

**Investigations on
male traits,
female mate choice,
and the role of parasites
in the three-spined stickleback
(*Gasterosteus aculeatus*)**

vorgelegt von Cathrin Kerstin Krobbach

Kiel 2006

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Darin besteht das Wesen der
Wissenschaft:
Zuerst denkt man an etwas, das wahr sein könnte.
Dann sieht man nach, ob es der Fall ist
und im Allgemeinen ist es nicht der Fall.

Bertrand Russel (1872-1970)

Contents

Summary.....	2
Zusammenfassung	4
Introduction	6
Sexual selection.....	6
Multiple sexual traits.....	8
Parasites in sexual selection.....	9
The model system.....	10
<i>Three-spined sticklebacks</i>	10
<i>Male traits in sticklebacks</i>	11
<i>Parasites in sticklebacks</i>	13
Outline	16
Chapter I Infection dose experiment.....	19
Chapter II Correlation of MHC to male and female traits.....	37
Chapter III Laboratory mate choice experiments.....	55
Chapter IV Field enclosure experiments	79
Conclusion	98
References.....	101
Danksagung	114
Curriculum vitae.....	117
Erklärung	118

Summary

Mate choice, based on secondary sexual traits is a common phenomenon in many animal species. These traits are exhibited by one of the partners, usually the male. If the traits reflect the ability of a mating partner to contribute direct or indirect benefits to the offspring, individuals (usually females) are able to increase their fitness by choosing these traits. Parasite load and parasite resistance are only two aspects of an animal that can be signalled by such traits. To investigate the effect of parasites and the MHC (Major Histocompatibility Complex) genetic background that is important for parasite resistance, on different male traits, I used the three-spined stickleback (*Gasterosteus aculeatus*) as a model organism.

In most cases, invasion of a host by a parasite is followed by a reaction of the host's immune system. Distinct parasite species have a different impact on their host which might result in different physiological effects. In Chapter I it was shown, in a controlled infection experiment, that two nematode parasites (*Camallanus lacustris* and *Anguillicola crassus*) differed in prevalence in their stickleback host. The corresponding immune reaction of the fish was also found to be different, which generally could result in alterations of male traits.

The immune reaction of the host does not only depend on parasite species, but also on its own immuno-genetic background. The specific, adaptive immune system in vertebrates is represented by MHC alleles (class I and class IIB). Do male traits also signal direct information about the MHC background of the fish irrespective of parasite load and do females base their mating decision on such traits? In Chapter II two sexually selected male traits, the red breeding colouration and nest quality were correlated to MHC allele number. We found that males with intermediate number of MHC class II alleles built nests that should be more attractive for females, while redness correlated positively with number of MHC I alleles.

Visual traits such as red colouration or nest quality seem to reflect parasite resistance of male sticklebacks. Laboratory experiments showed that females also choose mating partners for odour cues that are related to the MHC genetic background. If visual and olfactory cues do not carry the same

information, e.g. different aspects of a males' condition, this raises the question of female preference in situations where visual and olfactory traits are available at the same time. Through mate choice experiments (Chapter III) it was shown that, in this situation, females seem to use a combination of visual and olfactory traits. While they choose the redder male in the visual test and the best fitting one in the olfactory test, no choice for either the one or the other was found when both choice situations were combined. The females then preferred males for their overall condition, which was not reflected solely by either of the male traits investigated.

In order to test whether the results from my laboratory experiments are applicable to the natural situation in the field, outdoor enclosures were stocked with fish of known MHC background (Chapter IV). Parenthood analysis was performed for eggs collected from the males' nests. Reproductive success was estimated for males and females in combination with measurements of male traits and parasite load. Since it was not possible to identify a certain male trait the females always preferred, choice for a combination of traits also seems most probable in the field.

Zusammenfassung

Wählerisches Verhalten von Weibchen bei der Partnerwahl ist im Tierreich weit verbreitet und basiert auf sekundären Geschlechtsmerkmalen der Männchen. Wenn solche Merkmale Informationen darüber tragen, ob der Partner direkt oder indirekt einen Beitrag zur Fitness der Nachkommen leisten kann, können Weibchen durch Verpaarungen anhand dieser Merkmale ihre Fitness steigern. Beispielsweise können Informationen über Parasitenbelastung oder Parasitenresistenz eines Männchens in Form solcher Merkmale übermittelt werden. Um den Einfluss von Parasitierung und Resistenzgenen, hier in Form von MHC (Haupt Gewebekompatibilitäts Komplex), auf Männchenmerkmale zu untersuchen, habe ich den Dreistachligen Stichling (*Gasterosteus aculeatus*) als Modelorganismus verwendet.

Auf das Eindringen eines Parasiten in einen Organismus folgt in der Regel eine Immunantwort des Wirtes. Bestimmte Parasitenarten können dabei unterschiedliche physiologische Effekte in ihren Wirten auslösen. In Kapitel I wird anhand eines kontrollierten Infektionsexperimentes gezeigt, dass zwei parasitische Nematoden, *Camallanus lacustris* und *Anguillicola crassus*, sich in ihrer Prävalenz im Stichlingswirt unterscheiden. Auch die Immunreaktion des Fisches war in diesem Zusammenhang unterschiedlich, was zu einer Veränderung von Merkmalen bei Männchen beitragen könnte.

Die Immunreaktion eines Wirtes hängt aber nicht nur von der Parasitenart, sondern auch von seinen Resistenzgenen ab. Bei vertebraten Wirten wird diese spezifische, angepasste Immunantwort durch MHC der Klasse I und Klasse II repräsentiert. Können nun Merkmale von Männchen auch direkte Information über den MHC übermitteln? In Kapitel II zeigen wir anhand einer korrelativen Analyse, dass sowohl die rote Balzfärbung der Männchen als auch bestimmte Qualitätsmerkmale ihrer Nester tatsächlich im Zusammenhang mit der Anzahl von MHC Allelen stehen. Männchen, die eine mittlere Anzahl von MHC II Allelen trugen, bauten Nester, die für Weibchen attraktiver sein sollten. Darüber hinaus war ihre Balzfärbung positiv mit der Anzahl von MHC I Allelen korreliert.

Visuelle Merkmale von Männchen, wie die Balzfärbung oder Nestqualität, reflektieren demnach offensichtlich Resistenzgene beim Stichling. Aus früheren Laborstudien ist bekannt, dass auch olfaktorische Merkmale, auch im Zusammenhang mit MHC, von Weibchen für die Auswahl eines Partners herangezogen werden. Das wirft die Frage auf, an welchem Merkmal die Weibchen ihre Wahl orientieren, wenn visuelle und olfaktorische Merkmale nicht die gleiche Information beinhalten, aber gleichzeitig zu Verfügung stehen. In Kapitel III wurde mit Wahlexperimenten an weiblichen Stichlingen gezeigt, dass in diesem Fall visuelle und olfaktorische Merkmale für ihre Wahl kombiniert werden. Während im visuellen Test das röttere und im olfaktorischen das besser zu ihrem MHC passende Männchen bevorzugt wurden, konnte in der kombinierten Wahlsituation keine eindeutige Präferenz für eines der Merkmale gefunden werden. In diesem Fall wählten Weibchen solche Männchen, die eine bessere Gesamtkondition aufwiesen, was wiederum nicht eindeutig nur von einem Merkmal reflektiert wurde.

Um zu überprüfen, ob sich die im Labor gewonnenen Ergebnisse auf die natürliche Lebenssituation der Fische im See übertragen lassen, besetzten wir Außenkäfige mit Fischen, die einen bekannten MHC aufwiesen. Elternschaftsanalysen der gesammelten Eier, zusammen mit Analysen der Merkmale der Männchen und deren Parasitenbelastung sollten Aufschluss über ihren Fortpflanzungserfolg geben. Obwohl es uns nicht möglich war die Wahl der Weibchen einem bestimmten Merkmal der Männchen zuzuordnen, scheint auch im natürlichen Lebensraum die Nutzung einer Kombination von Merkmalen eine Rolle zu spielen.

Introduction

Sexual selection

In many animal species, males and females are phenotypically dimorph, e.g. the Ruff (Fig. 1). Darwin (1871) wondered about how this dimorphism might have evolved, and what the role and the origin of the, in some cases eye-catching traits could be. An intersexual conflict, based on anisogamy, was stated as one possible reason by Bateman (1948). Females produce few large eggs, enriched with nutrients, in contrast to males that have many small sperm. The gender with the lower potential reproductive rate, usually the females, should be more selective about the



Figure 1: female and male ruff (*Philomachus pugnax*), from Naumann, *Naturgeschichte der Vögel Mitteleuropas*, Band VIII, Tafel 23 Gera, 1902

mating partner since it faces higher costs of few or low quality offspring (Shuster & Wade 2003). Individuals of the other gender, usually the males, compete among each other for mating and exhibit traits to attract females. As a result, fuelled by choice and competition, sexual selection evolved (Andersson 1994). Males can enlarge their reproductive success by fertilisation of as many eggs, of different females, as possible, while females have to choose a male that provides direct or indirect benefits for the offspring (Clutton-Brock & Vincent 1991). Females use different male traits to judge a male for its ability to provide such benefits. For example a large territory or good nuptial gifts provide direct benefits that the female might be able to evaluate easily by inspection. Indirect benefits that are based on heritable, genetic advantages, such as parasite resistance, might be more difficult to detect and evaluate.

Preference for traits that indicate such indirect benefits could have evolved in at least three different ways (summed up in Stearns & Hoekstra 2005):

1. 'Honest signal' hypothesis: Costly, 'honest' male traits signal 'good genes' that promise better survival or other fitness benefits (e.g. Pomiankowski *et al.* 1991, Iwasa *et al.* 1991). If the costs for the exhibition of a trait are high, and only superior males can invest without suffering, these traits are called 'handicaps' (Zahavi 1975). This is especially true for signals that reflect parasite resistance (Hamilton & Zuk 1982). Such 'indicator mechanisms' (Andersson 1994) are for instance colour patterns in many birds or fish (e.g. Hill 1991).
2. Fishers' runaway selection (Fisher 1930): Females prefer males for a certain trait (e.g. exaggerated tail feathers). The resulting male offspring faces the benefit that it shows the trait and is preferred by females. The female offspring inherits the genes for this preference. The correlation of female choice and male trait could force evolution of exaggerated traits that might be disadvantageous but persist only because of the females' preference (Bakker 1993).

The 'honest signal' and Fishers' runaway hypothesis are not mutually exclusive and may partly act on mate choice depending on the costs for trait and preference (Kokko 2002). However, there is a third theory of how preference and traits might have evolved, which might also build the basis for preferences in general.

3. 'Sensory bias' hypothesis: Male traits show a normally distributed variation in the population. Females differ in their preference for the trait due to differences in their sensory system. Some females are able to detect traits only on the one end of the distribution and therefore prefer this trait while others might prefer those on the other end for the same reason. This preference is only based on the natural sensory bias of the females (Ryan 1985).

For many male traits it is difficult to disentangle which of these mechanisms might govern the corresponding preference. In most cases it is probably a combination of all of them.

Even though males face costs for production and exhibition of sexually selected traits only one successful mating can ensure their fitness. The costs for females are even more pronounced, not only due to the inter-sexual conflict, but

also due to the concern of low quality offspring. Mating an inferior male is better than not mating at all since it is not always clear whether a second mate will appear. Costs of female choice could decrease if male traits are reliable and less likely (since more costly) to be cheated. This is discussed as one reason for the evolution of multiple ornaments in mate choice.

Multiple sexual traits

In most species, females base their mating decision not only on one, but on a variety of traits. The evolution of such multiple traits, and their use in mate choice, has been discussed from many points of view and the main theories are reviewed by Møller & Pomiankowski (1993) and Candolin (2003). Females should avoid costs of mating an inferior male and use multiple, and thus more reliable, traits to avoid cheating. Some traits could act together as multiple messages reflecting different aspects of one individual (Johnstone 1996, McGraw & Hill 2000). The actual condition of a male might, for example, be reflected by one trait, while the investment into reproduction is reflected by another. Under limiting conditions with restricted resources, only superior males might be able to invest into both. Other males have to allocate the limiting resources into only one trait. Hence, females would mate the best male if they take both traits into account.

Other traits could act as redundant signals that signal information about the same aspect of a given male. Taken together, they function as a more reliable message for the female signalling e.g. the condition of a possible mating partner (Johnstone 1996, Møller & Petrie 2002, Partan & Marler 2005). However, male traits do not always carry information about the males' condition or possible direct and indirect benefits to the offspring. Some traits might also be uninformative with respect to the individual male but facilitate e.g. species detection or signal assessment (Pomiankowski & Iwasa 1993, Iwasa & Pomiankowski 1994, Candolin 2003). These and other theories are highly discussed, but since they are not mutually exclusive a concrete determination of the role of a signal might not be possible in every single case.

The variety of male ornaments is high between species. Anderson (1994) analysed 232 studies on 186 species that investigated sexually selected traits

ranging e.g. from insects, fish and birds and mammals. In some cases it might be difficult to disentangle traits used during mate choice or for male contest. This difficulty could result in overlooking traits that play no role for female choice but are important for successful male-male competition. However, in Anderson's table, the most prominent male traits that were used by females for mate choice are acoustic signals that carry information about the males' condition, like songs and calls in birds, amphibians and insects. In addition, some species, especially e.g. birds, show exaggerated or colourful cues (e.g. Hill 1991) which can reflect parasite resistance. Also, the female choice for olfactory signals was discovered in mice (e.g. Yamazaki 1976) and humans (e.g. Wedekind *et al.* 1995). Here, odour cues can signal e.g. hormone status (Willis & Poulin 2000), parasitism (Penn & Potts 1998) or immuno-genetic background (Wedekind 2004, Boehm 2006).

