixed factor: size of the added mating partner (same size as focal worm or half the size) (n = 19). See also Fig. II. 3b.

	F	NumDF	DenDF	Prob>F
Between subjects				
Size	0.001	1	17	0.98
Within subjects				
Time	82.70	1	17	< 0.0001
Worm	162.57	1	17	< 0.0001
Time x Worm	84.96	1	17	< 0.0001
Time x Size	1.55	1	17	0.23
Worm x Size	0.26	1	17	0.62
Time x Worm x Size	0.34	1	17	0.57

Outcrossing and Selfing Rates

To test whether worms increased their outcrossing rate after having reproduced alone for a longer time, we compared the outcrossing rates of focal worms where the second worm had been added after 70 hours with focal worms where the second worm had been added after 40 hours. 4 out of 19 clutches of the pairs where the second worm had been added after 40 hours consisted of both selfed and outcrossed offspring in measurement B. While the remaining 15 pairs only produced selfed eggs. In the pairs where the second worm had been added after 70 hours 5 out of 16 pairs produced selfed and outcrossed offspring and 11 pairs only selfed. Outcrossing was therefore not significantly more frequent in pairs if worms had to self for a longer time (Fisher's exact test: $p = 0.7$). There was also no difference in the mean outcrossing rate between the two samples of pairs that did actually outcross (Mann-Whitney U-test: $m = 5$, $n = 4$, $U = 11$, p = n.s.). This suggests that worms that have to self-fertilize for a longer time cannot compensate this by increasing their outcrossing rate compared to worms that self-fertilize for a shorter time.

Change in the Outcrossing Rate of Worm Pairs over Time

In the treatment group where focal worms were left to reproduce alone for 40 h before a second worm was inserted, outcrossing and selfing rates of were measured two times, at time B and C. Outcrossing and selfing rates were measured in the first 30 h the worms reproduced in pairs and in the second 30 h (sampling B and C respectively; for experimental design see Fig. II. 1). In the first 30 h worms reproduced in pairs, only 4 out of 19 worm pairs were outcrossing at all (outcrossing rate between 12 % and 25 %), the remaining pairs were selfing despite the opportunity to outcross. In the second 30 h of paired reproduction 17 out of 19 pairs were partly or fully outcrossing (outcrossing rate between 7 % and 100 %). Worm pairs increased their outcrossing rate significantly from sampling B to sampling C (Fig. II. 3a; paired t-test: $n = 19$, $t = 6.85$, $p < 0.0001$). The selfing rate of the worms that had reproduced alone for the first 40 h decreased while reproducing in pairs, while the selfing rate of the inserted worm slightly increased (Fig II. 3b and Table II. 3; one-way ANOVA with repeated measurements (focal worm / second worm of a pair and sampling B / C as paired repeats) and size of second worm as fixed factor: interaction of repeats: $n = 19$, $F_{1, 17} = 84.96$, $p < 0.0001$). In total, the focal worm selfed more eggs than the inserted worm (Fig. II. 3b; $F_{1, 17} = 162.57$, $p < 0.0001$), and worm pairs selfed less eggs at sampling C than at sampling B (Fig. II. 3b; effect of sampling time: $F_{1, 17} = 82.70$, $p < 0.0001$). The size of the second worm (either same

size as the focal worm or half the size of the focal worm) nor the interaction had a significant effect on selfing rates (F 1, 17 always < 1.55, p always > 0.23).

The Lower the Focal Worms' Investment in Selfed Eggs, the Higher is Their Investment in Outcrossing

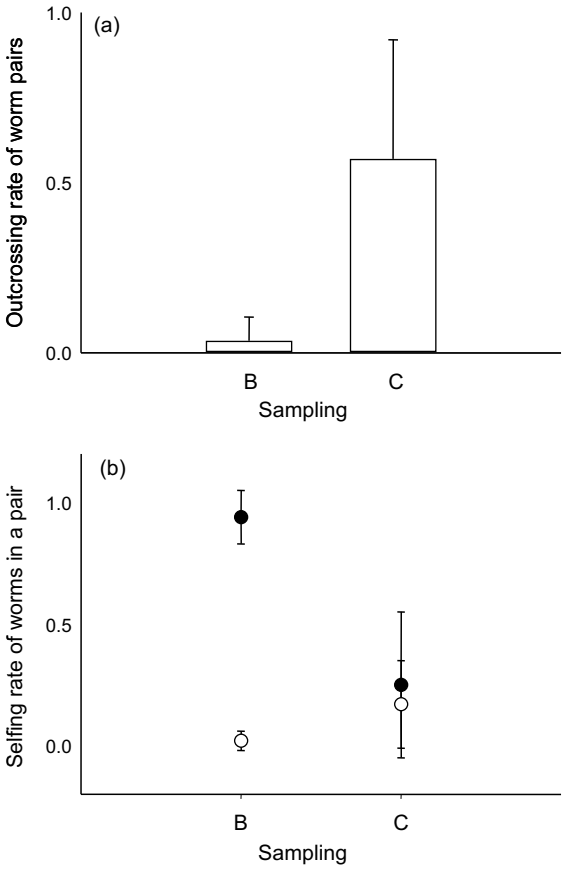


Fig. II. 3. Percentage of outcrossed and selfed offspring produced by worm pairs, where the second worm had been added to the focal worm 40 hours after the start of the experiment (n = 19). (a) Mean percentage of outcrossed offspring (± s. d.) of worm pairs during sampling B (40 to 70 hours after the start of the experiment) and during sampling C (70 to 100 hours after the start of the experiment). (b) Mean percentage of selfed offspring (± s. d.) of worms during sampling B and C. Filled circles = selfing rate of the focal worm; open circle = selfing rate of the partner worm (inserted to the focal worm 40 hours after the experiment started). For full model see Table II. 3.

We found a strong, significantly negative correlation between selfing rate of the focal worm and outcrossing rate at sampling C (Fig. II. 4a. n = 17, r = - 0.82, p < 0.0001). Because our methods unfortunately did not allow to amplify microsatellites on unhatched eggs, we had to determine the selfing and outcrossing rates of worm pairs from hatched eggs. Therefore we had to test whether the negative correlation between outcrossed and selfed offspring of focal worms was not a mere result of better or worse hatching rates of selfed eggs. As we measured hatching rates of selfed eggs of focal worms at sampling A we could estimate the investment in selfing of focal worms at sampling C by multiplying the measured selfing rate with the inverse of the hatching rate of selfed eggs from sampling A. If focal worms would have produced about equal numbers of selfed eggs the correlation between outcrossing rate and investment in selfing should have disappeared after adjusting the selfing rate as described above. This was not the case: there was also a significant negative correlation between outcrossing rate and the investment in selfing (adjusted selfing rate) of focal worms (Fig. II. 4b. n = 17, r = - 0.5, p < 0.05). Moreover, this adjustment is conservative, because the difference in

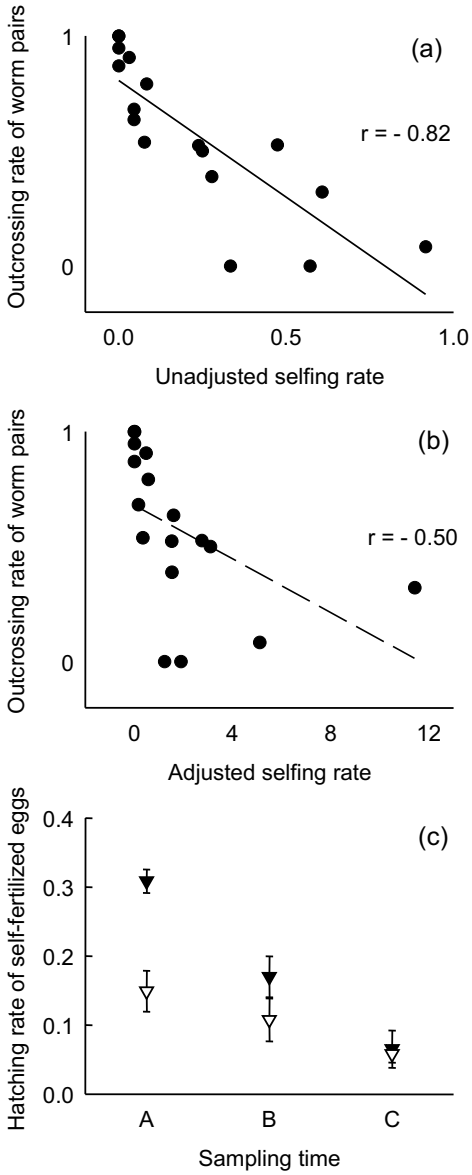


Fig. II. 4. (a) Percentage of selfed hatched offspring of focal worms plotted against the percentage of hatched outcrossed offspring of pairs at sampling C. Only data from worm pairs where the second worm had been added to the focal worm 40 hours after the start of the experiment are shown (n = 17). (b) Adjusted percentage of selfed hatched offspring of focal worms plotted against the percentage of hatched outcrossed offspring of pairs at sampling C. For further details see results section. (c) Hatching rate of self-fertilized eggs plotted against the three sampling times. Only data from worms that did not outcross in any of the three sampling times are shown (n = 12). Filled triangles = hatching rate of selfed offspring at sampling A was higher than the median of the hatching rate (> 0.26); open triangles = hatching rate of selfed offspring at sampling A was lower than the median of the hatching rate (< 0.26).

hatching rate between selfed eggs that have a high hatching rate (closed triangles in Fig. II. 4c) and those that have a low hatching rate (open triangles in Fig. II. 4c) at sampling A decreases over time. (In this last graph only data from focal worms were used that never outcrossed and where the second worm did not produce any offspring (n = 12). The samples were then assigned to the group with low hatching rate or high hatching rate respectively, depending on whether their hatching rate was smaller or larger than the median of the hatching rates.)

The Effect of Fitness of Selfed Offspring (Hatching Rate) on Outcrossing Probability

To investigate whether fitness through self-fertilization influenced the outcrossing rate of worm pairs, we tested whether the hatching rate of selfed offspring at sampling A and the individual heterozygosity of the added worms at sampling B influenced the outcrossing rates of pairs at sampling C. (Only worm pairs where the second worm had been added already after 40 hours (sampling B) were used for this analysis). 17 worm pairs were used in this analysis from the starting total of 20: in two cases it was not possible to measure the hatching rate at sampling A, due to a fungal contamination and in one case none of the used primer pairs allowed an unequivocal assignment of offspring to either of the two parent worms. Hatching rate of selfed offspring at sampling A significantly influenced the outcrossing rate at sampling C (Fig. II. 5a, Table II. 4; multiple regression,

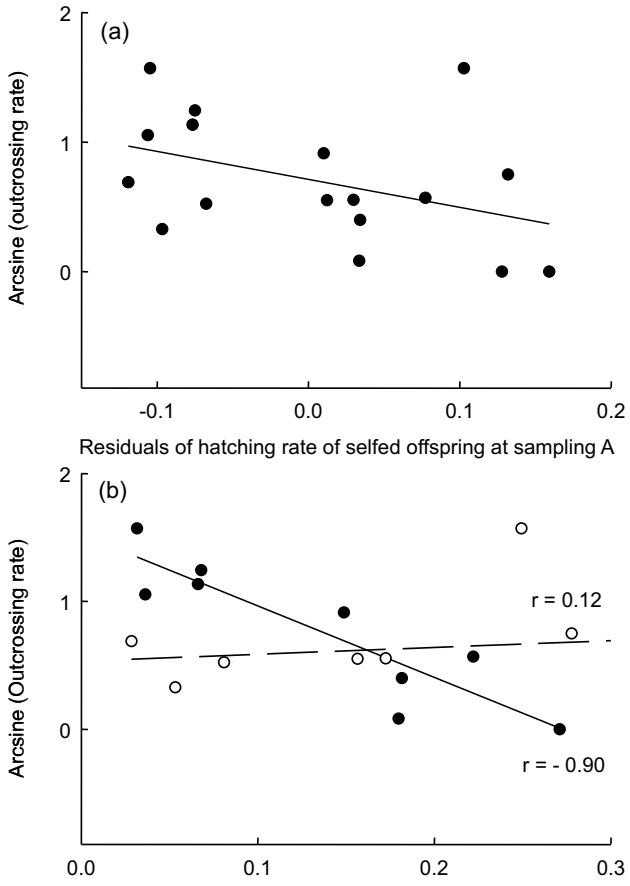


Fig. II. 5. (a) Percentage of outcrossed offspring produced by worm pairs during sampling C (70 to 100 hours after the start of the experiment) plotted against the partial residuals of the hatching rate of selfed offspring from sampling A (0 to 40 hours after the start of the experiment; heterozygosity of added worms removed). Only data from worm pairs where the second worm had been added to the focal worm 40 hours after the start of the experiment are shown (n = 17). For details on the multiple regression see Table 4. (b) Hatching rate of selfed offspring plotted against percentage of outcrossed offspring during sampling C. Filled circles = individual heterozygosity of the second worm was higher than the median of individual heterozygosity (> 0.63); open circles = individual heterozygosity of the second worm was lower than the median of individual heterozygosity (< 0.625). This graph visualizes the significant interaction term in the multiple regression analysis (see Table II. 4).

effect of hatching rate on outcrossing: $F_{1, 15} = 4.96$, $p = 0.04$). There was no significant influence of individual heterozygosity of the added worm on outcrossing rate (Table II. 4; effect of individual heterozygosity of the added worm: $F_{1, 15} = 0.13$, $p = 0.73$), but there was a significant interaction of the two independent variables (Fig. II. 5b, Table II. 4; interaction: $F_{1, 15} = 7.89$, $p = 0.015$). Fig. II. 5b visualizes this interaction: the effect of hatching rate of selfed eggs on outcrossing rate is stronger if heterozygosity of the added worm is high. This suggests that worms can indeed adaptively adjust their outcrossing rate depending on their fitness as selfers and on the genetic quality of the offered partner.

Table II. 4. Multiple regression table with outcrossing rate as dependent and hatching rate of selfed eggs and individual heterozygosity of the second worm as independent variables (n = 17). See also Fig. II. 5.

	Estimate	Std Error	t Ratio	Prob>t	Std Beta
Mean Heterozygosity	-0.32	0.91	-0.35	0.73	-0.09
Hatching Rate	-2.09	0.94	-2.23	0.04	-0.4
Interaction	-27.51	9.79	-2.81	0.01	-0.69

DISCUSSION

The hatching success of eggs produced by the focal worm through selfing at sampling A significantly influenced its outcrossing rate when it later reproduced in a pair at sampling C: the lower the hatching success of selfed eggs at sampling A, the higher the outcrossing rate at sampling C (Fig. II. 5a). This result suggests that worms which have a high fitness gain by reproducing through selfing invest less in outcrossed offspring than worms which gain less by reproduction through selfing. This finding is in line with the predictions for this system mentioned in the introduction which point out that it may be advantageous for *S. solidus* to be adapted to selfing due to fluctuations in mating partner availability (see introduction (Pennycuick 1971b; Cheptou and Dieckmann 2002)). Worms may sometimes have to reproduce entirely by self-fertilization if they end up alone in the final host. Therefore worms which have a high fitness gain through self-fertilization should preserve these highly fit genotypes by selfing a large part of their eggs instead of breaking the linkage between genes by outcrossing all the eggs.

However, outcrossing rate at sampling C was significantly influenced not only by the hatching rate of selfed eggs produced by the focal worm at sampling A, but also by the individual heterozygosity of the added mating partner: the relationship between hatching rate of selfed eggs and outcrossing rate was very clear when the added mating partner had a high individual heterozygosity and less so when its heterozygosity was lower (Fig. II. 5b). How could the individual heterozygosity of the added mating partner influence the outcrossing rate? The reward that a focal worm gains from outcrossing its eggs may be twofold: (1) outcrossed offspring have a higher fitness than selfed offspring (see introduction (Wedekind *et al*. 1998; Wedekind and Rüetschi 2000; Christen *et al*. 2002)), (2) outcrossed offspring inherited new alleles from the added worm which had not been present in the focal worm before. These new alleles may increase the fitness of selfed offspring in future selfing events. Therefore worms may not only outcross because of the immediate fitness reward, but also because of new alleles that may be beneficial for future selfing events. This may exactly be the point where individual heterozygosity becomes important: more heterozygous individuals have a higher diversity of alleles and therefore the probability of inheriting a new beneficial allele for selfing is highest in the most heterozygous individuals.

If our interpretation of the observed results is correct, we have to postulate the presence of two prerequisites: (1) worms have to be able to remember their inbreeding history, in order to "know" whether their fitness to be gained through selfing is high or low, and (2) worms have to be able to measure the individual heterozygosity of a mating partner. This may appear

somewhat far-fetched at first sight, however, we still know very little about chemical communication in this species (which could be used to measure heterozygosity of mating partners) and even less about a possible kind of genomic imprinting with which inbreeding history might be measured. The only evidence pointing to the existence of a chemical recognition system comes from a mate choice experiment with these worms, where small worms preferred bigger worms, without direct contact (Lüscher and Wedekind 2002).

Two worms that meet for reproduction may not have the same optimal outcrossing rate. In our experimental set-up we could not measure the hatching success of selfed eggs of the second, added worm, because it was immediately in the presence of the focal worm and had no preceding period of self-fertilization. The experimental design was explicitly chosen that way. We wanted to simulate the situation where first one worm ends up in the birds gut and then some hours later a second worm from a freshly caught infected fish joins the first one, which already had reproduced alone. The different optima of paired worms concerning the amount of outcrossed offspring are expected to increase variance in our data, which renders our measurements conservative.

Worms which could dynamically adjust their egg production rate, would have a considerable advantage assuming that there is an optimal outcrossing ratio for every worm. Worms that happen to be alone in the final host could start reproducing by selfing, until they have reached their optimal amount of selfed eggs. If worms could then slow down egg production while waiting for a mating partner, they could still reach their optimal outcrossing and selfing rate, despite the fact that they had to reproduce alone first.

We tested this in two experimental situations: In the first situation a focal worm had to reproduce alone for 40 hours before a mating partner was added and in the second situation focal worms had to reproduce alone for 70 hours. From the first situation we took one egg sample from the focal worms reproducing alone (Fig. II. 1 sampling A) and two from the focal and the added worm reproducing in pairs (sampling B and C). From the second experimental situation we took two egg samples from the focal worms reproducing alone (sampling A and B) and one from the focal and the added worm reproducing in pairs (sampling C).