Parasites in sexual selection

Price (1980) stated that parasites affect life and death of practically every organism. They have a strong impact on their host's fitness due to various effects, e.g. direct pathology or alteration of host behaviour (reviewed in Barber *et al.* 2000). For the latter, it is not always clear whether the effects are induced by parasite manipulation, or as a by-product of the infection (Poulin 1995). Due to the parasite's profound negative effects on its host, information about parasite load or resistance seems likely to be mediated by sexually selected traits. Females benefit from this information about the males and thus avoid the costs of mating inferior males.

Most of the effects that parasites cause on hosts are due to the defence mechanisms against the invading parasite. Parasites counter evolve mechanisms to circumvent this defence and better exploit the host. The interaction of host and parasite with evolving adaptation and counter adaptation was compared with an arms race termed the 'Red Queen hypothesis' (e.g. van Valen 1973). This analogy was adopted from Carroll's (1871) 'Through the looking glass' where the Red Queen explains to Alice that it takes 'all the running you can do to stay in the place you are'. Sexually reproducing hosts are able to evolve more rapidly than asexual ones (Lively 1996). The resulting

faster evolution of adaptations is discussed as one of the major reasons for the maintenance of sexual reproduction (Lively 1992) since mate choice enables reproduction with a high quality partner that provides good parasite resistance resulting in better adapted offspring and higher fitness.

The model system

Three-spined sticklebacks

The model organism I used in my investigations is the three-spined stickleback (*Gasterosteus aculeatus*, L.). This small, promiscuous fish inhabits brackish and fresh waters as well as the sea on the northern hemisphere. The reason why sticklebacks became a pet to scientists of many different fields in biology might have been due to the eye-catching behaviour and conspicuous colouration of the males (Fig. 2, right).



Figure 2: left: a school of female three-spined sticklebacks (*Gasterosteus aculeatus*), right: male three-spined stickleback with red breeding colouration in front of its nest. (pictures from: SWR, www.kindernetz.de)

The start of the breeding season in early spring depends on temperature and photoperiod (e.g. Wootton 1976, 1984a, Mori 1985). During this time, males exhibit a typical breeding colouration with a red throat and blue eyes. They establish territories in the shallow water of lakes and small rivers where they build nests on the ground using plant material. To fix the nest, males use Spiggin which is produced by the trunk kidney and released via the urinary bladder (van Iersel 1953). At the same time, females' eggs begin to mature, and are then spawned (Fig. 2, left) into the nest of a well chosen male. The spawning behaviour follows a discrete scheme (Fig. 3) starting with the female approaching the male at his nesting site, with the so-called head-up posture,

presenting her egg packed belly. The male reacts with a zig-zag courtship dance (Tinbergen 1951) and finally, sometimes only seconds later, leads the female to the entrance of his nest. When the female has entered the nest, the male encourages the spawning by hitting the base of the female's tail fin, then banishes her and swims through the nest release the sperm. After collection of 3-6 egg clutches from different females, the male cares for the eggs. He fans water through the nest and removes moulded eggs until the fry hatch after 5-8 days, depending on the temperature.

Apart from collecting clutches into his own nest, another not mutually exclusive strategy of male sticklebacks to gain fertilisations is through sneaking behaviour (van den Assem 1967, Kynard 1978a). Sneaker males can have their own territory and nest. They manage to change their colouration suddenly to mimic females and then swim near the bottom close to the nest of a neighbouring male that recently received eggs from a female. The sneaker swims through the foreign nest, sometimes even before the nest owner himself and adds his sperm. Thereby sneaking males gain additional fertilisations without having the costs of brood care.

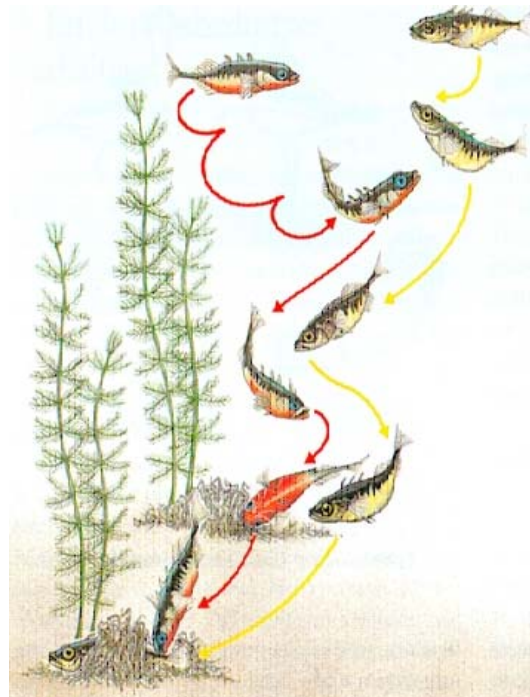


Figure 3: Mating behaviour of the three-spined stickleback. (picture from Linder *Biologie*, Metzler Schulbuchverlag GmbH, 20. Auflage, Stuttgart, 1992)

Male traits in sticklebacks

Female sticklebacks base their mating decision on both, visually and olfactory male traits. Redness (Bakker & Milinski 1991, Milinski & Bakker 1990, 1992, Bakker 1993, Bakker & Mundwiler 1994, Wedekind *et al.* 1998, Barber *et al.* 2001b), symmetry (Mazzi *et al.* 2003, 2004) and zig-zag courtship dance (Rowland 1995, Bronseth & Folstad 1997, Ishikawa & Mori 2000) are only some

of the visual traits that were shown to play a role in female mate choice. The typical red breeding colouration of the males is based on carotenoids (Brush & Reisman 1965). Carotenoids are not only used for colouration but also as radical scavengers during innate immune reactions (e.g. Kurtz 2003). Since carotenoids are limited in the diet of the stickleback, redness might reflect the physical condition of the male especially according to the parasite defence ability. Female sticklebacks spent more time in front of the redder male when presented two alternatives (Milinski & Bakker 1990) and they are able to distinguish even small differences in colouration (Künzler & Bakker 1998). Another colour trait that might play a role for female mate choice is the blue iris of the male, but investigations on this are sparse (Rowland 1984, McLennan & McPhail 1989). In addition to colouration, other traits are also important for visual mate choice e.g. nest characteristics. Females choose mating partners for nest organisation, visibility of the entrance or loose ends of used plant filaments (Barber 2001a, Östlund-Nilsson & Holmlund 2003) and they are more likely to spawn into nests that already contain eggs (Belles-Isles *et al.* 1990). Clutches spawned in high quality nests might face higher survival due to good male broodcare.

However, in *G. aculeatus* female choice could be demonstrated not only for visual, but also for olfactory traits (Reusch *et al.* 2001, Aeschlimann *et al.* 2003, Milinski *et al.* 2005, McLennan 2003). Here, chemical cues associated with genes of the major histocompatibility complex (MHC) caught most attention. MHC genes play a major role in initiating the acquired immune response by presenting peptides derived from pathogens (Klein 1994) and contain the most polymorphic gene loci known in vertebrates (e.g. Janeway *et al.* 2001). In sticklebacks, there are probably six loci of MHC class IIB and twelve loci of MHC class I (Sato *et al.* 1998). Evidence for the existence of an optimal rather than a maximal (i.e. 12 alleles) individual MHC class IIB diversity is mounting. In sticklebacks, females use a self-referential process, signalled by peptide ligands, to assess MHC class IIB alleles of a possible mating partner (Milinski *et al.* 2005). They choose the male, optimal with respect to their own MHC, leading to an intermediate number of around 5-6 MHC sequence alleles in their offspring (Aeschlimann *et al.* 2003, Reusch *et al.* 2001, Milinski *et al.*

2005). Fish with an intermediate number of MHC sequence alleles have a clear fitness advantage, because they are less likely to get infected under experimental conditions as well as in the wild (Wegner *et al.* 2003a, b), and suffer less from the infection (Kurtz *et al.* 2004). This suggests that a link exists between female mate choice and parasite resistance. From mice it is known that females can smell a possible parasitisation directly in the odour marks of the male (Kavalier & Colwell 1995). This ability to detect infection might also play a role in sticklebacks. Further information signalled via the odour of a male could include e.g. hormones or immune parameters but more research is necessary to disentangle different odour contents and their role in the context of mate choice (Ferkin & Zucker 1991, Westneat & Birkhead 1998).

Parasites in sticklebacks

Among the various macro parasites that can be found in the three-spined stickleback, I chose the three most abundant species in the lakes around Plön, Northern Germany. Namely, these were the two nematodes *Camallanus lacustris* and *Anguillicola crassus* and the trematode *Diplostomum pseudospathaceum*.

C. lacustris and *A. crassus* are transmitted when sticklebacks prey upon copepods. Copepods, which are first intermediate hosts (Moravec 1994), become infected by ingesting parasite larvae. In both parasites' life cycles sticklebacks serve as paratenic hosts until a more suitable host preys upon it. Several predatory fish species like pike (*Esox lucius*) or perch (*Perca fluviatilis*) (Moravec 1969) are final hosts of *C. lacustris*, whereas in Europe *A. crassus* reproduces only in the eel (*Anguilla anguilla*).

C. lacustris (Fig. 4) is a long-established parasite in lakes of Northern Germany that, after tropical transmission, stays in the anterior part of the intestine of the fish host. There, *C. lacustris* anchors in the intestinal mucosa and feeds on blood and tissue (Stumpp 1975, Lodes & Yoshino 1985, Meguid & Eure 1996).



Figure 4: left: *Camallanus lacustris* adults in the intestine of a stickleback, right: head capsule of a *C. lacustris* larvae. (pictures: M. Kalbe)

A. crassus (Fig. 5) was introduced to Germany recently in 1982 (Neumann 1985). It came from Japan where it is a common parasite of the Japanese eel (*Anguilla japonica*). It penetrates the intestine before it migrates through the body cavity into the wall of the swim-bladder. There, it feeds on blood and can cause major damage to the tissue (Würtz & Taraschewski 2000).



Figure 5: left: *Anguillicola crassus* adults in the swim bladder of an European eel (*Anguilla anguilla*) (picture from: www.abo.fi/institut/fisk/Swe), right: *A. crassus* larvae (picture: M. Kalbe).

The digenean trematode *D. pseudospathaceum* (Fig. 6) is not transmitted to the stickleback by ingestion of an intermediate host. Free-swimming larvae, released from *Lymnaea stagnalis* snail hosts, penetrate the skin of the fish. Thereafter, they migrate into the eye-lenses of the fish ('eye-fluke') to evade the immune system (Chappell *et al.* 1995). Sight ability is reduced in infected fish that therefore face higher predation risk or decreased prey finding ability (Crowder & Broom 1980, Owen *et al.* 1993). The fish is then preyed upon by the definitive host (gull), in which *D. pseudospathaceum* matures and finally releases its eggs into the water.

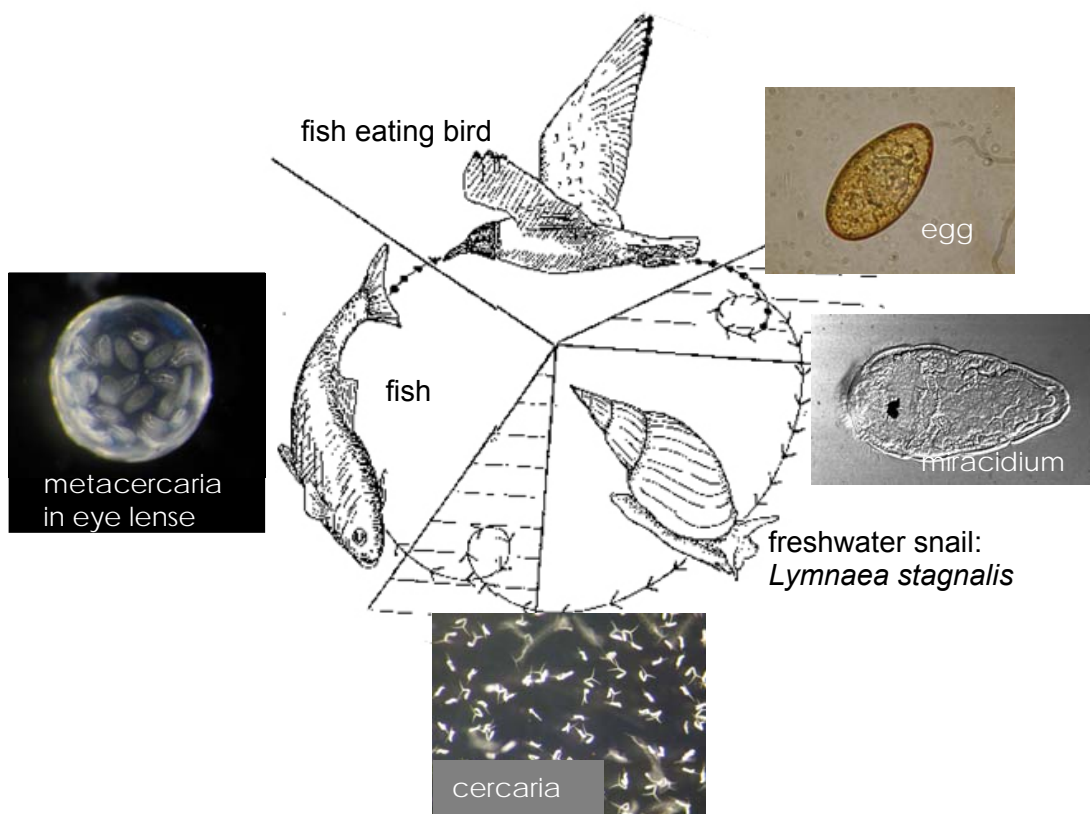


Figure 6: Life cycle of *D. pseudospathaceum* including *Lymnaea stagnalis* as specific first intermediate host, a fish as second intermediate host and a fish eating bird as final host. Denomination of life stages is given within the pictures (pictures by M. Kalbe, lifecycle by W. Haas).