To test whether worms could adjust their egg production depending on whether a mating partner was present or not, we had to calculate expected egg numbers for each of the 6 sampling points mentioned above. We calculated these expected numbers from data of a previous experiment. We used those data, because in this previous experiment worms of similar sizes were reproducing in pairs during the whole 100 hours and the *in vitro* system was exactly the same. We could therefore calculate the expected egg production for each of the 6 sampling times based on data from worm pairs. This allowed us to test whether the egg produc-

tion of the worms in this study which have been in the two mentioned experimental situations differed from the expected values based on calculations of worm pairs. If worms could adjust their egg production to reach an optimal ratio between outcrossing and selfing we would expect the following results: worms reproducing alone for 70 hours should produce less eggs than expected at sampling A and B (reproducing alone) and more eggs than expected at sampling C (reproducing in pairs). For the focal worms where a second worm was added already after 40 hours a similar pattern should be visible: they should produce less eggs than expected at sampling A (reproducing alone) and more eggs than expected at sampling B and C (reproducing in pairs). However, the effects in the situation where the second worm was added to the focal worm already after 40 hours should be less pronounced than in situations where the second worm was added only after 70 hours. However our experimental test of these predictions found no evidence that worms could adjust their egg production depending on their experimental situation. The results rather suggest that egg production was completely additive, mainly depending on the weight of worms as was suggested by Wedekind *et al*. (1998) and Lüscher and Milinski (subm.). The influence of worm weight is clearly visible at sampling C, where equally sized worm pairs with the second worm added after 40 hours (Fig. II. 2. filled squares) produce more eggs than the worm pairs where the added worm had only half the size of the focal worm (open squares). Egg production rate may be mainly triggered by temperature in the final host (about 41°C) and a mechanism (besides temperature) a worm could apply to slow down or increase these physiological processes may be difficult to achieve. Similarly, egg size was not influenced by our treatment and decreased steadily over the three sampling times. This is in line with results from another study (Lüscher and Milinski subm.), and may be an adaptation to the risk of being excreted by the bird host before egg production has finished. Yet it remains unclear whether bigger eggs actually produce fitter offspring.

Bigger worms of *S. solidus* are expected to be of higher female quality than small worms, because female fecundity is strongly positively correlated with body size (Wedekind *et al*. 1998; Schärer and Wedekind 1999). The higher female quality and attractiveness of big worms compared to that of small ones was confirmed in a mate choice experiment, where small worms preferred high fecundity, potential sperm acceptors (big worms) (Lüscher and Wedekind 2002). Moreover, worms produce more outbred offspring with decreasing weight difference between two paired worms (Lüscher and Milinski subm.). In the present study we tested if we find the same result in worm pairs where one of the two worms had already reproduced alone by selfing before the pairs were formed. In the first treatment group the second worm had the same weight as the focal worm and in the second treatment group the second

worm had only half of the weight of the focal worm (See also Fig. II. 1). Outcrossing rates of worm pairs increased from sampling B to sampling C (Fig. II. 3a) not only in the mean outcrossing rate, but also in the numbers of pairs reproducing through outcrossing. The selfing rates of the focal worms decreased from sampling B to sampling C, while the selfing rates of the second worm were low at both sampling times (Fig. II. 3b). The weight of the second worm (either same weight or half the weight of the focal worm) had no significant influence on the selfing rates of the two worms in a pair. This suggests that lower outcrossing rates of pairs due to a large weight difference within pairs may not be relevant in this experimental situation. Due to the preceding period of obligate selfing the focal worm may accept any partner to outcross irrespective of the partner's weight.

The rate of egg production is not the only trait that worms could adjust to reach an optimal ratio between outcrossing and selfing. Worms that first have to reproduce alone could compensate the obligate selfing period by fully outcrossing as soon as a mating partner becomes available. To test this we looked at the outcrossing rates in the first 30 hours of reproduction as worm pairs. We expected worm pairs where the focal worm had to reproduce alone for 70 hours to produce more offspring through outcrossing (sampling C) compared to worm pairs where the second worm had been added already after 40 h (sampling B). There was neither a significant difference in the number of worm pairs actually reproducing through outcrossing, nor was there a difference in the outcrossing rate. This suggests that worms do not dynamically adjust their outcrossing rate depending on the amount of selfed eggs they have already produced.

Environmental fluctuations leading to unpredictable density fluctuations in definitive hosts may be the selective pressure which can lead to adaptive mixed-mating in *S. solidus*. Here we tested and showed for the first time that individuals adaptively adjust their outcrossing rate depending on their fitness as selfers.

ACKNOWLEDGEMENTS

We thank Wolf-Rüdiger Wulff and Sybille Liedtke for lab assistance and several people from the Department of Evolutionary Ecology at the MPI Plön generously helped with catching and dissecting sticklebacks. We thank August Hämmerli for helpful comments.

Brackish water bay near Neustadt (Schleswig-Hostein), where infected sticklebacks were caught (Photo by G. Augustin)

Comparative genetic variability in a natural vertebrate - endoparasite system

SUMMARY

Few studies have investigated the genetic structure of both hosts and parasites at the population and the individual level. We investigated the genetic variability of the three-spined stickleback (*Gasterosteus aculeatus*) and its helminth parasite, the cestode *Schistocephalus solidus*. Population genetic parameters of hosts and parasites in two consecutive years were estimated using microsatellite markers. Parasites showed a significant heterozygote deficiency, resulting in a mean selfing rate (S) of 0.27. This high selfing rate reflects this hermaphrodites' reproductive mode, namely mixed-mating (i.e. reproduction through selfing and outcrossing). In the host, individual heterozygosity did not differ between infected and uninfected fish. Even though the body condition of infected fish was severely reduced, there was no relation between individual heterozygosity of infected fish and its condition. However, condition in uninfected fish correlated significantly with individual heterozygosity. This suggests that there is a general heterozygote advantage in terms of fitness (e.g. higher fecundity). There was however, no evidence for a heterozygote advantage reducing the susceptibility to infection with *S. solidus* in more heterozygous fish.

INTRODUCTION

Parasites have long been considered to play an important role in the evolution of their hosts. Parasites are under constant selection to increase the ability to infect hosts, and hosts under constant selection to avoid parasitism. This coevolutionary arms race can favour genetic recombination (sexual reproduction) which allows fast adaptation to new environments (the red queen hypothesis (Jaenike 1978; Hamilton 1980; Bell 1982)). However, coevolutionary dynamics cannot be understood without a clear view on the genetic variability of wild host and parasite populations. Major aspects for further understanding are: (1) Models predict that the relative rate of migration of hosts and parasites determines the outcome of local adaptation of hosts to parasites (Gandon *et al*. 1996; Gandon *et al*. 1998). (2) Environmental fluctuations acting on population dynamics can have considerable influence on the breeding system of hermaphrodite species. Cheptou and Dieckmann (2002) showed that mixed-mating (i.e. reproduction through both self-fertilization and cross-fertilization) can become evolutionarily stable under fluctuating population densities. (3) Several studies have shown that individual fitness partially depends on individual heterozygosity (i.e. individual inbreeding) (David 1998) suggesting that any reduction in individual heterozygosity is indicative for selectively relevant genes as well (Hedrick 1999; Sunnucks 2000). Resistance of hosts towards parasites has also been shown to be a function of individual inbreeding (Coltman *et al*. 1999). Similarly the virulence of parasites might be related to individual inbreeding (Christen *et al*. 2002; Christen and Milinski subm.).

Although knowledge of the genetical structure of populations is important for the understanding of their ecology and evolution, only few studies have investigated the genetic structure of both hosts and parasites at the same time (Mulvey *et al*. 1991; Dybdahl and Lively 1996; Delmotte *et al*. 1999; Jobet *et al*. 2000). In this paper we investigate the genetic structure of the stickleback *Gasterosteus aculeatus* and its helminth parasite *Schistocephalus solidus*. The life cycle of this hermaphroditic endoparasite includes two intermediate hosts (a cyclopoid copepod and the three-spined stickleback) and one final host (any fish eating bird). Sticklebacks are parasitised by eating infected copepods and infection is causing severe fitness reduction in the fish (Arme and Owen 1967; LoBue and Bell 1993). Prevalence of *S. solidus* in sticklebacks is often high, sometimes up to 100% (Dick 1816; Smyth 1947; Hopkins and Smyth 1951; Arme and Owen 1967). The distribution of *S. solidus* in sticklebacks is overdispersed with most infected fish containing only one worm (Smyth 1994). Reproduction of the parasite takes place in the gut of the final host by cross- or self-fertilization. In the final host, up to around 300 adult worms per individual host have been observed (Nybelin 1919; Vik 1954), but these numbers probably

represent extreme cases. Low densities in their final host due to strong fluctuations in prevalence between years (Pennycuick 1971b), may sometimes force them to self-fertilize as last resort.

This parasites life cycle as well as population fluctuations between years and the mode of reproduction may have an impact on host and parasite genetic diversity. In this study we investigated host and parasite population genetic diversity as well as individual host and corresponding parasite genetic heterozygosity using 6 highly polymorphic microsatellite markers for the sticklebacks (Largiadèr *et al*. 1999) and 7 for *S. solidus* (Binz *et al*. 2000). 3 of the 7 markers used for *S. solidus* are new and first characterised in this paper.