Outline

This thesis is divided into four chapters. The chapters represent independent studies and are structured into abstract, introduction, methods, results and discussion. In Chapter II results and discussion are combined. The first two chapters address the effect of parasites and immuno-genetic background on physiology and male traits of three-spined sticklebacks. Both aspects were incorporated in a laboratory mate choice experiment (Chapter III) which was supported by data from a field enclosure experiment (Chapter IV). This outline gives a short overview of the motivation for the distinct experiments.

Contribution to the chapters:

My contribution to the single chapters was as follows: Chapter I: experiment performance, data analysis, and manuscript preparation. Chapter II: photographs of the fish, analysis of redness, and help with manuscript. Chapter III: performance and analysis of experiment, preparation of manuscript, Chapter IV: help during data collection, analysis of the data, and preparation of the manuscript.

Chapter I: Infection dose experiment

To use parasites in laboratory infections, it is important to know about their infection ability and the immune reaction they activate. This is especially true for my experiments including sexually selected traits that might reflect parasite load or resistance. In the first chapter of this thesis, it was therefore investigated whether two different nematode parasites elicit different immune reactions in their paratenic stickleback host. Therefore, sticklebacks were exposed to different doses of *C. lacustris* and *A. crassus* and immune activation was measured at a specific time point 18 weeks after exposure. As expected the two parasite species varied in the immune response of the host, possibly either because they have different target organs, or due to different duration of their host-parasite co-evolution.

Chapter II: Correlation of MHC to male and female traits

As showed in Chapter I, different parasites cause different reactions in their stickleback host. This might be due to the immuno-genetic background of the fish. In Chapter II, it was investigated whether male traits, namely red breeding colouration and nest quality, were thus correlated to the MHC class I and class IIB allele number of the fish. Both male traits are known to be involved in mate choice and might enable females to evaluate possible mating partners not only for parasitisation, but also for the MHC background, and according parasite resistance.

Chapter III: Laboratory mate choice experiments

On the basis of the two former chapters, mate choice of female sticklebacks for parasitisation and MHC class IIB alleles of males (Chapter III) was experimentally tested. For this purpose, a set-up was developed that tested visual and olfactory female preference separately or in combination.

Apart from preference for males that are MHC optimal with respect to their own MHC background in the olfactory choice situation, we expected females to choose non-parasitised males. In visual choice situations this might be due to changes in red colouration, while nothing is known for parasite induced changes in olfactory choice in sticklebacks so far. Although several traits might have evolved for different functions, females probably include multiple traits into their mating decision to obtain a reliable overall picture of the males' condition and parasite resistance. Therefore, this experimental approach with a combined choice situation resembles the natural situation where females are confronted with visual and olfactory cues at the same time.

Chapter IV: Field enclosure experiments

In Chapter IV of this thesis, I present a field enclosure experiment that was performed to verify the results and conclusions from the laboratory experiments. The experiments were performed with a controlled stocking of fish that were selected for their MHC background. After final dissection and parasite determination, male traits, parasite load and MHC class IIB alleles were correlated to the number of eggs the males had collected. Using parenthood

analysis it was possible to determine which males were chosen by the females, and whether the visual and olfactory male traits, or their MHC background, might have played a role for this decision. How these traits, as well as parasitism of the males, might influence the female reproductive success can only be assumed. However, the results point in the same direction as those from laboratory experiments: also in the field, females base their mating decision on multiple visual and olfactory traits to increase their fitness.

Chapter I

Infection dose experiment



The nematode parasites, *Camallanus lacustris* and *Anguillicola crassus*, operate differently in a paratenic host, the three-spined stickleback (*Gasterosteus aculeatus*)

Abstract

Three-spined sticklebacks (*Gasterosteus aculeatus*) are frequent paratenic hosts of the nematode parasites *Anguillicola crassus* and *Camallanus lacustris*. Both parasites feed on blood and tissue, *A. crassus* in the wall of the swim bladder, and *C. lacustris* on the intestinal mucosa. The latter is a long time established parasite in freshwater fish stocks of Europe, but *A. crassus* was introduced to Europe recently, about 20 years ago. This implicates a potential difference in host-parasite adaptation, which might influence the infection ability. In the present study, a paratenic host of the two parasites, *G. aculeatus*, was for the first time infected under controlled laboratory conditions. Exposure to doses of 6, 12, 18 and 24 parasites resulted in significantly higher infection rates (18-49%) for *C. lacustris* compared to *A. crassus* (4-14%). Highest infection rate was observed in sticklebacks exposed to 12 *C. lacustris* larvae. Exposure to 18 *C. lacustris* larvae did not increase the infection rate and it even decreased in fish exposed to 24 *C. lacustris* larvae. In *A. crassus* infected sticklebacks, no significant effect of administered parasite dose on infection rates was observed. Respiratory burst activity and lymphocyte proliferation of head kidney leukocytes did not show any alteration by either parasite or exposure dose 18 weeks post exposure, indicating that the infections might have entered a chronic phase.

Keywords

Anguillicola crassus, *Camallanus lacustris*, *Gasterosteus aculeatus*, infection dose, neozotic parasite, immune response, G/L ratio, respiratory burst, lymphocyte proliferation

Introduction

In natural habitats animals are exposed to a variety of parasite species that can cause inflammation, drainage of nutrients and even changes in the hosts' behaviour. The possible effects often vary between parasite species (Combes 2001, Moore 2002, Hoole *et al.* 2003). Characteristics of the immune defence of the hosts may depend on infection route, target organ and pathogenicity of the invading parasite species (Buchmann *et al.* 2001, Tully & Nolan 2002, Wiegertjes *et al.* 2005, Roberts *et al.* 2005, Reite & Evensen 2006).

The three-spined stickleback (*Gasterosteus aculeatus*) is intermediate, paratenic or final host for several parasite species and is investigated as a model organism for host-parasite interaction. Two of the most abundant parasites in sticklebacks in northern Germany are the nematodes *Camallanus lacustris* and *Anguillicola crassus* (Kalbe *et al.* 2002). Both parasites are transmitted by various copepod species, as first intermediate hosts (Moravec 1994). For *C. lacustris* final hosts are predatory fish like pike (*Esox lucius*) or perch (*Perca fluviatilis*) (Moravec 1969b), whereas in Europe *A. crassus* reproduces only in the eel, *Anguilla anguilla* (Moravec & Taraschewski 1988). Direct transmission to final hosts is possible but frequently small planctivorous fish (e.g. sticklebacks) are paratenic hosts in which both parasite species persist without reproduction.

In the final as well as in the paratenic host, *C. lacustris* stays in the anterior part of the intestine where it anchors in the mucosa, feeds on blood and tissue, and causes inflammatory reactions (Stumpp 1975, Lodes & Yoshino 1985, Meguid & Eure 1996). *A. crassus* penetrates the intestine of its fish host, migrates into the wall of the swim-bladder where it feeds on blood. In its final eel host *A. crassus* causes serious damage to the swim bladder tissue (Taraschewski *et al.* 1988, Molnar *et al.* 1995, Würtz & Taraschewski 2000) and elicits cellular and humoral immune responses (e.g. Haenen & Van Banning 1990, Knopf *et al.* 2000, Mulcahy *et al.* 2005).

The three-spined stickleback *G. aculeatus*, among several fish species, is a frequent paratenic host for *A. crassus* (Haenen & Van Banning 1990,

Thomas & Ollevier 1992); a constitutive route of infection next to direct transmission by crustaceans (Haenen & Van Banning 1990). Abundance of *A. crassus* larvae varied among possible paratenic host fish species, but higher prevalence was observed in physoclist fish (Thomas & Ollevier 1992). Among these more susceptible fish (e.g. river goby, common perch) were distinguished from not susceptible fish (several cyprinids). The latter are more effective in a cellular immune response against *A. crassus*, resulting in encapsulation of invading larvae and formation of granulomas in which the parasites are killed (Székely *et al.* 1996). The capability of paratenic hosts to control their parasite load thus can significantly influence infection pressure of parasites to their final host.

C. lacustris is a long-established parasite in lakes of northern Germany, probably since the last glacial phase, whereas *A. crassus* was introduced to Germany in 1982 (Neumann 1985). The two parasites might perform differently when infecting three-spined sticklebacks as paratenic hosts. In wild caught sticklebacks indeed lower abundance of *A. crassus* compared to *C. lacustris* was found (Kalbe *et al.* 2002). If this is due to differences in the infection pressure in wild populations is not clear yet. Furthermore differences in the immune response of stickleback against the two parasites might play a role. However, information about possible effects of infection dose and dynamics of host-parasite interaction of *C. lacustris* and *A. crassus* in paratenic hosts is limited at the moment.

A major part of innate immune defence of fish hosts against helminth parasites is the activation of granulocytes (Whyte *et al.* 1989, Zapata *et al.* 1996, Nie & Hoole 2000, Kurtz 2004, Scharsack *et al.* 2004). In addition to the innate line of defence (Jones 2001) fish hosts possess adaptive immunity that produces specific antibodies against parasite antigens (Roberts *et al.* 2005, Wiegertjes *et al.* 2005). Clonal expansion of lymphocytes is a fundamental part in a specific immune response of fish (Rijkers *et al.* 1980) and lymphocyte proliferation is used as a measure for activation of the specific immune system against helminth (Nie *et al.* 1996) and protozoan (Hamers & Goerlich 1996, Scharsack *et al.* 2000) parasites. To determine the relative activity of the innate

versus the adaptive immunity, the ratio of granulocytes to lymphocytes (G/L ratio, Birkhead 1998, Kurtz 2004) is assessed.

In the present study, we exposed sticklebacks to infective stages of *A. crassus* and *C. lacustris* respectively under laboratory conditions, in order to determine differences in infection ability between the two parasite species. Infection was allowed to persist for 18 weeks for a strong discriminatory power on parasite survival. Varying infection doses were used for exposure to check for a threshold in parasite number for optimal infection success. Body condition of infected fish was recorded and activation of their cellular immune system was measured. Furthermore, respiratory burst activity, proliferation of lymphocytes and G/L ratio was used to quantify activation of innate and adaptive immunity. We intended to detect differences in performance of the neozoic *A. crassus* and the long established parasite *C. lacustris* in the three-spined stickleback.

Methods

Parasites

A. crassus and *C. lacustris* parasites used for the infection experiments originated from host fish caught in the Grosse Plöner See (Germany) in autumn 2003 and spring 2004. *A. crassus* (L2) larvae were washed out from freshly isolated swim bladders of eels (*Anguilla anguilla*) with PBS buffer and *C. lacustris* larvae were dissected from gravid female parasites collected from the intestinal blind sacks of perch (*Perca fluviatilis*). All infective stages were stored overnight in glass petri dishes with 0.64% NaCl at 6°C.

Infection of copepods

Macrocyclops albidus copepods were obtained as intermediate hosts from a parasite free laboratory culture and prepared for infection as described in Hammerschmidt & Kurtz (2005a). On the day of infection, each copepod was assigned to either *C. lacustris* or *A. crassus* treatment and was offered six actively moving larvae of the respective parasite. Infected copepods were kept singly in wells of 24-well micro-titre plates at 18°C under a 16:8h light: dark cycle for 3 weeks. They were fed three times a week with 5 living *Artemia* nauplia. One week before they were fed to the fish, the number of parasites in the body cavity of each copepod was counted using a microscope. Copepods infected with six living (moving) parasites were exclusively selected for the fish exposure.

Infection of sticklebacks

Three-spined sticklebacks were caught in the Grosse Plöner See in 2003. For the infection experiments, six fish families were bred from these parental fish under parasite free laboratory conditions and raised for 7 months within their family groups. The groups were maintained in separate tanks (16L) with continuous water supply and aeration at 18°C under a 16:8h light: dark cycle. After initial feeding with living *Artemia* nauplia, the fish were fed *ad libitum* three times a week with a mix of frozen chironomid larvae and daphnids.