MATERIALS AND METHODS

Sampling of hosts and parasites

Sticklebacks were caught during September and October 2000 and 2001 in a brackish water bay near Neustadt (Schleswig-Holstein), Germany. Shoaling sticklebacks were dip-netted from a 1 km long shore stretch. The fish were kept in 194 l tanks for six weeks in 2000 and for five month in 2001 before dissection. Fish were killed by a cerebrospinal cut, weighed and checked for infection status. If a fish was infected, the worm was aseptically removed from the stickleback. The worms were weighed to the nearest mg and stored in 70 % Ethanol. In 2001 each worm was assigned to its host fish to allow for linking parasite data to corresponding host data.

DNA extraction and microsatellite amplification

DNA extraction of both fish and worms followed the Chelex © extraction method (Walsh *et al*. 1991). 1 mm^3 of worm tissue or a 1-2 mm piece of spine, respectively was mixed in a 100 µl 5 % Chelex © suspension and stored at –20 °C. 1µl of Proteinase K solution (Roche) was added to the sample in suspension and incubated at 55 °C for 15 min followed by another 15 min at 100°C. Samples were placed on ice, centrifuged for 30 seconds at 2000 rpm, and stored at –20°C.

Four microsatellite loci SsB6, SsA22, SsB24 and Ss25 of the cestode *S. solidus* were amplified according to the protocol given in Binz *et al*. (2000) and Lüscher and Milinski

Table III. 1. Characterization of three new microsatellite loci in the tapeworm *S. solidus*

Locus	Primer (5' to 3')	PCR product size of the sequenced allele (bp)	Observed allele numbers	Observed allele sizes (bp)	Repeat structure	GenBank accession no.
SsCA76	F: GGC AAC CTT CCA ACC GC R: CAC TCG GGG CAA TAA AAG G	210	6	166 - 180	$(CA)_{27}$	AY158698
SsCA89	F: CAC TCT CTT CTC CTC GCC C R: CAA ATG CTA ACA CAC TCG GGG	167	11	145 - 191	$(CA)_{20}$	AY158699
SsCA77	F: ATT CAC AAA CCG CAT CAG TG R: TGT GGA GTA AGT TTT CGG GTG	121	21	95 - 153	$(CA)_{13}$	AY158700

(subm.). To enhance the resolution needed for this study we isolated and characterized three new microsatellite loci. Methods for isolation and later amplification of tapeworm microsatellite loci followed exactly the protocol given in Binz *et al*. (2000). All three loci were polymorphic and their characteristics are summarized in Table III. 1.

To asses the genetic variability of sticklebacks we used six microsatellite loci (GAC1097, GAC1125, GAC2111, GAC4170, GAC5196, GAC7033) developed by Largiadèr *et al*. (1999) and amplified them according to the protocol given in Reusch *et al*. (2001).

Statistical analysis

The data were analysed with the JMP 4.0.4. statistics package (Sall and Lehman 1996) where not specified differently. Fish condition was calculated as

$$c_F = \frac{m_F}{l_F^{\,3}}$$

where m_F is the weight of the fish and l_F the corresponding length (Pennycuick 1971a; Bolger and Connelly 1989). Individual heterozygosity of fish and worms were expressed as proportions of loci heterozygous and therefore arc sine transformed.

Genetic data analysis

Basic data on the genetic composition (microsatellite allele frequencies, no. of alleles/locus, heterozygosity, Hardy-Weinberg-Equilibrium (HWE)) were computed using the software GENETIX (Belkhir *et al*. 1998). Significance of the F-Statistics was determined by permutating the data set 1000 times. We tested for linkage disequilibrium according to the method of Black and Kraftsur (1985) implemented in GENETIX.

Analysis of molecular variance

In order to compare the variance components contributed by infection status of the fish (infected/uninfected) and sampling year (2000/2001), total molecular variance was subdivided using nested analysis of molecular variance AMOVA (Weir and Cockerham 1984; Excoffier *et al*. 1992) using the software ARLEQUIN v 2.001(Schneider *et al*. 1997).

RESULTS

Characteristics of the parasite

All loci used showed considerable amounts of polymorphism (alleles / locus : 16.14).No significant linkage could be detected between any locus pair (P > 0.05 in all comparisons, Bonferroni correction not applied). The parasite index (worm weight divided by fish weight; (Arme and Owen 1967; Pennycuick 1971b)) was not significantly influenced by the individual heterozygosity of the worms (Fig. III. 1, n = 42, t = 0.93, r = 0.15, p = 0.36). This is not surprising, because worm

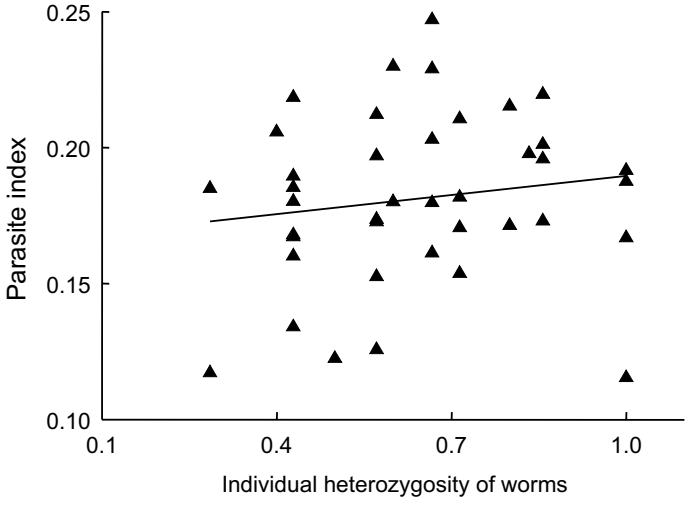


Fig. III. 1. Parasite index plotted against the individual heterozygosity of worms. The figure gives the regression line.

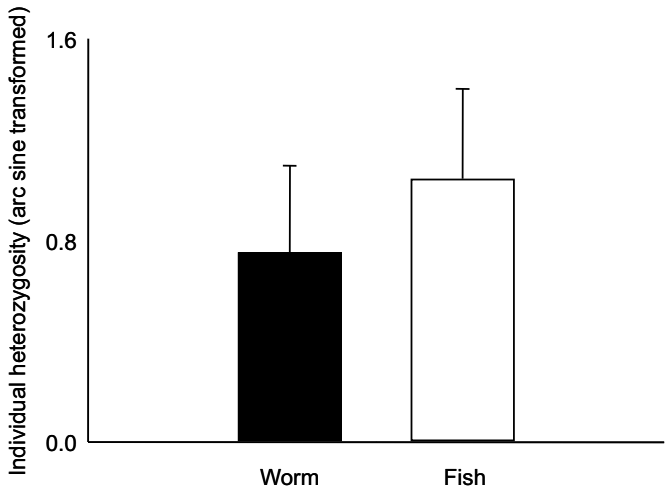


Fig. III. 2. Mean individual heterozygosity (± s.d.) of fish and worm.

Fig. III. 3. Mean condition (± s.d.) of infected and uninfected fish.

weight is strongly correlated with fish weight ($n = 42$, $t = 12.95$, $r = 0.90$, $p < 0.001$; (Lüscher and Milinski subm.)).

In both years we found a significant positive deviation for *S. solidus* populations from HWE (year 2000: $n = 60$, $F_{IS} = 0.17$, $p < 0.05$; year 2001: $n = 45$; $F_{IS} = 0.14$, $p < 0.05$). There was no significant difference in genetic variability between the two sampling events ($F_{ST} = 0.003$, p = n.s.).

Characteristics of the host

Microsatellite loci of *G. aculeatus* were slighty more polymorph than the loci used for *S. solidus* (alleles / locus: 22.5). Across all groups of sticklebacks we found a weak positive deviation from HWE (year 2000, uninfected: $n = 30$, $F_{IS} = 0.034$, $p = 0.09$; infected: $n = 33$, $F_{IS} = 0.029$, $p = 0.11$; year 2001, uninfected: $n = 89$, $F_{IS} = 0.073$, $p < 0.05$, infected: $n = 42$, $F_{IS} = 0.081$, $p < 0.05$). This finding is in line with previous study on *G. aculeatus* populations which show a weak positive deviation from HWE for all populations examined in North Germany (Reusch *et al*. 2001).

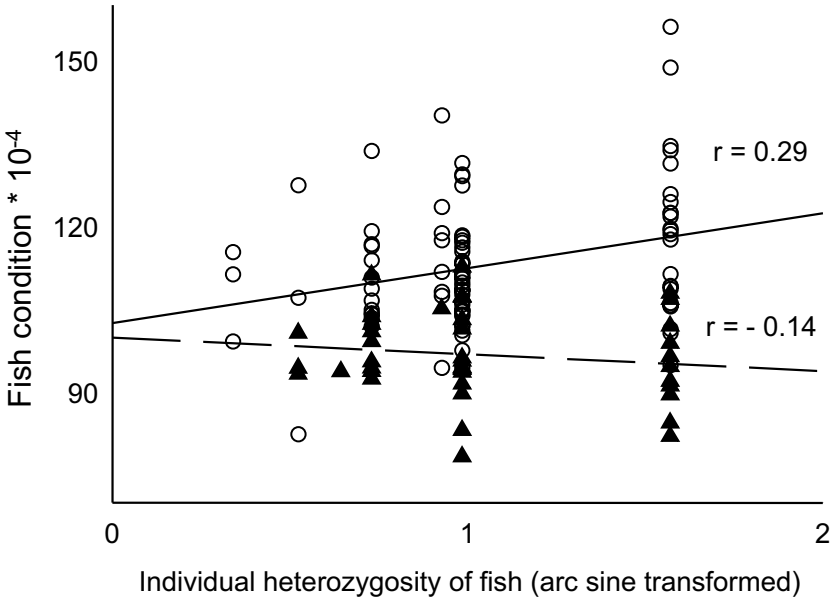


Fig. III. 4. Condition of fish plotted against their individual heterozygosity. Open circles = uninfected fish and continuous regression line; closed triangles = infected fish and dotted regression line. Pearson correlation coefficients are shown.