Prior to exposure fish were marked according to their infection dose (by clipping 0 to 2 spines: 1st dorsal spine- 6 parasites, 2nd dorsal spine- 12 parasites, both dorsal spines- 18 parasites, one dorsal and one pectoral spine- 24 parasites and no spine for control fish). Wedekind & Little (2004) found an effect of spine clipping on infection rate and if so, we would expect an increase in immune reaction for the fish with one or more spines clipped. Since we found no significant difference in hepatosomatic index or G/L ratio between the control and the fish which had at least one spine cut (ANOVA: both $P>0.2$) we conclude that the clipping had no effect in our experiment. To enhance the consumption of the respective number of copepods the marked fish were put individually in aquaria with 1L of water and starved for three days. From each family we draw blind 20 individuals of which we assigned 10 to *C. lacustris* treatment and 10 to *A. crassus*. Within each of these groups, two fish were offered 1, 2, 3 or 4 copepods (each containing six parasite larvae) respectively, according to the infection dose they were assigned to and formerly marked for. This procedure resulted in 8 exposed fish per group and the remaining two fish served as controls that were fed no copepods. The groups of 10 fish were kept together in 16L aquaria until dissection.

Dissection of the sticklebacks

Of the 120 experimental fish, 14 died during the experiment due to technical problems with the water supply of two aquaria. In total, 106 fish were available for dissection with N=46 in the *A. crassus*, N=40 in the *C. lacustris* treatment group and N=20 controls. For each parasite group and the controls from the same aquaria, dissection took place on two consecutive days 18 weeks post exposure. Fish were weighed and measured for length and then dissected. Livers were removed and weighed to calculate the hepatosomatic index (W_{liver}/W_{fish})*100 where W is the fish wet weight. The hepatosomatic index is a measure for the fish's metabolic condition including fat reserves and carbohydrates (Chellappa *et al.* 1995). At the same time head kidneys, the major lymphatic organs in teleost fish (Press & Evensen 1999) were prepared for immunological assays (see below). To count infested *A. crassus* parasites, the fish's swim bladder was removed and placed on a glass slide for

microscopic examination. For the *C. lacustris* treatment group the guts of the fish were squeezed between two glass plates and screened for parasites under a dissection microscope. Individual infection rate was calculated as the number of successfully established relative to the number of offered parasites. Since the absolute number of parasites found in the fish did not show any significant effect on any of the investigated traits we included only the parasite dose we used for exposure into the statistics.

Immunological assays

Leukocytes were isolated from the head kidney of all dissected sticklebacks (N=106) as described in Kurtz *et al.* (2004). Numbers of viable leukocytes (exclusion of propidium iodide positive cells) were enumerated by means of flow cytometry (FACSCalibur, Becton and Dickinson, USA). Total cell numbers in head kidney leucocyte (HKL) suspensions were determined with the standard cell dilution assay (SCDA, Pechhold *et al.* 1994) in a modified form (Scharsack *et al.* 2004; Kurtz *et al.* 2004). Propidium iodide (2 mg/L) and green fluorescent standard particles (3×10^4 , 4 μ l, Polyscience, USA) were added to the suspensions to discriminate standard particles (green fluorescence positive) from viable HKL (propidium iodide-negative, green fluorescence negative). Distinct leukocyte sub-sets were identified according to their characteristic FSC/SSC (forward scatter/side scatter) profiles as described by Scharsack *et al.* (2004) and analysed as described in Hammerschmidt & Kurtz (2005a). Proportions of granulocytes (FSC/SSC^{high}) and lymphocytes (FSC/SSC^{low}) of total viable cells in individual HKL samples were used to calculate granulocyte/lymphocyte ratio (G/L-ratio) for each fish.

As an important effector-mechanism of the innate immune system respiratory burst activity of HKL was quantified in a lucigenin-enhanced chemiluminescence assay. The assay was modified after Scott & Klesius (1981) and cell densities were adjusted as described by Kurtz *et al.* (2004). Cell suspensions (160 μ l/well with 2×10^5 HKL) were added to 20 μ l lucigenin solution (2.5mg/ml PBS) per well in 96-well flat bottom micro titre plates and incubated for 30min at 18°C for lucigenin uptake of the cells. Phagocytosis and production of reactive oxygen species was initiated by addition of 20 μ l zymosan

suspension (7.5mg/ml PBS). Control cultures received 20µl of PBS without zymosan. Plates were measured for 3 h at 20°C using a micro titre plate illuminometer (Berthold, Germany). Relative luminescence was calculated as relative luminescence units (RLU) for each sample using the WinGlow software.

As a parameter for activation of the adaptive immune system, we determined the relative number of proliferating lymphocytes in the S and G_{2-M} phase of the cell cycle by quantification of DNA content. HKL were fixed with ethanol (100µl cell suspension, 1.25 x 10⁶/ml, as described above in 900µl ice cold Ethanol 98%) and stored at 4°C. Cells were centrifuged (550 x g, 10min, 4°C) and supernatant ethanol was removed. Re-suspended HKL were incubated with RNase (500 µg/ml PBS) for 10min at room temperature to remove background labelling of RNA. Propidium iodide (Sigma Aldrich) was added to a final concentration of 7.5 µg/ml and cells were incubated for 10min at room temperature. For individual samples, events were measured for three minutes or up to 30.000 events with a flow cytometer (Becton Dickinson FACSCalibur). Red fluorescence (propidium iodide) was measured in linear mode. Data were evaluated with the CellQuest Pro 4.02 software. Lymphocytes were identified according to their characteristic FSC/SSC profile. Doublet cells were subtracted from single cells as described by Wersto *et al.* (2001). Frequencies of lymphocytes in G₀₋₁, S and G_{2-M} phase were acquired by DNA content analysis of red fluorescence intensity (propidium iodide labelling) of single cells from the lymphocyte gate.

Statistical analysis

We analysed the differences in infection rate and the immune parameters due to parasite species and infection dose. For all analyses, residuals were used to eliminate possible effects of fish family and fish gender. Since the residuals were not normally distributed, we transformed the raw data of infection rate logarithmically, RLU was square-root transformed and for G/L ratio and hepatosomatic index Box-Cox transformation was used.

We used full-factorial ANOVA models with parasite species and infection dose as independent variables. We also tested for differences between the infection-treatments (*C. lacustris*, *A. crassus* and control). Interactions between factors

were not found to be significant in any of the models and were therefore excluded from the analyses. The post-hoc Tukey-Kramer HSD test was used to detect within-group differences in cases where the ANOVA was significant. All tests were performed as two-tailed tests using JMP v.5.0.1.2 (SAS) with a significance level of $P < 0.05$.

Results

Out of 86 surviving fish exposed to doses of 6-24 of either *A. crassus* or *C. lacustris* infective larvae, 56 were found to be infected 18 weeks post exposure. In the *A. crassus* group 22 fish were exposed but not infected (47.8%), whereas in the *C. lacustris* group only 8 fish were not infected (20%). We found no significant difference in any of the investigated traits (hepatosomatic index, G/L ratio, RLU and S+G_{2M} lymphocytes) between exposed but not infected, and infected fish for both parasite species (*t*-test: $N=105$, all $P=0.216$). Infection with *A. crassus* resulted in a maximum number of 6 parasites per fish, whereas a maximum number of 17 parasites per fish were found for *C. lacustris*. Over all infection doses the number of infested *C. lacustris* was significantly higher compared to *A. crassus* (χ^2 test: $\chi_1^2 = 21.5$, $P<0.001$). In the *A. crassus* group the average number (Fig. 7a) of parasites per fish did not vary significantly between infection doses (effect of infection dose: $F_{42,3}=0.7541$, $P=0.5262$). In *C. lacustris*, the number of infested parasites tended to increase for exposure to 6, 12 and 18 parasites respectively, but tended to decrease for the dose with 24 parasite larvae (Fig. 7a) (effect of infection dose: $F_{3,36}=2.2096$, $P=0.1038$).

The infection rates (Fig. 7b) differed significantly between the two parasite species (effect of parasite species: $F_{1,54}=6.5923$, $P=0.0130$). Comparison of the least square means for the *C. lacustris* group showed that the infection rate tended to be highest for 12 parasites (mean 48.8%) and lowest for the 24 parasite dose (effect of infection dose: $F_{1,17}=3.7114$, $P=0.0709$). In the *A. crassus* group no significant difference of infection rate between infection doses could be detected (effect of infection dose: $F_{3,42}=1.3313$, $P=0.2770$).

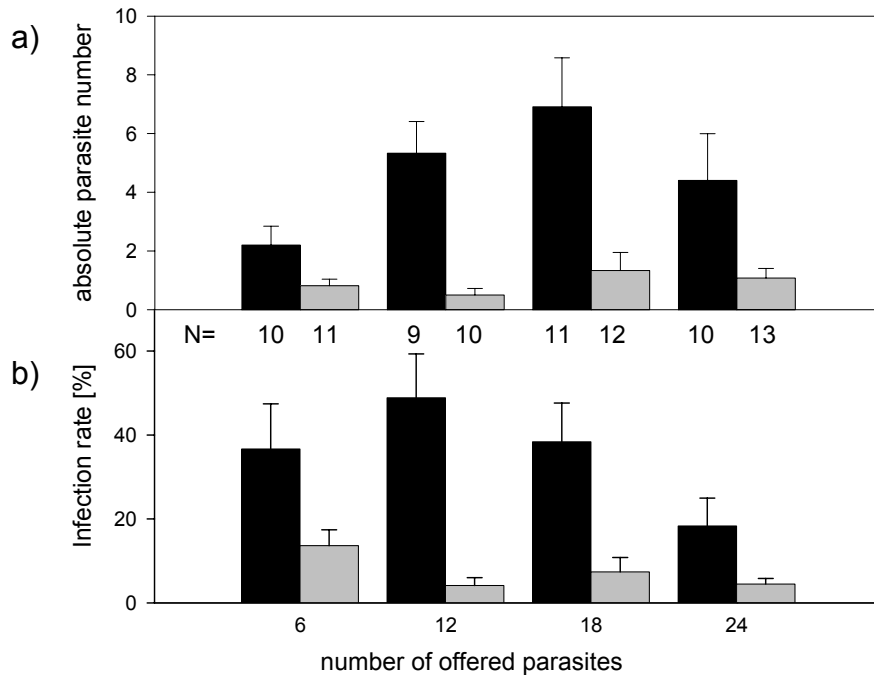


Figure 7: Absolute (mean ± SE) number of parasites (a) and infection rate (b) found in laboratory infected sticklebacks 18 weeks post exposure to four different doses of either *C. lacustris* (black bars) or *A. crassus* (grey bars). In all infection doses, *C. lacustris* shows significantly higher infection rates and higher total numbers than *A. crassus* (ANOVA: $F_{1,54}=6.5923$, $P=0.0130$). Error bars represent standard errors.

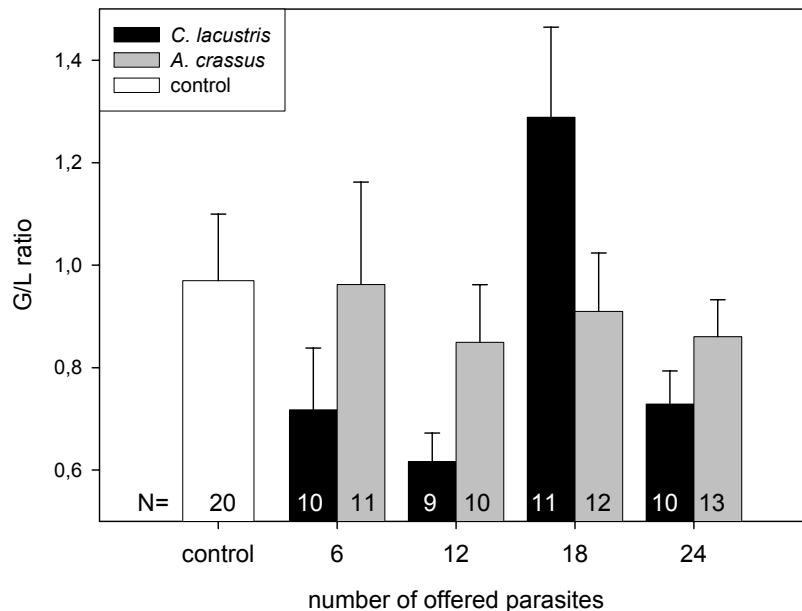


Figure 8: Granulocyte to lymphocyte ratio (G/L ratio, mean ± SE) for fish exposed to four different doses of both *A. crassus* or *C. lacustris* and the non-exposed control group.

To determine the relative activation of innate vs. adaptive immune system, the granulocyte to lymphocyte ratio (G/L ratio) in head kidney leukocytes was measured. The *C. lacustris* group had lower G/L ratios than both the *A. crassus* and the control group for the treatments with 6, 12 and 24, but not for 18 parasites administered (Fig. 8). Within the *C. lacustris* group infection with 18 parasites showed a higher G/L ratio than the other parasite doses which was significant for 6 and 12 offered parasites (effect of infection dose: $F_{3,36}=4.2629$, $P=0.0113$, post-hoc test significant). In the *A. crassus* group, no effect on the G/L ratio of the parasite dose was observed (effect of treatment: $F_{3,42}=1.3313$, $P=0.2770$).

The innate immune responses of sticklebacks against *A. crassus* and *C. lacustris* were tested using the respiratory burst activity (Tab.1, RLU) of head kidney leukocytes. All fish showed elevated respiratory burst activity in cultures with zymosan stimulation compared to not stimulated controls. No significant differences in respiratory burst activity (including spontaneous (no zymosan) and zymosan-induced reaction) were detected for the control group and the two parasite treatment groups (effect of treatment: $F_{3,74}=0.5291$, $P=0.6636$). Also, no effect was found between the different infection doses (*A. crassus*: $F_{3,41}=1.4683$, $P=0.2373$, *C. lacustris*: $F_{3,33}=0.5181$, $P=0.6728$). Proliferation of head kidney lymphocytes (% S+G2M lymphocytes) did not show significant differences between treatment groups either (Tab. 1). Absence of significant effects of the parasite infections on respiratory burst activity and lymphocyte proliferation might indicate that the infection had entered a chronic phase after 18 weeks post exposure.

As a measure for energy stored by the fish we determined their hepatosomatic index. There was no significant difference in energy status between the control and the *A. crassus* and *C. lacustris* groups (effect of treatment: $F_{2,102}=0.5573$, $P=0.5745$). Exposure to different parasite doses had no significant effect on the condition of the fish independently of parasite species (Fig. 9, effect of treatment: $F_{4,100}=1.3657$, $P=0.2513$). However, although not significant in the ANOVA model, fish with the highest parasite load in the group of *C. lacustris* (18 parasites offered, infested: mean: 7 ± 1.66) tended to have the lowest body condition.

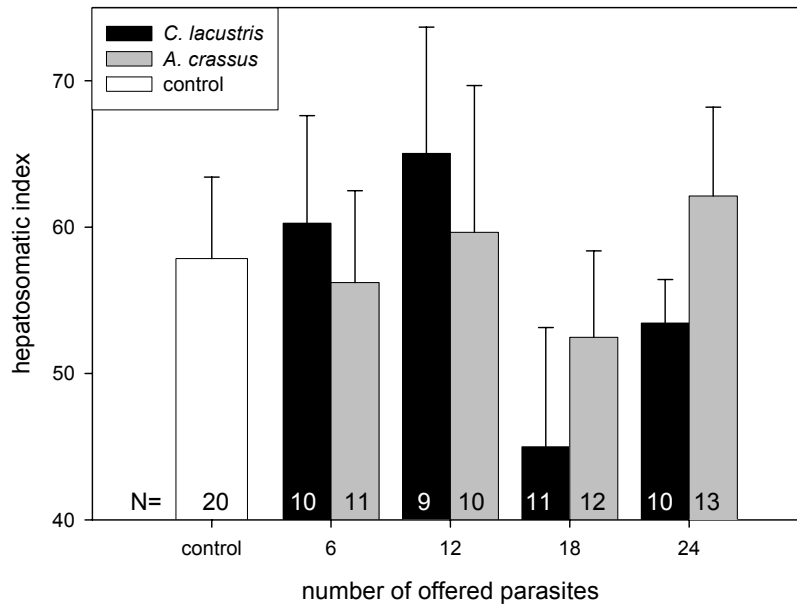


Figure 9: Fish condition as calculated by the hepatosomatic index (mean ± SE) for fish exposed to four different doses of either *C. lacustris* or *A. crassus* and the non-exposed control group.

Table 1: Mean values (mean ± SE) of the investigated immune traits respiratory burst reaction (RLU, $\times 10^5$) and S+G2M lymphocytes (% of total lymphocytes). Significant P values for the ANOVA models were not detected.

Immune trait	Number of exposed parasites:					ANOVA			
	control	6	12	18	24	F	df/error	P	
<i>C. lacustris</i>	RLU	43.02 ± 4.68	44.10 ± 8.15	47.48 ± 4.87	49.13 ± 6.55	42.10 ± 7.68	0.518	3/33	0.673
	lym S+G _{2M}	3.46 ± 0.60	3.21 ± 0.64	3.12 ± 0.50	3.49 ± 0.48	3.22 ± 0.64	0.536	4/34	0.711
<i>A. crassus</i>	RLU		47.66 ± 6.840	45.57 ± 7.66	34.39 ± 3.90	43.52 ± 4.49	1.468	3/41	0.237
	lym S+G _{2M}		3.80 ± 0.75	3.80 ± 0.91	2.73 ± 0.53	2.65 ± 0.65	1.067	4/42	0.385

Discussion

We investigated the infection of three-spined sticklebacks (*Gasterosteus aculeatus*) with the helminth parasites *Camallanus lacustris* and *Anguillicola crassus* under defined laboratory conditions in a dose depended manner. In the laboratory, infection total numbers of infested *A. crassus* (Haenen & van Banning 1990, Thomas & Ollevier 1992) and *C. lacustris* (Kalbe *et al.* 2002) were in the range observed in wild stickleback populations. In our experiment, exposure to equal doses of the two parasites resulted in significantly higher numbers of infested *C. lacustris* than *A. crassus*. Thus, higher numbers of infested *C. lacustris* than *A. crassus* observed in wild caught stickleback (Kalbe *et al.* 2002) are probably not caused by differences in the infection dose. In *C. lacustris* infected sticklebacks, infection rate and total number of infested parasites showed dose depended maxima at 12 and 18 exposed parasites respectively. In the *A. crassus* group, infection rate was low and dose effects were not detectable. The immune reaction of the stickleback host seems to be dose dependent in the case of *C. lacustris*, whereas effects of infection dose were not observed in *A. crassus* infected fish. In *C. lacustris* infected sticklebacks highest G/L ratio was detected in the group exposed to 18 parasites with the highest total number of infested parasites. Both parasite infections had no significant effect on respiratory burst activity when tested in head kidney leukocytes 18 weeks post exposure (Tab. 1). The observed elevated G/L ratio in the *C. lacustris* 18 group did not result in increased respiratory burst capacity as relatively higher numbers of granulocytes were used in the *in vitro* tests. Thus, increase of the G/L ratio could be due to an increase of premature granulocytes in the head kidney, due to elevated consumption of granulocytes at sites of infestation in the gut. Analysis of total cell counts in head kidney leukocytes did not reveal significant differences between treatment groups (data not shown).

At the relatively late time point (18 weeks p. e.) we investigated, the infection had probably come to a chronic stage in which innate immunity was levelled out. Influence of either parasite, or infection dose on the trait of adaptive immunity recorded, the lymphocyte proliferation, was not detectable.

Again, the late (18 weeks p. e.) sampling time point has to be taken into account here. In fish, depending on the environmental temperature, peak antibody response is present two to several weeks after an infection (Rijkers *et al.* 1980). Thus, in the present experiment, lymphocyte proliferation already might have decreased again. Analysis of immune parameters at earlier time-points during both infections is necessary to reveal the underlying mechanisms and kinetics. However, European sticklebacks are new paratenic hosts for *A. crassus*, but seem to be more successful defending the neozoic parasite than the established *C. lacustris*.

Comparisons of pathological effects of *C. lacustris* and *A. crassus* are however not unequivocal because the parasites inhabit different organs of the fish. Furthermore, competition for energy resources between parasite larvae might play an important role for infection success in the different hosts. The copepods we used were chosen with an infection rate of six parasites per copepod, which is a high parasite number compared to the much lower natural infection rates (~ 1 , Sysoev 1985). However, for higher infection doses more than one copepod was fed to the fish, but we did not find proportionally higher infection rates and thus, competition within the copepod seems rather unlikely. Limitation of resources and also direct interaction between parasites could shape the development of simultaneously infecting parasite larvae as Lello *et al.* (2004) state for inter-species interaction. Paterson & Viney (2002) suggest density dependent effects of intraspecific competition to regulate nematode infections. In addition to living *A. crassus* larvae, dead parasites can be found in the swim bladder of infected fish (Haenen & van Banning 1990). Although we did not distinguish dead and alive parasites, parasite-parasite interactions might also have occurred in our experiment. Low infection rates and absence of dose effects in the *A. crassus* group indicate that with 3-4 infested larvae already a maximum is reached. In wild caught stickleback up to 15 infested *A. crassus* were found in single individual stickleback (Kalbe, unpublished), suggesting that in the laboratory infection immune defence is responsible for low infection rates of *A. crassus*.

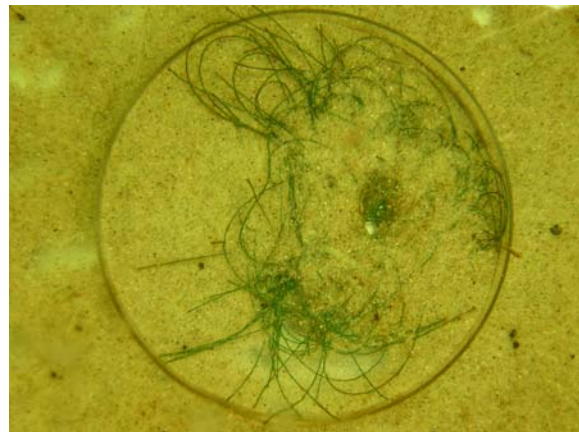
Knopf & Mahnke (2004) investigated the infection success of *A. crassus* in its natural final host, the Japanese eel (*Anguilla japonica*) and the recently

affected European eel (*Anguilla anguilla*). They found the latter, new host to be more susceptible to *A. crassus* infection than the Japanese eel. This might indicate, together with our observations, that *A. crassus* has evolved strategies to evade the immune response of its final eel host, but not to such a high extent of its paratenic stickleback host.

Although we found changes in innate immune parameters that indicate activation of the immune system of the *C. lacustris* infected fish, there was no significant difference in body condition due to exposure or infection. We expected a decrease in the condition of the stickleback since immunity is supposed to be costly (Moret & Schmidt-Hempel 2000, Kurtz *et al.* 2006). Sticklebacks face a variety of parasite species and limited resources in the field, whereas laboratory reared fish might be able to compensate for the impact of an infection under less restricted conditions e.g. with *ad libitum* feeding (Candolin & Voigt 2001). In contrast, Wegner *et al.* (2003b) found a condition decrease in highly parasitised sticklebacks in the field. In summary, the neozoic *A. crassus* was less successful infecting its paratenic host the three-spined stickleback compared to the established *C. lacustris*. *A. crassus* appears to be specialised in adaptation to its final host, the eel and therefore might lack the ability to be adapted to a broad range of paratenic hosts too. For *C. lacustris* however, the high infection rate and the dose response observed in our experiments suggest a high degree of adaptation to the stickleback as paratenic host.

Chapter II

Correlations of MHC with male and female traits



Individual MHC class I and MHC class IIB diversity are associated with male and female fitness traits in the three-spined stickleback

Abstract

Genes of the major histocompatibility complex (MHC) play a major role in pathogen resistance. Sexual selection combined with an ever-changing pathogen community has been hypothesized to maintain the striking polymorphism of those genes. In the three-spined stickleback (*Gasterosteus aculeatus*) there is strong evidence that an intermediate rather than a maximal individual MHC class IIB allele diversity is optimal with regard to parasite resistance. Here, we related individual MHC diversity to fitness traits likely to be affected by parasitization, namely the number of egg clutches laid by a female, male nest quality and the intensity of male breeding colouration. For the first time, MHC class I was investigated in three-spined sticklebacks as well. The sticklebacks under investigation were subjected to either high or low food conditions. The number of egg clutches as well as male nest quality was significantly higher under good food conditions. Whereas for male nest quality an optimum concerning MHC class IIB diversity was found, male breeding colouration was most intense at a maximal MHC class I diversity. Two MHC class I alleles were not only associated with a higher intensity of red colouration but were also more likely to be carried by individuals with a higher allele diversity, possibly explaining the positive correlation. It seems likely that these alleles confer resistance to a specific and presumably common intracellular pathogen.

Introduction

The major histocompatibility complex (MHC) has received much attention with respect to important questions of evolutionary ecology like parasite-mediated selection (e.g. Doherty and Zinkernagel 1975; Hedrick and Kim 1999; Wegner *et al.* 2003a, b) and sexual selection (e.g. Yamazaki *et al.* 1976; Potts *et al.* 1991; Reusch *et al.* 2001; Bonneaud *et al.* 2006; reviewed in Penn and Potts 1999; Milinski 2006). The MHC is central to the specific antibody-based responses of the adaptive (or acquired) immune system of vertebrates and contains the most polymorphic gene loci known in vertebrates (e.g. Janeway *et al.* 2001). MHC molecules are involved in pathogen recognition by presenting pathogen-derived peptides to T-cells that subsequently initiate an immune response. There are two classes of MHC molecules, class I and II. While MHC class I molecules bind peptides derived from intracellular pathogens, e.g. viruses, MHC class II molecules recognize peptides from extra cellular pathogens. The striking polymorphism within those genes might be attributed to the balancing selection of an ever-changing pathogen community (Takahata and Nei 1990; Slade and McCallum 1992). In order to be able to fight a broad range of pathogens, one would expect selection to favour individuals with a high individual MHC diversity, i.e. high individual MHC heterozygosity. However, an increased number of MHC variants may not only bind more foreign antigens but also a greater variety of self-derived peptides, which may lead to an elevated risk to initiate an autoimmune response. T-cell clones that recognize self-peptides bound to MHC molecules are eliminated from the initially highly variable repertoire, thereby reducing the T-cell clone repertoire that is finally available for parasite recognition (Goldrath and Bevan 1999). A balance between the two opposing selective forces of broader pathogen resistance on one hand and increased T-cell clone elimination on the other hand might lead to an optimal rather than a maximal individual MHC diversity (Nowak *et al.* 1992; De Boer and Perelson 1993; Milinski 2006; but see Borghans *et al.* 2003).