Interaction of parasite and host genetics

The worms had a significantly lower genetic diversity than the fish hosts they infected (Fig. III. 2. Paired t-test: n = 40, t = - 6.72, p < 0.001). Genetic diversity did not significantly differ between infected and uninfected sticklebacks as revealed in a nested AMOVA (Table III. 2). However, the condition of infected fish was significantly lower than the condition of uninfected fish (Fig. III. 3; MANOVA with infection status and individual heterozygosity; effect of infection status: n = 128, $F_{1, 124}$ = 65.91, p < 0.001). Moreover, the influence of individual heterozygosity on fish condition differed depending on infection status of the fish (Fig. III. 4;

Table III. 2. Analysis of molecular variance AMOVA.

Effect	d.f.	% variation	P
Among infection status	1	0.02	n.s.
Among years / within infection status	2	-0.01	n.s.
Within populations	384	99.99	< 0.0001

MANOVA with infection status and individual heterozygosity; infection status X individual heterozygosity: n = 128, $F_{1, 124} = 5.10$, $p < 0.03$). Individual heterozygosity alone had no significant effect on fish condition (MANOVA with infection status and individual heterozygosity; effect of individual heterozygosity: n = 128, $F_{1, 124} = 1.44$, $p = 0.23$). Following this analysis we tested the correlations of individual heterozygosity on fish condition by splitting the data set in to the two infection statuses (infected/uninfected). We did not find a significant correlation in infected fish (Fig. III. 4. n = 40, r = - 0.14, t = - 0.87, p = n.s. Bonferroni corrected), but we found a significant correlation in the uninfected fish (Fig. III. 4. n = 88, r = 0.29, t = 2.78, p < 0.02 Bonferroni corrected). This suggests that very heterozygous fish generally have a higher fitness in terms of condition compared to more homozygous fish.

DISCUSSION

Parasite populations showed an excess of homozygotes, as demonstrated by the significant F_{IS} for the years 2000 and 2001. From these values we can estimate the selfing rates (S) using the classical formula: $F_{IS} = S/(2 - S)$ (Hartl and Clark 1988; Jarne and Lagoda 1996). Which gives a selfing rate of 0.29 for the year 2000 and a selfing rate of 0.25 for the year 2001. Positive deviations from HWE can result from processes other than selfing. Especially the presence of Null alleles will result in elevated values. The multilocus F_{IS} values determined here did however not vary markedly when we jackknifed over loci (jackknifed F_{IS} range: 0.20 - 0.26), meaning that there was no single locus with Null alleles contributing a major proportion. Rather, all loci contributed equally towards total F_{IS}. This suggests that *S. solidus* individuals were reproducing through both self-fertilization and cross-fertilization. Some worms may have had to self-fertilize their eggs when ending up alone for reproduction in the final host. However, reproducing through both outcrossing and selfing may be adaptive even when a mating partner would be available. Recently, Cheptou and Dieckmann (2002) have shown that environmental fluctuations leading to fluctuating population densities can favour the evolution of mixed-mating systems (i.e. reproduction through selfing and outcrossing). The deviation from HWE found for *S. solidus* is in line with the results of an experimental study on this parasite. *S. solidus* individuals were partly reproducing through selfing even when they were provided with a mating partner (Lüscher and Milinski subm.). The finding that *S. solidus* had a lower individual genetic diversity than its fish host (Fig. III. 2) is probably reflecting the differing breeding systems of the two species. The mixed-mating system exhibited by these parasites may also blur the relationship between individual heterozygosity of worms and their fit-

ness (parasite index) (Fig. III. 1). Offspring resulting from several generations of selfing would be of very low individual heterozygosity, but may be as fit as the mean of the population due to purging of deleterious recessives (Charlesworth and Charlesworth 1998).

In the host we found a significant correlation between individual heterozygosity of uninfected fish and their condition. The genetic basis of body condition is complex with influences of numerous genes spread over all chromosomes. Therefore, the number of homozygous deleterious recessives will negatively correlate to genome-wide heterozygosity and thus a general heterozygote advantage appears. In sticklebacks fish condition is a measure of individual fitness, because males in better condition are preferred by females (Milinski and Bakker 1990) and females in better condition produce more eggs (Wootton 1976). This suggests that fish with high individual heterozygosity may produce relatively more offspring, than do more inbred ones. Interestingly we did not find such a heterozygosity - fitness relationship in infected fish (Fig. III. 4), nor was there an overall difference in individual heterozygosity between infected and uninfected fish (Table III. 2). The body condition - heterozygosity relationship maybe blurred by the varying dates on which individual fish were infected. However, the condition of infected fish was severely reduced compared to uninfected ones (Fig. III. 3). In extreme cases *S. solidus* individuals can grow nearly twice as heavy as their hosts, because they are more efficient in energy transformation (Clarke 1954; Walkey and Meakins 1970).This shows the severe consequences of infection by *S. solidus*, as even superior host genotypes (i.e. the ones with highest heterozygosity) do not gain from their beneficial genetic preposition. Differences in parasite susceptibility might rather be influenced by the polymorphism in immunologically relevant genes as the MHC (Major Histocompatibility Complex) (Kurtz *et al*. subm.), which is initiating specific immune responses against pathogens and parasites in vertebrates by presenting antigens to T cells (Klein 1986; Edwards and Hedrick 1998). Variability in this restricted chromosomal region is not correlated to genome-wide heterozygosity in sticklebacks (Wegner *et al*. subm.), and a general heterozygote advantage can therefore not be expected to influence resistance to a specific parasite.

ACKNOWLEDGEMENTS

We thank Wolf-Rüdiger Wulff, Sybille Liedtke and Catha Schmuck for lab assistance and several people from the Department of Evolutionary Ecology at the MPI Plön generously helped with catching and dissecting sticklebacks. We thank Thomas Binz for the introduction to microsatellite development. We thank Manfred Milinski and August Hämmerli for helpful comments.

Freshly dissected heavily infected *G. aculeatus*.

Schistocephalus solidus: a molecular test of premature gamete exchange in the intermediate host *Gasterosteus aculeatus*

SUMMARY

We tested the hypothesis that the cestode *Schistocephalus solidus* is capable of premature gamete exchange as a plerocercoid in the last intermediate stickleback host. The existence of such a reproductive mode is suggested by the highly advanced gonadal development in the plerocercoid and the large fitness gain of outcrossing. In addition, eggs from selfing cestodes have a higher hatching rate when the cestode originated from a doubly infected stickleback than when it came from a singly infected fish. We hatched eggs from 10 singly breeding cestodes that originated from doubly infected sticklebacks with the prediction that some should be outcrossed and share alleles with both the breeding cestode and the second cestode in the coinfection if the hypothesis is correct. However, all of the 430 tested larvae matched only the alleles of the breeding cestode. It is therefore very unlikely that *S. solidus* engaged in gamete exchange in its fish host. We suggest an alternative hypothesis to explain the higher hatching rate of eggs produced by cestodes from doubly infected fish as compared to those from single infections.

MAIN TEXT

The indirect life cycle of the trophically transmitted pseudophyllidean cestode *Schistocephalus solidus* Müller, 1776 involves three larval stages (Smyth and McManus 1989). Eggs pass into water with guano from the final bird host and hatch into free-swimming coracidia. These develop into procercoids in the first intermediate host, a cyclopoid copepod, while the last larval stage, the plerocercoid, develops in the three-spined stickleback, *Gasterosteus aculeatus*. When the plerocercoid stage is ingested by the final bird host the short-lived adult stage develops within a few days in the intestine and continuously produces its lifetime production of eggs over a period of a few weeks.

S. solidus is a simultaneous hermaphrodite capable of both selfing and outcrossing. However, eggs produced by outcrossing cestodes have a higher hatching rate than those produced by selfing ones (Wedekind *et al*. 1998; own observation). This difference in hatching rate is likely to translate into a substantial fitness benefit of outcrossing.

Multiple infections of *S. solidus* often occur in the intermediate fish host (Vik 1954). Cestodes from doubly infected fish produce the same size and number of eggs as similar sized ones from singly infected fish. However, the hatching rate of eggs from cestodes originating from doubly infected fish is significantly higher, even when they are bred singly (Fig. IV. 1b, method described later). Considering, in addition, the high fitness benefit of outcrossing and the fact that *S. solidus* is unusual among cestodes because of its advanced gonadal development at the plerocercoid stage (Smyth 1946), we hypothesise that *S. solidus* may be capable of premature gamete exchange already in the fish host.

It is of great interest to test this hypothesis because premature gamete exchange would have important consequences for an understanding of this cestode's sexual adaptations in addition to the evolution of its complex life cycle.

The hypothesis of premature gamete exchange in the fish host predicts that singly breeding cestodes originating from multiply infected sticklebacks should produce a certain proportion of outcrossed offspring. We tested this prediction by hatching eggs from singly breeding cestodes that originated from doubly infected sticklebacks. We extracted DNA from the coracidia and from both cestodes in the doubly infected fish, and then compared their allelic composition using the molecular markers developed by Binz *et al*. (2000).

Specifically, the sticklebacks were caught in the late summer of the year 2000 in Northern Germany in the brackish water lagoon of Neustadt. The fish were killed by a cerebrospinal cut and the cestodes carefully removed from the body cavity. One of the two cestodes in a doubly infected fish was randomly assigned to breeding while the other was fixed

in 70% ethanol for later DNA extraction. For each of the 10 cestodes thus assigned to breeding a match of similar weight (max weight difference: 4 mg, weight range: 132-333 mg) was selected among cestodes from singly infected sticklebacks. These 20 cestodes were then allowed to breed singly in an *in vitro* system that simulates the intestinal environment of the bird host. This *in vitro* system has been modified and improved from Smyth (1954), Wedekind *et al*. (1998) and Schärer and Wedekind (1999). Shortly, the cestode is introduced to a bag of nylon net, which is then suspended in the lid of a 250 ml polypropylene bottle filled with a culture medium resembling the chemical environment in the bird intestine. After introducing the worms, the bottles were kept at 40 °C for 8 days. The eggs that the cestode produces pass through the nylon net and could be collected from the bottom of the bottle.

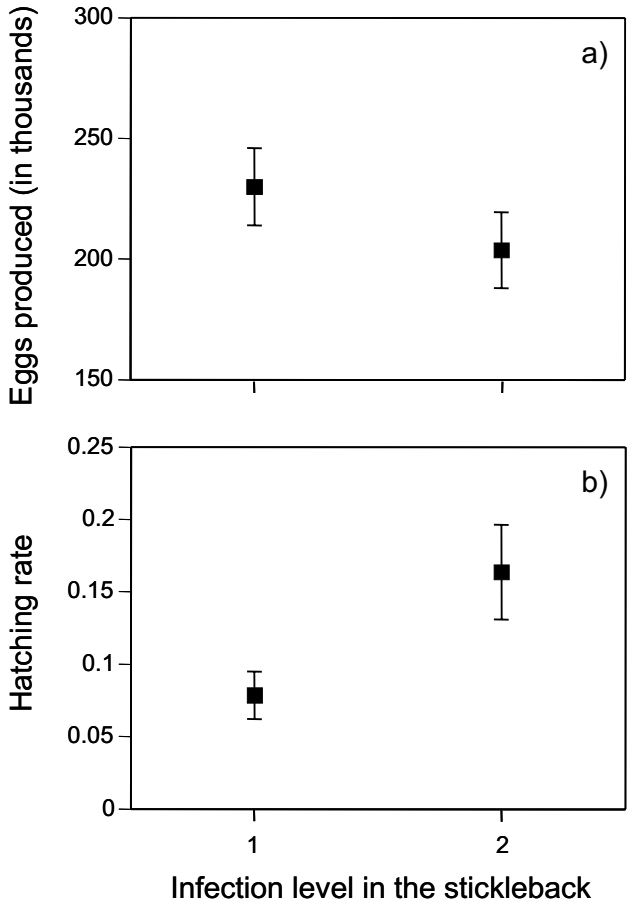


Fig. IV. 1: Number (a) and hatching rate (b) of eggs produced by singly breeding S. solidus that originate from either singly or doubly infected sticklebacks. ± 1 SE is indicated with vertical lines. N=10 for each infection level.

We counted the eggs by applying a known proportion from each sample to a petri dish. 15 areas, each of 0.605 mm^2, were randomly chosen to be counted in each petri dish. The eggs within each area were counted with the image analysis program NIH Image (http://rsb.info.nih.gov/nih-image/), and we estimated the total number of eggs within each petri dish by extrapolation assuming an even distribution of eggs. Using the same program, the mean size of the eggs was calculated by measuring the length and width of 50 randomly chosen eggs, assuming an ellipsoid shape. While there was no significant difference in the size of the eggs produced by cestodes from singly and doubly infected fish (two-tailed paired t-test: ts=0.390, df=9, P=0.706), cestodes from singly infected fish produced close to significantly more eggs than cestodes from doubly infected fish (two-tailed paired t-test: ts=2.169, df=9, P=0.058, after Bonferroni correction for multiple testing: P=0.174, Fig. IV. 1a).

In order to determine the hatching rate, a sample of the eggs were hatched by incubating them for 10 weeks at 20 °C with alternating 12 hours light and 12 hours darkness. The hatching rate was determined by noting for 100 randomly selected eggs whether they had hatched (i.e. operculum open or removed (Swiderski 1994)) or not. The hatching rate of the eggs produced by cestodes originating from double infections was significantly higher than that of eggs produced by cestodes from singly infected sticklebacks (two-tailed paired t-test: ts=2.922, df=9, P=0.016, after Bonferroni correction for multiple testing: P=0.048; Fig. IV. 1b).

A different sample of eggs from the cestodes from doubly infected fish was used to hatch coracidia for DNA extraction. These eggs developed at 20 °C in darkness for three weeks, after which they were exposed to a period of 8 hours light and 8 hours darkness. Hatching could then be induced by a short exposure to light. We collected 46 coracidia from 9 of the clutches while a low hatching success only allowed us to collect 16 coracidia from the 10th clutch. DNA was extracted from the coracidia and from the worms that had been breeding and those that had been fixed in ethanol (see Binz *et al*. (2000) for a description of the method). For each pair of cestodes that had been in the same fish we compared the alleles at two highly variable loci with the composition of the coracidia from the one of the two cestodes which had been allowed to breed.

In all 10 samples, the two cestodes that had been together in the fish had clearly distinguishable alleles. Furthermore, in all cases, the alleles of the coracidia only matched the composition of the breeding cestode. Thus, all of the 430 tested coracidia were the result of selfing. It therefore seems very unlikely that *S. solidus* engaged in premature gamete exchange in its fish host.

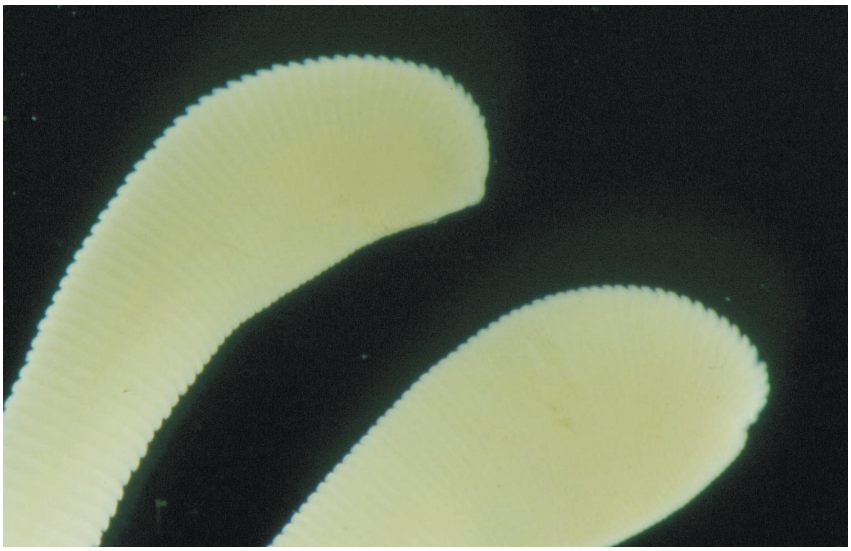
This result lends strong support to earlier conclusions based on histological examinations of plerocercoids of *S. solidus*. Although the gonads reach a very advanced developmental stage in the plerocercoid they appear immature (Smyth 1946), and an increase in temperature equivalent to that in the final host seems imperative for normal gametogenesis (i.e. meiosis) to take place (Smyth 1952).

The negative result begs an alternative explanation for the higher hatching rate of cestodes originating from doubly infected fish. In a recent study of *S. solidus*, Schärer *et al*. (2001) showed that this cestode is likely to experience sperm competition in its final bird host; the amount of sperm received from mating partners increased when the size of the mating group in the final host increased. Thus, if the number of coinfective cestodes in the fish host is a good predictor of the size of the mating group in the final host then cestodes may benefit from investing relatively more in the male part of the reproductive system in order to enable a larger sperm production. The larger amount of sperm produced per egg by cestodes from

double infections could enable them to fertilise a larger proportion of their eggs when breeding alone than cestodes from single infections, resulting in a higher hatching rate of eggs produced by the former (Fig. IV. 1b). The tendency of cestodes from doubly infected fish to produce a lower amount of eggs than those from singly infected fish (Fig. IV. 1a) suggests that the hypothesised increase in male allocation could happen at the expense of female function.

ACKNOWLEDGEMENTS

We thank M. Milinski, J. C. Koella, J. Kurtz and M. Kalbe for useful comments on earlier drafts of the manuscript.



The anterior part of two *S. solidus* individuals.