The three-spined stickleback (*Gasterosteus aculeatus*) has been in focus of many MHC related investigations (e.g. Reusch *et al.* 2001, Aeschlimann *et al.* 2003, Wegner *et al.* 2003a,b; Kurtz *et al.* 2004; Kurtz *et al.* 2006; Wegner *et al.*

2006). There are probably six loci of MHC class IIB and twelve loci of MHC class I (Sato *et al.* 1998). Unlike in other vertebrates, the MHC class I and class II regions are not linked in teleost fish (Sato *et al.* 2000). In the stickleback, evidence for the existence of an optimal rather than a maximal (i.e. 12 alleles) individual MHC class IIB diversity is mounting, whereby the optimal number of variants seems to be around six alleles. The mean individual diversity in a natural population was found to be 5.8 alleles (Reusch *et al.* 2001). Correlating individual MHC diversity of wild caught sticklebacks with their parasite load revealed a minimal parasite burden in fish with 5.2 MHC class IIB alleles (Wegner *et al.* 2003a). This correlative observation was later confirmed in two experimental studies, in which individuals with an intermediate number of MHC class IIB alleles (i.e. 5-6 alleles) suffered least from parasite infection and were able to reduce parasite growth (Wegner *et al.* 2003b; Kurtz *et al.* 2004). As yet, MHC related studies in the stickleback were restricted to MHC class IIB, thereby missing out on the whole range of intracellular pathogens.

As in many other vertebrate taxa (e.g. reviewed in Penn and Potts 1999 and Penn 2002; Bonneaud *et al.* 2006) mate choice in the three-spined stickleback has been shown to be related to MHC (Reusch *et al.* 2001; Aeschlimann *et al.* 2003; Milinski 2003). In contrast to many other studies, which found disassortative mating preferences (e.g. Yamazaki *et al.* 1976; Wedekind *et al.* 1995; Olsson *et al.* 2003; Richardson *et al.* 2005), stickleback females did not prefer MHC dissimilar mating partners to MHC similar ones (Reusch *et al.* 2001). Rather they chose partners such that, in self-reference to the number of her own alleles, the mean MHC IIB allele diversity of the offspring would be 5.2 (Aeschlimann *et al.* 2003). So far, MHC based female mating preference has only been related to odour signals. However, stickleback mate choice possibly depends on numerous male secondary sexual traits like breeding colouration (Milinski and Bakker 1990; Bakker and Milinski 1991; Milinski and Bakker 1992), nest quality (Barber *et al.* 2001a) and zigzag dance (Rowland 1995). Females are expected to assess their mates by traits honestly reflecting their condition (Zahavi 1975; Hamilton and Zuk 1982). Indeed, the intensity of the male red breeding colouration has been shown to reveal condition and parasitization, and females chose, as expected, the redder

unparasitised male (Milinski and Bakker 1990). Despite this close connection between the immune status of a male and its breeding colouration, male redness has not been related to MHC based pathogen resistance yet. Similarly, other fitness related traits like the male secondary sexual traits mentioned above or female traits such as the number of egg clutches might reflect an individual's immune status.

MHC based pathogen resistance might be more important under poor food conditions since trade-offs are often weaker or sometimes not even visible at all under good environmental conditions (Stearns 1992). If food is limited, a potentially costly immune response can possibly not be compensated by a higher food intake.

In the present study, we related individual diversity of MHC class IIB and MHC class I to relevant fitness traits of the three-spined stickleback, namely the number of egg clutches laid by a female, male nest quality and the intensity of male breeding colouration. This is the first study to investigate MHC class I in the three-spined stickleback. Investigating sticklebacks under high and low food conditions might enable us to evaluate the relative importance of MHC diversity for individual fitness.

Materials and Methods

Study animals

Three-spined sticklebacks were caught in the Große Plöner See (Northern Germany) in December 2003. In the laboratory, they passed through spring temperature and light conditions before they were kept under summer conditions (18°C, 16:8 h light: dark cycle) for the rest of the time. Each fish's length (to the nearest mm) and weight (to the nearest mg) was measured about six weeks after catching (when the fish were transferred from spring to summer conditions). A body condition factor was calculated as $100 \times \frac{W}{L^b}$ where W is the fish weight in g, L the fish length in cm and b the regression coefficient calculated from the logarithm-transformed values of length and weight (Frischknecht, 1993). In the following, this body condition index is referred to as initial body condition. Under summer conditions, fish were fed *ad libitum* with frozen chironomid larvae and additional live food every day (well-fed group) or only frozen chironomid larvae every second day (poorly fed group). Individual fish were randomly assigned to the two food groups.

MHC class I and class II genotyping

Genomic DNA was extracted from a single dorsal spine with a DNA extraction kit (Invitex, Germany) from each individual fish. The MHC class IIB exon 2 ($\beta 1$ domain) diversity was determined by using capillary electrophoresis (CE) single strand conformation polymorphism (SSCP) as described in Binz *et al.* (2001). Recently, CE-SSCP genotyping has been extended to determine individual MHC class I exon 2 ($\alpha 1$ domain) diversity in the three-spined stickleback (Schaschl & Wegner in press). In brief, PCR was carried out by using the MHC class I forward primer GAAC3 (Sato *et al.* 1998) and the following three class I sequence-specific reverse primers (primers were differently labelled on the 5' end with either FAM, HEX or TET): GalRo01 (5'-AGT TTG GTT GAA GCG TTG TTT TGC-3'), GalRo02 (5'-AGT TTG GTT GAA GCG TYG TTT CAC-3'), and GalRo03 (5'-TTG GTT GAA GCG TYG TTT TAG-3'). The forward primer GAAC3 was used in combination with the dye labelled primer GalRo01 in a first PCR reaction and with a GalRo02/GalRo03 primer mix in a second one. The

thermal cycling profile for both PCRs was: initial heating at 95°C for 15 min (hot-start polymerase activation), followed by 30 cycles of denaturation at 94°C for 35 s, annealing at 56°C for 35 s, extension at 72°C for 1 min and ending with a 10 min extension step at 72°C. Fluorescent-labelled PCR samples were prepared for electrophoresis by combining 1 µl PCR product with 9 µl loading mix (8.35 µl Hi-DI formamide, 0.35 µl Genescan ROX 350 standard (Applied Biosystems) and 0.3 µl 0.3N NaOH). The mixture was heated for 5 min at 92°C to separate the complementary DNA strands, chilled on ice and analysed by capillary electrophoresis on an ABI PRISM® 3100 automated DNA Sequencer (Applied Biosystems). The separation of the class I allelic variants was achieved under native capillary electrophoresis by a run temperature of 22°C. The retention times of the MHC allelic variants were identified relative to the ROX 350 standard. The GeneScan and Genotyper software packages (Applied Biosystems) were used to process the obtained SSCP data.

Number of egg clutches, nest quality and male breeding colouration

During 25 days, starting approximately four weeks after fish had been moved to summer conditions, each female's gravidity was assessed every day except day 15 and 20. A female was assumed to have spawned when assessed as being very ripe on one day but not on the following. When ripe, female sticklebacks eventually spawn their eggs even if no male is available.

Assessment of male nest quality took place on day 25, the last day of female assessment. Nests were classified according to five categories: 0 – no nest; 0.5 – nest messy, no visible entrance; 1 – nest messy, entrance vaguely visible; 1.5 - nest neatly glued, but entrance only vaguely visible; 2 - nest neatly glued and entrance clearly visible. The rationale behind this classification was to assess how well the nest was maintained, i.e. if it was glued regularly, also by the male creeping through. An only vaguely visible entrance represents an opening that is not regularly used and glued. All nests were assessed by the same person.

The red breeding colouration was analysed about one week after the assessing period. For that, males were photographed ventrally after having

been stimulated with a ripe female for 15 min the day before. The males were gently fixed with a sponge in a water-filled plastic box, which had a window of filter glass (high resolution skylight filter, Hama, Germany) to ensure that the whole colour spectrum was transmitted. The pictures were taken within a dark box using a digital camera (Olympus E20-p) with a 36 mm macro-objective. Closing time of the lens was 1/60 sec with aperture 7. For illumination, we used 4 cold lights (KL 1500 LCD, Leica) with 3300 K colour temperature according to daylight illumination. Intensity analysis of the red colouration was performed with IP Lab 3.6.2 for Mac OS 9.2.2 (Scanalytics, Inc.). On each picture, we selected an area of the throat covering the majority of the red parts (ROI, region of interest). The mean red intensity was determined using the R/RGB model (8-bit red-green-blue colour model, Frischknecht 1993). All pictures were measured twice in random order, on two consecutive days. The marking of the area and the resulting red values were highly repeatable: 95.9% for the marked area as well as 99.98% for the mean colouration.

Parasites

Only males were dissected and screened for parasites. Since not all dissections could be done on the same day, only parasite species for which we were sure that they could not be eliminated during that time or during the assessment period were taken into account: the three trematode species *Diplostomum sp.*, *Cyathocotyle prussica* and *Echinochasmus sp.* as well as the nematode *Contracaecum sp.* Individuals of the respective parasite species were counted and an overall parasite load was calculated for each fish using the following approach (Rauch *et al.* 2006): for each parasite species, a relative number of individuals per fish was determined (number of individuals per fish divided by the maximum number of individuals of that respective species found on a single fish). The relative numbers of the four parasite species were summed up and averaged over the four species.

Statistical analysis

To analyse the influence of food group and individual diversity of MHC class I and MHC class IIB on the number of egg clutches, an analysis of

variance (ANOVA) was conducted. Parasite load could not be considered since there was no data available for the females. Their initial body condition was not taken into account either because it could not be excluded that some individuals had already developed eggs at the time of measurement.

Males were tested for significant effects on nest quality with a generalized linear model (GLM) incorporating food group, number of MHC class I alleles, number of MHC class IIB alleles and parasite load as independent variables and initial body condition as a covariate. The same factors were considered with respect to male breeding colouration performing an analysis of covariance (ANCOVA).

All interactions were initially included in the models and non-significant ones subsequently removed (at the 10% significance level) by performing backward elimination. Significant single factors that are also part of significant interactions are considered when a post-hoc test (t-test or Chi-square test) verified its sole significance.

Chi-square tests - or two-tailed Fisher's Exact Tests when sample sizes were too small - were used to test for significant differences in allele frequencies when comparing the reddest and dullest quarter of the test fish. To reveal whether the presence of the alleles identified by the above analyses increases with increasing individual allele diversity regression analyses were conducted. To account for the fact that only few individuals were available carrying allele numbers at the marginal ends of the allele range, the regression analyses were weighed by sample size. Finally, Oneway-ANOVAs were performed to test for significant effects of the presence of those alleles on male redness. A post-hoc test (Student's t-test) was applied to obtain the respective significance level.

All statistical analyses apart from the GLM were performed using the software JMP 5.0.1.2 for Macintosh (SAS Institute Inc. 2003). The GLM was conducted in R statistical package version 2.1.1.

Results and Discussion

Number of egg clutches

Females under good food conditions laid significantly more egg clutches than poorly fed females (ANOVA, $N = 75$, $df = 1$, $F = 43.04$, $P < 0.0001$, Fig. 10). Wootton (1994) and Fletcher and Wootton (1995) concluded that the number of clutches laid per breeding season is dependent on the current food supply, whereas clutch and egg size are a function of female size at the start of the breeding season, thereby being only little affected by food availability. This would mean that egg size and the number of eggs per clutch are fixed before the start of the breeding season on the basis of current condition and food shortages occurring afterwards are then traded off by reducing the number of spawnings. However, current food availability seems to have a higher impact on female fitness since reducing the number of clutches also reduced the total number of eggs spawned within a breeding season (Wootton 1994). The poor food conditions applied in this study are not insufficient and probably still better than the food conditions encountered in nature; nevertheless, we found a big difference between the two food groups. This clearly emphasizes the importance of diet on individual performance and proposes to cautiously think about the feeding regime when conducting laboratory experiments (see also Barber *et al.* 2000; Candolin and Voigt 2001).

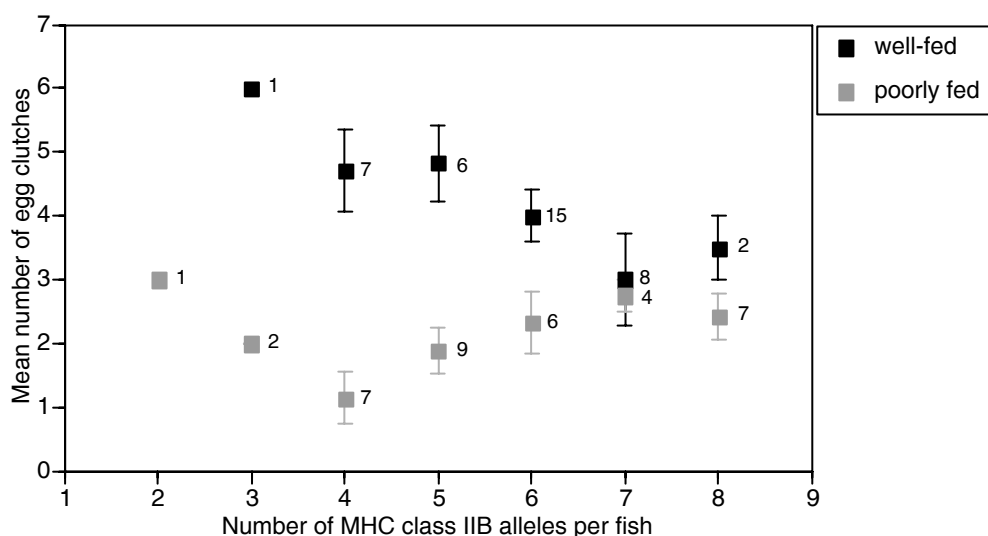


Figure 10: Mean number of egg clutches laid by female sticklebacks during the 25-day-assessment time in relation to individual MHC class IIB allele diversity. Sample sizes are given besides each data point. Error bars indicate one standard error.