Hermaphroditic endoparasites, the ideal model organisms for the investigation of mating system evolution

Fisher (1941) showed that a selfer gene which is introduced into an outcrossing population at low frequency will have a 50% fitness advantage and therefore spread rapidly. This would soon lead to complete inbreeding if it were not counteracted by inbreeding depression. Most models on mating system evolution predict either one of two extremes to be evolutionarily stable: complete selfing or complete outcrossing (e.g Lande and Schemske 1985; Charlesworth *et al*. 1990). Which of the two is predicted by a model depends mainly on whether inbred progeny are assumed to have a disadvantage through inbreeding depression of less or more than 50 % compared to outcrossed progeny. In contrast to these predictions however, data from wild populations show that many hermaphroditic plants and animals are reproducing through a mixture of both, selfing and outcrossing (mixed-mating) (Lande and Schemske 1985; Charlesworth *et al*. 1990; Jarne and Charlesworth 1993). This is why the conditions under which mixed-mating could also be evolutionarily stable has come into focus of evolutionary biologists in the past decades.

Fryxell (1957) estimated that 10 % of flowering plant species reproduce by a mixture of selfing and outcrossing. Although most studies on intermediate selfing report rates near zero and one rather than 0.5 (Schemske and Lande 1985), several species seem to maintain mixed-mating as a stable system (e.g. *Impatiens capensis* (Schemske 1978), *Lamium amplexicaule* (Lord 1979) or *Viola canina* (Darwin 1877). In animals, hermaphroditism is probably more prevalent than usually assumed considering that 21 out of 32 phyla contain hermaphroditic species (Jarne and Charlesworth 1993). Data on outcrossing rates for hermphroditic animals is still scarce (Bell 1982). The only relatively well documented cases are those of pulmonates (Jarne *et al*. 1993), ascidians (Ryland and Bishop 1990; Bishop and Ryland 1993) and platyhelmithes (Trouvé *et al*. 1996; Nollen 1997; Lüscher and Milinski subm.), while for a rich variety of species selfing and outcrossing rates are still unknown (e.g. cnidarians, sponges, brachiopods, bryozoans annelids, isopods).

Traditionally, evolutionarily stable mixed-mating systems are predicted only for the following special cases: (1) when both self-fertilization and biparental inbreeding occur simultaneously (Uyenoyama 1986), and (2) when selfed progeny are less well dispersed than outbred progeny (Holsinger 1986). Yet, these models still leave a diversity of mixed-mating systems unexplained, because these systems do not fit the rather specialized assumptions. A major limitation of the traditional models is their simplified treatment of population dynamics.

Inbreeding depression lowers population growth rates and thus must be expected to have an impact on population dynamics (Halley and Manasse 1993; Saccheri *et al*. 1998). A recent model by Cheptou and Dieckmann (2002) now assumes explicitly fluctuating populations, which is in contrast to the traditional approaches. They could show that both, deterministic and stochastic environmental fluctuations can also lead to mixed-mating systems, which are evolutionarily stable under a wide range of conditions. This has brought up the need for suitable study organisms. Fluctuating population sizes are common in nature and are expected to be influenced mainly by density and stochastic variation in environmental conditions (Murdoch 1994; Saether 1997).

Promising organisms to test this new hypothesis are the large groups of endoparasitic trematodes (ca 6500 species) and cestodes (ca 3000 species), because (a) most of them are simultaneous hermaphrodites, capable of both selfing and outcrossing (Smyth 1994), (b) parasite densities in the final host where reproduction takes place are known to fluctuate stochastically (Shaw and Dobson 1995; Shaw *et al*. 1998), and therefore individuals may sometimes be forced to self-fertilize their eggs due to the lack of mating partners, (c) for several species *in vitro* systems and lab cycles have been established (for about 30 trematode species and 30 cestode species) (Smyth and McManus 1989; Smyth 1994) which allow for experiments under controlled conditions with manipulations of traits of interest such as mating group size, weight of mating partners or inbreeding history. The following is a list of species for which parasitologists have established laboratory conditions to cover the whole life cycle. For the reasons mentioned above they seem to constitute promising candidates for the study of mating system evolution in the future.

(1) *Ligula intestinalis*, a cestode with two intermediate hosts (copepod and fish) and one final host (bird) (Smyth 1994). The species is common all over the world, with prevalences varying from year to year (e.g. Szalai *et al*. 1989). Reported numbers of worms in the final host are rather low (1-3) (Dubinina 1966), and therefore worms have to self-fertilize sometimes due to a lack of mating partner. *In vitro* systems (Smyth 1990) and lab cycles (Orr and Hopkins 1969) are well established and worms start to produce eggs readily after 72 hours at 40°C (either in the *in vitro* system or in the duckling, which can serve as final host in the lab).

(2) *Schistocephalus solidus* is the smaller relative of *Ligula intestinalis*. *In vitro* systems for this species (Smyth 1946) have been used with remarkable success for experiments in evolutionary ecology (Wedekind *et al*. 1998; Schärer and Wedekind 1999; Schärer *et al*. 2001; Lüscher and Wedekind 2002). The parasite is common only on the north-

ern hemisphere, as it is rather specific on its second intermediate host, the three-spined stickleback (*Gasterosteus aculeatus*).

(3) Although in *Hymenolepis diminuta* a large number of insects can act as intermediate host, the flour beetle *Tribolium confusum* serves as convenient laboratory host. The final host in the lab is the albino rat, while in the wild also other rodentia serve as hosts. Besides the well established and commonly used lab cycle for *H. diminuta* (Arai 1980), remarkable success has been achieved with *in vitro* cultures: the entire life cycle of this species can be completed *in vitro* (Arme 1987; Smyth 1990). This cosmopolitan species has long been used as a model organism in medical and veterinary research. Therefore vast information exists on this species life cycle, development biology, morphology and biochemistry (reviewed in (Arai 1980)), which is extremely valuable when designing experiments in evolutionary ecology.

(4) The cestode *Echinococcus granulosus* has one intermediate host (sheep or other grazing mammals) and one final host (dogs or other canidae). *Echinococcus sp*. is of great medical importance because its larvae are causing the hydatid disease in humans. However, *E. granulosus* is also an unusually interesting species from the evolutionary biologists point of view, as it serves as a superb model for the study of asexual / sexual differentiation *in vitro* (Smyth 1987). In the final host either many thousands of genetically identical individuals can be produced by asexual budding of a single larvae or the larvae directly differentiate in sexually mature adult worms. Unfortunately this parasite is a human pathogen, which renders its establishment in the lab and thus in experiments difficult.

(5) The life cycle of the trematode *Echinostoma caproni* includes three successive hosts (Huffmann and Fried 1990). A mollusc serves as first intermediate host, while the second host can be again a mollusc or an amphibian. The final host is a bird or a mammal. In the laboratory, *E. caproni* is routinely cycled through two hosts: the snail *Biomphalaria pfeifferi* and the mouse (Huffmann and Fried 1990). Experimental work using radioactive isotopes (Nollen 1983; Nollen 1997) and allozymes (Trouvé *et al*. 1996) respectively, showed that worms self and cross-fertilize when they mate in groups. This host-parasite system seems promising to test in further experimental situations the causes for varying selfing and outcrossing rates.

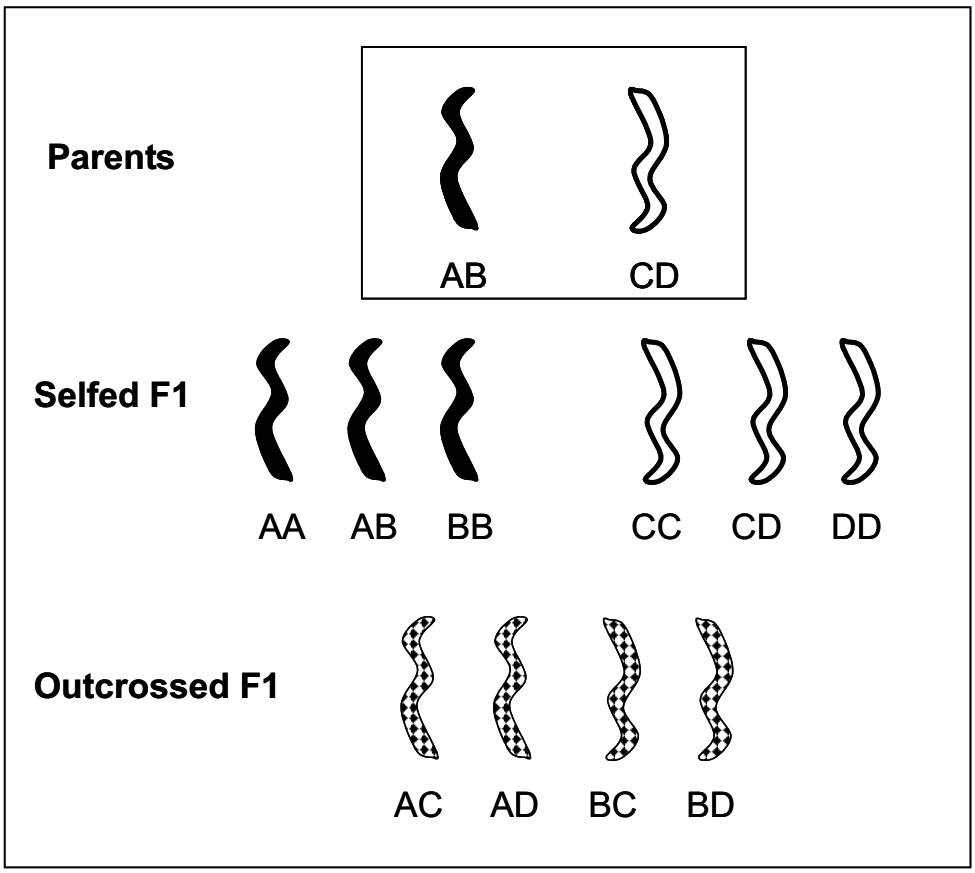


Fig. V. 1: Offspring of two hermaphrodite parents sharing no allele for a particular microsatellite locus. Every possible genetic combination in the offspring can be unequivocally assigned to one (selfing) or both of the parents (outcrossing).

Recent rapid development of molecular techniques will further facilitate research in this field, since fine scale genetic differences in offspring (i.e. selfed or outcrossed) can now be detected easily (Queller *et al*. 1993; Jarne and Lagoda 1996). Several genetic markers have been widely used in population genetic studies. Microsatellites, single sequence repeats on the DNA, possess a great advantage in terms of polymorphism compared to allozymes or RAPD's (random amplified polymorphic DNA) (Jarne and Lagoda 1996). For example in *S. solidus* no polymorphism could be detected with allozymes, suggesting reproduction by 100 % self-fertilization (McManus 1985), while microsatellites were highly polymorphic and revealed an outcrossing rate of about 0.7 (Lüscher and Wegner subm.). Especially if experimenters are interested in the genetic source of an offspring, highly variable markers are unavoidable.

If two hermaphrodites meet for reproduction, their offspring can either stem from self-fertilization by one or the other parent, or they can result from cross-fertilization between the two parents. It is only possible to assign an offspring to one of these three ways of reproduc-

tion, if the two parents each possess two different alleles at one specific locus, i.e. they do not share an allele at that locus (Fig. V. 1). For this kind of experiments microsatellites are most suitable, despite the fact that they are more expensive and laborious and need more development time than for example Allozymes or RAPD's (Jarne and Lagoda 1996).

The evolution of mixed-mating systems has long been and obviously still is a challenge for evolutionary biology. A stimulating interaction between theorists and empiricists may promote the discovery of most interesting phenomena in this vivid field of research.

Worms in alcohol (Phto by A. Hämmerli)

CONCLUSION

My first encounter with worms and fish was on a summer holiday in 1984 standing on the mole in the harbour of Naples dangling a thin nylon thread into the turbid water. The excitement of that huge fish that would eventually swallow the worm on my hook and the anticipation of a nice barbecue made me come back every day. Finally, one day I caught a fish. It was however, so tiny and bony that even the cat I fed it to didn't become very enthusiastic. Back then I only felt somewhat gloomy about the dark future of the worm on my hook. It would never come to my mind that worms would actually feed on fish and live inside them, and even less did I believe that I would once study them. At that time I was sure to become a postman like my grandfather.

What convinced me to work with the cestode *S. solidus* some years later (instead of becoming a postman) was the well established *in vitro* system simulating the final host and the fact that this animal was a hermaphrodite. All the theory and hypotheses I was taught on the evolution of sex and sexual selection during my studies needed rethinking if I tried to formulate them from a hermaphrodites' and not from a gonochores' point of view. Now, after 4 years of studying *S. solidus* individuals (diploma time included), I can say, that I didn't regret to have stayed within the context of my early hobby: worms and fish.

In the following paragraph I want to resume the main results of this thesis, discuss their possible implication for a better understanding of the biology of *S. solidus* and point at some new questions that have arisen during the course of this work. Experiments in the lab (Chapters I + II) as well as the field study (Chapter III) show that this species is reproducing by mixed-mating (i.e. reproduction through both, selfing and outcrossing). The proportion of outcrossing and selfing of worm pairs was influenced by their mean weight as well as weight difference (Chapter I). Moreover, worms adaptively adjusted their outcrossing rate depending on whether their selfed progeny had a high or a low hatching rate (Chapter II). Other studies on this species showed that inbreeding depression is severe and is therefore expected to be a strong force selecting against self-fertilization. Why are worms then self-fertilizing a certain proportion of their eggs even when a mating partner is present? Worm densities in the final host are small in some years. Therefore, the probability that some worms have to self-fertilize their eggs in some years, due to a lack of mating partners is high. Selfed offspring of worm lineages which have always selfed a certain proportion of their eggs (despite the availability of a partner), may produce fitter progeny than do offspring from worms that have always outcrossed when both have to self in a year of low worm density. Moreover, worms that adaptively adjust their outcrossing rate depending on their potential fitness gained through self-fer-

tilization are at a selective advantage compared to worms that outcross and self at random. Therefore, the selective pressure to always outcross is maybe not as strong as previously thought, and did therefore not lead to the evolution of a gamete exchange already in the fish (Chapter IV). Moreover, the probability to end up in the same final host is probably quite high for worms in a multiply infected stickleback, and this further lowers the pressure to already exchange gametes in the intermediate host.

The studies in this thesis have shown that *S. solidus* is a suitable model system to study mating system evolution. Future experiments exploring this species are expected to discover further fascinating aspects that led to the evolution of a mixed-mating system. However, the investigation of similar questions in other model systems will be of substantial importance if we want to understand the evolution of mixed-mating systems. In an essay I propose several helminth model systems which seem to be suitable for such purposes (Chapter V). The possibility to keep the animals in lab cycles is of major importance if we want to conduct experiments under controlled conditions. Molecular techniques are developing incredibly fast, which opens unimaginable possibilities in the future. However, good model systems and fancy techniques do not automatically make a good experiment. The crucial idea for an experiment may sometimes be found in articles on the species in question which are more than 50 years old. I'm sure the numerous articles on *S. solidus* published only in Russian would be a most interesting source of information on this species biology. Unfortunately, I do not understand Russian.

Many questions are open and many more may rise. I would like to close this conclusion with the quotation of Friedrich Nietzsche "You have made your way from worm to man, and much within you is still worm." (from Zarathustras' Prologue 3). Reading this phrase some weeks ago, my first thought was that Zarathustra must be quite a lucky man with part of him still being worm. In the course of doing my experiments for this thesis I became incurably fascinated by the multifaceted biology of *S. solidus* and I have a deep respect for all worm creatures. Only at the second thought I realized that this was probably not what Nietzsche intended to express with this phrase.

Ein kapitaler Dorsch, gefangen an einem kalten Märztag in der Ostsee.

DANKSAGUNG

An erster Stelle möchte ich Prof. Dr. Milinski herzlich danken für die Betreuung der Doktorarbeit. Insbesondere habe ich die bei der Durchführung der Arbeit eingeräumten Freiräume und die ständige Diskussiosnbereitschaft sehr geschätzt. Mein besonderer Dank gilt Wolf-Rüdiger Wulff, der für meine Experimente über 300 Liter Medium angerührt hat. Ausserdem möchte ich Sybille Liedtke und Catha Schmuck dafür danken, dass sie mir einige PCR's abgenommen haben. Herzlichen Dank auch an Dr. Thomas Binz, der mich in die Geheimnisse des Microsatelliten-Fischen eingeführt hat und mich auch in den ersten Monaten hier in Plön von Bern aus in molekularbiologischen Fragen fernberaten hat. Vielen Dank auch an Derk Wachsmuth, der auch bei den übelsten Computerproblemen immer die Ruhe bewahrt hat und die Kisten wieder wie gewünscht zum laufen gerbacht hat. Danken möchte ich auch August Hämmerli für das kritische durchlesen meiner Arbeiten und die moralische Unterstützung. Ausserdem möchte ich mich bei Dietmar Lemke, Elsbeth Hammerich, Edeltraut Lesky, Brigitte Lechner, Monika Möller, Lore Jahnke, Gerd Augustin, Horst Hansen, Harald Deiwick, und Brigitte Albrecht bedanken, die mich in vielfältige Weise bei meiner Arbeit unterstützten. Ich möchte mich auch herzlich bedanken bei allen Mitgliedern unserer Arbeitsgruppe Dirk, Solveig, Martin, Joachim, Maze, Thorsten, Ineke, Lena, Marilyn, Mira, Pierre und Michu, die mir immer wieder mit Rat und Tat zur Seite standen. Herzlichen Dank auch an meine Denkzellennachbarin Mira mit der es nie langweilig war.

Mein ganz besonderer Dank gilt meinen Freunden hier in Plön für so manchen Abend der mich vom Alltagsstress abgelenkt hat (Thank you, grazie, gracias!). Und ich danke natürlich auch meiner Familie und Freunden in Bern für ihre vielseitige Unterstützung.

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ERKLÄRUNG

Hiermit versichere ich, dass diese Abhandlung - abgesehen von der Beratung durch meine akademischen Lehrer - nach Inhalt und Form meine eigene Arbeit ist und dass ich keine als die angegebenen Hilfsmittel und Quellen verwendet habe. Die Arbeit hat bisher weder ganz noch zum Teil and anderer Stelle im Rahmen eines Prüfungsverfahrens vorgelegen. Teile dieser Arbeit wurden als Manuskripte bei Zeitschriften eingereicht. Kapitel I wurde beim Journal of Evolutionary Biology eingereicht mit Manfred Milinski als Koautor. Kapitel II wurde bei Evolution eingereicht mit Manfred Milinski als Koautor. Kapitel III wurde bei Molecular Ecology eingereicht mit Mathias Wegner als Koautor. Kapitel IV wurde bei Experimental Parasitology eingereicht mit Solveig Schjørring als Erstautorin und Kapitel V wurde bei Trends in Ecology and Evolution eingereicht.

Plön, den 1. November 2002 Annelis Lüscher