It seems that it is not current food availability alone that determines the number of egg clutches a female lays. We found a significant interaction between food level and individual MHC class IIB diversity on the number of egg clutches (ANOVA, $N = 75$, $df = 1$, $F = 11.10$, $P = 0.001$, Fig. 10). It might be that the importance of parasite resistance provided by adaptive immunity is higher when current energy availability is low. Due to the high immuno-pathology caused by, for instance, oxidative stress, innate immune mechanisms are thought to be very costly (von Schantz *et al.* 1999; Graham *et al.* 2005). When MHC-based adaptive immunity is efficient, an individual could be able to down-regulate the costly innate immune response (Kurtz *et al.* 2004; Kurtz *et al.* 2006). While a non-optimal individual encountering good food conditions might still be able to compensate the costs of a higher innate immune response, it might have to re-allocate energy to the costly innate immune response under poor food conditions. Since the number of egg clutches is highly affected by current energy income (Wootton 1994) such re-allocation might result in a reduced number of clutches in females with a non-optimal number of MHC IIB alleles. This might explain the increase in clutch number at an intermediate number of MHC class IIB alleles (5 to 7 alleles) in the poorly fed group (Fig. 10). Considering the above hypothesis, one would not expect a decrease in clutch number at an intermediate allele number in the well-fed group (Fig. 10). However, the 25 days during which female clutches were surveyed do not represent a whole stickleback breeding season. It might be that individuals with non-optimal allele diversity, although being able to compensate costly innate immune responses, invest more in early reproduction, whereas individuals with an optimal number invest during the whole breeding season.

Nest quality

As in females, food condition significantly affected male performance. Well-fed males maintained a nest of significantly higher quality than poorly fed males (GLM, $N = 72$, $df = 1$, $F = 5.88$, $P = 0.019$). Maintaining a high quality nest involves regular glueing, which might require a higher glue production. The glue is produced in the males' kidneys and they were significantly bigger in

males that owned a nest of high quality (Barber *et al.* 2001a) as well as in males that were kept under good food conditions (Wootton 1994).

There was a significant effect of individual MHC class IIB diversity on nest quality (GLM, $N = 72$, $df = 1$, $F = 5.82$, $P = 0.019$). This effect is due to the fact that most of the individuals maintaining a high quality nest were carrying an intermediate number of MHC class IIB alleles, whereas most individuals with a low or high allelic diversity had nests of lower quality (Fig. 11). The optimum, i.e. the number of alleles at which the highest proportion of individuals owned a nest of the highest quality, was 6 alleles (Fig. 11). This is well in accordance with previous correlative and experimental studies that also found an optimal individual diversity of around six MHC class IIB alleles (Reusch *et al.* 2001, Aeschlimann *et al.* 2003; Milinski 2003; Wegner 2003a, b; Kurtz *et al.* 2004).

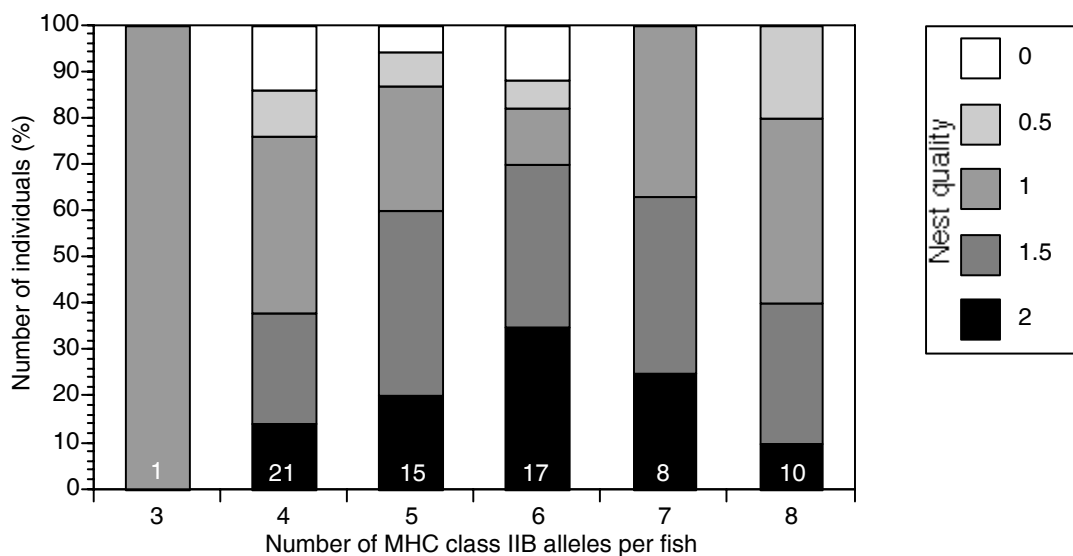


Figure 11: Proportion of male sticklebacks maintaining nests of different qualities ranging from 0 (low nest quality) to 2 (high nest quality) in relation to individual MHC class IIB allele diversity. See text for details on how nest quality was assessed. Sample sizes are given at the bottom of the bars.

Regarding MHC class I, there was a significant interaction between food level and allele diversity on nest quality (GLM, $N = 72$, $df = 1$, $F = 4.99$, $P = 0.029$). However, the range of individual allelic diversity was much smaller in the poorly fed than in the well-fed group (a range of 6 – 10 different MHC class I alleles in the poorly fed versus a range of 4 – 12 different alleles in the well-fed group). Thus, this result should be treated with caution. Food level also

interacted significantly with parasite load on nest quality (GLM, $N = 72$, $df = 1$, $F = 17.02$, $P < 0.001$) and there was also a significant interaction with initial body condition and parasite load on nest quality (GLM, $N = 72$, $df = 1$, $F = 6.69$, $P = 0.012$). It thus appears that factors affecting nest quality are more complex. A fish's condition (initial and current), MHC class I diversity as well as its parasite load seem to have an influence.

Male breeding colouration

Data of the two food groups were pooled because an analysis of covariance showed no statistical difference between them ($N = 73$, $df = 1$, $F = 0.92$, $P = 0.341$). We found a strong positive correlation of male breeding colouration with individual MHC class I diversity, i.e. the higher a male's number of MHC class I alleles the redder it is (ANCOVA, $N = 73$, $df = 1$, $F = 9.29$, $P = 0.003$, Fig. 12).

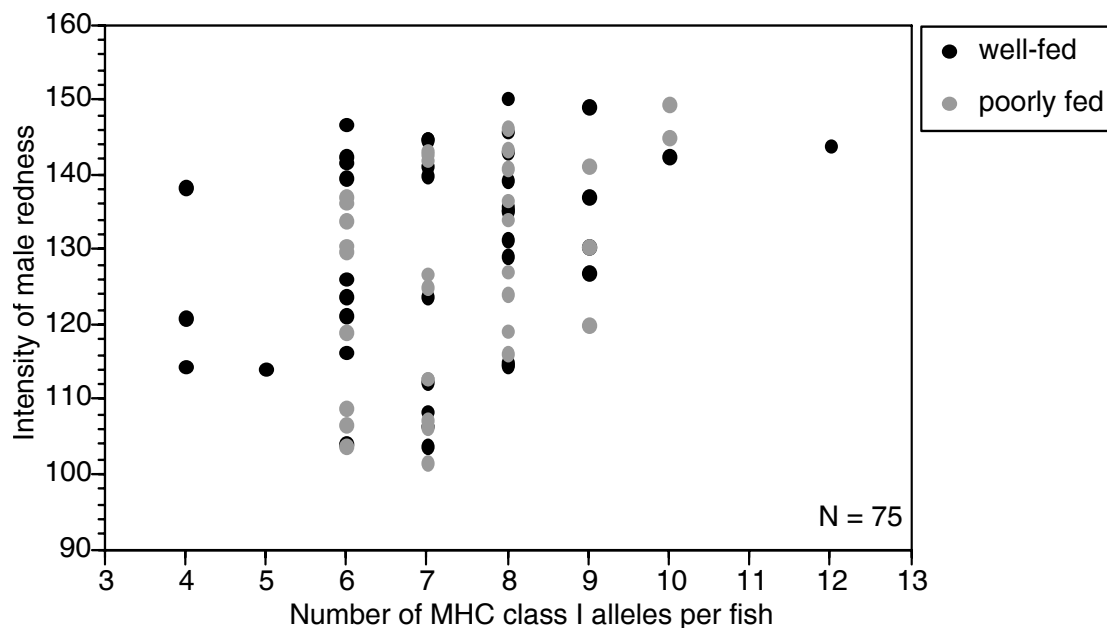


Figure 12: Intensity of male breeding colouration in relation to individual MHC class I allele diversity.

Due to the hypothesis of the two opposing selective forces acting on the number of MHC alleles –increased pathogen recognition versus increased auto-reactivity and thus increased de-selection of T-cell clones - we expected the males carrying an intermediate rather than a high number of MHC class I

alleles to express the most intense colouration. However, this assumes the two selective forces to act on a purely quantitative basis, i.e. the number of alleles being selected regardless of the nature of the respective alleles. If there is, however, an allele providing strong resistance to a predominant pathogen, one could imagine a net benefit of having this allele even in individuals with a super-optimal allele number. Since the likelihood to possess a specific allele should increase with the number of alleles per individual, we suspect that the males that are redder having a high diversity of MHC class I might carry such alleles. To test this hypothesis and to potentially identify such specific alleles, we compared the reddest quarter of the test fish with the dullest quarter according to their MHC class I allele composition.

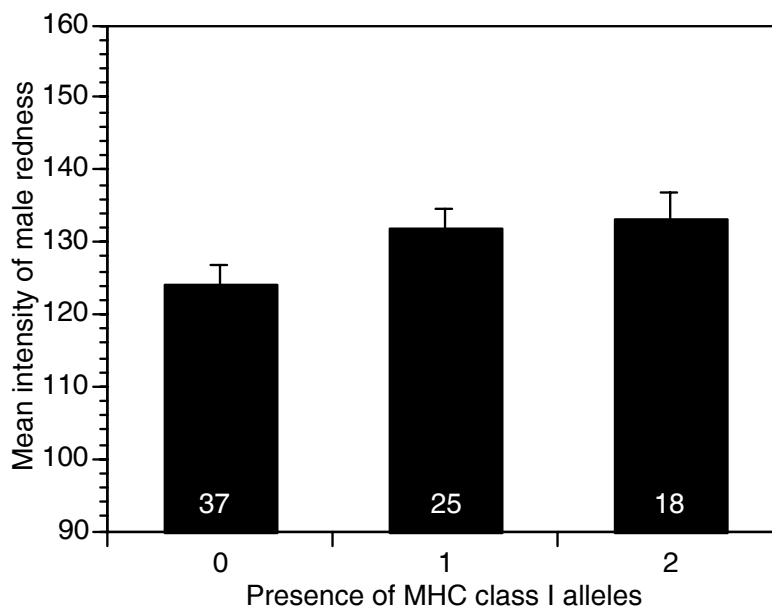


Figure 13: Mean intensity of male breeding colouration in relation to the presence of none (0), one (1) or both (2) of the identified specific MHC class I alleles. The two alleles were found to be carried significantly more often by individuals belonging to the reddest quarter when compared to the dullest quarter of the test fish. Sample sizes are given at the bottom of the bars. Error bars indicate one standard error.

Two alleles were found to be present significantly more often in red males than in dull males. 65% of all red males carried Allele 16, while it was only 20% of the dull males (Chi-square test, $N = 40$, $df = 1$, $\chi^2 = 8.29$, $P = 0.004$); Allele 31 was present in 60% of the red males as opposed to 20% of the dull males (Chi-Square test, $N = 40$, $df = 1$, $\chi^2 = 5.01$, $P = 0.025$). Interestingly,

when the data set was split up with respect to the two food groups, significance only remained in the poorly fed fish group (two-tailed Fisher's Exact Test, Allele 16: low food: $N = 18$, $P = 0.009$, high food: $N = 22$, $P = 0.395$; Allele 31: $N = 18$, $P = 0.0498$, high food: $N = 22$, $P = 0.67$). Within all test fish, the presence of at least one of these alleles was related to a more intense colouration (ANOVA, $N = 80$, $F_{2,77} = 3.68$, $P = 0.03$, Fig. 13). Again, after splitting up the data set, this relationship only remained significant for the fish under low food conditions (ANOVA, low food: $N = 37$, $F_{2,34} = 3.44$, $P = 0.044$; high food: $N = 43$, $F_{2,40} = 0.794$, $P = 0.459$). Furthermore, for both alleles, the likelihood for an individual to carry them increased with increasing individual allele number (Weighted linear regression, $N = 165$, Allele 16: $r^2 = 0.83$, $F_{1,7} = 35.39$, $P = 0.0006$; Allele 31: $r^2 = 0.61$, $F_{1,7} = 10.96$, $P = 0.013$). It thus seems that certain alleles can indeed achieve such an importance that the advantage of carrying it outweighs the disadvantage of being above the optimal allele number. Various studies have found specific MHC alleles associated with resistance to a certain pathogen (e.g. Langefors *et al.* 2001, Grimholt *et al.* 2003, Bonneaud *et al.* 2005; reviewed in Milinski 2006) and also in the stickleback, four MHC class IIB alleles were found that were significantly associated with the resistance against one specific macroparasite species (Wegner 2004a). It seems likely that the two MHC class I alleles that correlate with intense breeding colouration provide resistance against a specific and presumably common pathogen. Since MHC class I is involved in the recognition of intracellular pathogens such as viruses, identification and quantification of the pathogens was not feasible. Male sticklebacks showing a higher MHC based resistance could have a lower demand for innate immune activity, thus be less prone to oxidative stress (von Schantz *et al.* 1999; Graham *et al.* 2005), and therefore be able to allocate the available antioxidants to the carotenoid-based breeding ornamentation (Brush and Reisman 1965) instead. Experimental evidence for the existence of such a trade-off comes from a study on blackbirds, in which males immunised with an antigen showed a significant decrease in the intensity of the carotenoid-based colour of their bill (Faivre *et al.* 2003). Also three-spined sticklebacks reduced their carotenoid-based red colouration after being exposed to parasites (Milinski and Bakker 1990). Furthermore, it has been shown that sticklebacks with an

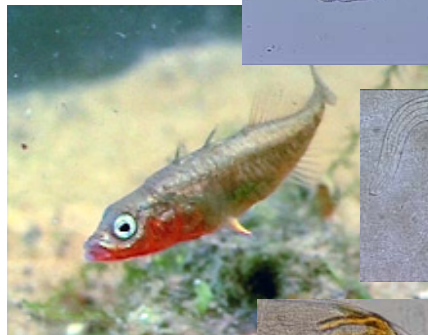
optimal number of MHC class IIB alleles show the lowest respiratory burst reaction, an innate immune mechanism that generates reactive oxygen molecules to kill pathogens (Kurtz *et al.* 2004). Moreover, sticklebacks with a non-optimal MHC class IIB diversity have higher levels of MHC gene expression (Wegner *et al.* 2006), which correlates with increased oxidative stress (Kurtz *et al.* 2006). Hence, the above finding of males being redder when carrying at least one of the MHC class I alleles could be explained by the lower demand of radical scavengers due to decreased oxidative stress. The importance of the trade-off might, however, depend on the energy income of the fish as indicated by the fact that the allele associations only remained significant in the poorly fed group. As already discussed for the females, well-fed males that do not carry at least one of those specific alleles might still be able to compensate a higher innate immune response and nevertheless become red, whereas poorly-fed males might not.

In conclusion, although just correlative, our study underlines the general importance of MHC allele diversity with respect to individual fitness. It does not only support the hypothesis of an allele optimum for MHC class IIB, which for the three-spined stickleback is at about six alleles, it also, for the first time, finds MHC class I diversity to be related to individual fitness. Two MHC class I alleles were identified potentially providing resistance to a specific and presumably common pathogen, possibly explaining the unexpected finding of a maximal instead of an optimal allelic diversity with relation to male breeding colouration. It has previously been shown that the red throat is an honest signal revealing a male's health (Milinski and Bakker 1990). Our findings might take this a bit further in that not only a male's condition but also the genetic value of its immune system is expressed by the intensity of the red ornamentation supporting the good-genes hypothesis (Hamilton and Zuk 1982). Females choosing the reddest male would then not only gain fitness benefits through a healthy mating partner taking care of the offspring, but also by ensuring that the offspring will be endowed with a good immune system. This is in accordance with the finding that offspring of brighter males were more resistant to infections with the cestode *Schistocephalus solidus* (Barber *et al.* 2001b). Furthermore, when redness can be linked to resistance against presumably common

pathogens, olfactoric mate choice based on MHC allele number could be improved by visual mate choice guaranteeing that not only the optimal MHC allele number but also relevant resistant alleles are provided for the offspring as had been suggested by Aeschlimann *et al.* (2003) and Milinski (2006).

Chapter III

Laboratory mate choice experiments



Differences in female sticklebacks' preference between visual, olfactory and combined mate choice situations

Abstract

We analysed mating preferences of female three-spined sticklebacks (*Gasterosteus aculeatus*) for non-infected versus infected males depending on their MHC classIIB variant number. With a new developed experimental setup we could test visual and olfactory choice independently. In addition, it was possible to combine the two and test the natural combination. We recorded several male traits that might play a role for the female mating decision such as redness, condition index, hepatosomatic index (HSI) and gonad index (GSI). In the visual choice situation, females significantly preferred the redder male of a given pair. However, females did not prefer males with respect to their infection status in either of the test situations. In the olfactory choice situation, females significantly preferred the males optimal according to their own MHC. These results underline those of earlier studies that found redness to be a mediator of male parasitisation, although redness was not correlated with infection in our experiment. In the combined choice situation, when the female could see and smell the two males, it significantly preferred the one with the higher HSI. HSI might be a reliable measurement of the overall condition of a male as we detected a correlation with condition, infection and via the GSI also with MHC allele number.

Keywords

mate choice, olfactory, visual, combined, multiple ornaments, parasitism, three-spined stickleback

Introduction

Females prefer certain males that provide direct or indirect fitness benefits for the offspring and these benefits are often reflected by the males' secondary sexual traits (Kirkpatrick & Ryan 1991, Anderson 1994). In the three-spined stickleback, *Gasterosteus aculeatus*, many studies experimentally proved females' preference for males' secondary sexual traits. Among others, it was shown that in visual choice situations, females preferred redder males (Milinski & Bakker 1990, 1992, Bakker & Milinski 1991, Bakker 1993, Bakker & Mundwiler 1994, but see also Nilsson & Nilsson 2000). Male redness can reflect the parasite load of the males (Milinski & Bakker 1990, Folstad *et al.* 1994) and potentially signals detailed information e.g. male condition to females and competing males (Wedekind *et al.* 1998). Another visual trait females base their mating decision on is the pectoral spine symmetry (Mazzi *et al.* 2003, Mazzi *et al.* 2004) which is a measure for an individuals' ability to persist environmental disturbances during development (van Valen 1962). Furthermore, the zig-zag courtship dance and the pectoral fin size reveal paternal quality (Künzler & Bakker 2000) and are traits the females use for their mating decision (Rowland 1995, Bronseth & Folstad 1997, Bakker & Mundwiler 1999, Ishikawa & Mori 2000, Barber 2001a). Only recently also UV reflectance was shown to affect female mate choice in sticklebacks (Rick *et al.* in press).

But not only visual traits seem to be important for the female choice in sticklebacks, also olfactory mate choice could be detected (Reusch *et al.* 2001, Aeschlimann *et al.* 2003, Milinski 2003, McLennan 2003, Häberli *et al.* 2004). In this context, chemical cues associated with genes of the major histocompatibility complex (MHC) caught most attention (Yamazaki *et al.* 1976, Milinski *et al.* 2005). MHC molecules play a major role in initiating the acquired immune response by presenting peptides derived from pathogens (Klein 1994). Due to specific binding capacities of single MHC molecules, heterozygous individuals are able to detect more pathogens at the same time than others (Doherty & Zinkernagel 1975). Since mammalian females tend to increase MHC heterozygosity by choosing mates with MHC genotypes different from their own (Yamazaki *et al.* 1978, Wedekind & Furi 1997, Potts *et al.* 1991), one

hypothesis for the functional basis of MHC based mate choice is an increased parasite resistance in offspring (Wegner *et al.* 2003a, b, Kurtz *et al.* 2004). However, too many different MHC alleles within an individual could lead to an increased risk of autoimmune disease (Janeway 2001). Therefore, one would expect optimal rather than maximum MHC heterozygosity (Nowak *et al.* 1992, DeBoer & Perelson 1993, Borghans *et al.* 2003). In sticklebacks, females use a self-referential process signalled by peptide ligands to assess MHC class IIB variants of a possible mating partner (Aeschlimann *et al.* 2003, Milinski *et al.* 2005) and choose that male with which they can reach an intermediate number of around 5-6 MHC alleles for their offspring (Reusch *et al.* 2001, Milinski 2003). Fish with an intermediate number of MHC sequence variants have a clear fitness advantage, because they are less likely to get infected under experimental conditions as well as in the wild (Wegner *et al.* 2003a, b, 2004a) and suffer less from the infection (Kurtz 2003, Kurtz *et al.* 2004). This suggests that a direct link exists between female mate choice and parasite resistance. In mice it was found that females can smell a possible parasitisation directly in the odour marks of males (e.g. Kavalier & Colwell 1995, Penn & Potts 1998, Zala *et al.* 2004) and this function might also play a role in sticklebacks. It is not yet clear which other information could be signalled via the odour of a male, but condition traits (Candolin 1999a) could also be involved or hormones as shown for humans (Rikowski & Grammer 1999).

However, in most species females base their mating decision not only on one, but on different, multiple traits which also is hypothesised for sticklebacks (Kraak *et al.* 1999, Künzler & Bakker 2001). Such male traits need not always carry information they can also be uninformative with respect to the fitness of the bearer but facilitate for instance partner detection or signal assessment (Iwasa & Pomiankowski 1994, Candolin 2003). How the use of multiple traits evolved and what these traits indicate is a highly discussed topic in the area of mate choice resulting in three main theories (reviewed in Møller & Pomiankowski 1993, Candolin 2003):

- 1) Evolution of multiple message cues (Johnstone 1996), which can apart reflect different components of the males' condition or changes on a different time-scale. Some cues could reflect the general condition which

is important as a long-time reserve but others might reflect the actual condition as reaction to sudden changes of the environmental conditions.

- II) Multiple ornaments might have evolved as redundant signals correlated with condition and therefore serving in combination as more reliable cues for the female (Rowe 1999). This includes the decreased possibility of cheating, since it should be less costly to actively change one cue compared to changing more than one.
- III) At last, some signals might be unreliable and not used any more but maintained because they are not costly or they evolved by Fisher's selection (Fisher 1930)

Which of these hypotheses for ornaments and mate choice in sticklebacks is more likely has not been studied so far and they might not be mutually exclusive (Evans & Magurran 2000). One of the goals of our study was to test which of these theories is the most probable in our model organism.

To our knowledge, this is the first study which tested in one experiment which male of a pair a certain female chooses if visual or olfactory traits are available exclusively and in combination. For that, we used a setup which enabled us to test the olfactory and visual choice independently but also in combination so that the female could see and smell the male at the same time. Furthermore, we were interested in whether the infection with a natural occurring parasite combination alters the ornamentation and if females thereby are able to detect the parasitisation. We recorded male traits that might play a role for the female mating decision such as redness, hepatosomatic index (HSI, Wootton 1977), gonad index (GSI, Stockley *et al.* 1997) and condition index (Bolger & Conolly 1989). With this comprehensive approach we want to shed more light into the complexity of female mate choice in general and their ability to use multiple traits in particular.

Methods

Experimental animals

Three-spined sticklebacks (*Gasterosteus aculeatus*) were laboratory bred offspring from 7 pairs of genetically different parents from the Grosser Plöner See population (Schleswig-Holstein, Germany) caught in 2003. After initial feeding of the fry with live *Artemia*, the fish were fed mixed frozen food (chironomids, *Daphnia*, *Artemia*) until the beginning of the experiment. Fish were maintained in full-sib groups of 20 fish in 16 L aquaria. To simulate a full season, fish were transferred to summer conditions (18°C, 18:6h light: dark cycle) after passing lab winter (6°C, 6:18h light: dark cycle) and spring conditions (12°C, 12:12h light: dark cycle), where they stayed until the end of the experiment. Under summer conditions, males were singly maintained in 16 L aquaria with an 8 cm Petri-dish filled with sand and supplied with green standard-length synthetic threads as nesting material. Males were positioned next to each other within pairs of brothers and according to their treatment for the choice test (group 2 and 3: optimal next to suboptimal, with respect to a certain MHC of the same family, group 1: two optimal, e.g. MHC similar males next to each other). Opaque partitions prevented sight between aquaria. Females were maintained in 16 L aquaria that were lengthwise split into two halves by solid, opaque partitions in groups of 3, respectively. During the experiment all fish were fed mixed frozen food and additionally live *Daphnia*.

Only males actively caring for a nest and reacting with zigzag courtship dance to a ripe female the day before the experiment were used for the choice trials. Females were used only if they were in spawning condition, indicated by head-up posture to a courting male. To test for the reaction to courtship only such males and females were used that were not included in the later tests. For the transport of the females to the test aquaria a glass pipe filled with water was used (Milinski & Bakker 1992, Wedekind & Milinski 1996) to prevent major disturbance.

For the exposure of the fish, the three most abundant parasite species, *Camallanus lacustris*, *Anguillicola crassus* and *Diplostomum pseudospathaceum*, in lakes in Northern Germany were used (Kalbe *et al.*

2002). Three-spined sticklebacks serve as paratenic host to the two nematodes *C. lacustris* and *A. crassus*, both transmitted by ingestion of intermediate hosts. As intermediate hosts, copepods (*Macrocyclops albidus*) were taken from a laboratory culture and prepared for infection as described in Hammerschmidt & Kurtz (2005a). Infection and maintenance of the copepods were performed as described in Wegner *et al.* (2003b). Each copepod was singly exposed to six actively moving larvae of the assigned parasites (*C. lacustris* or *A. crassus*). One week before exposure to fish, the number of larvae within each copepod was determined using a microscope. The third parasite species, *D. pseudospathaceum*, is released into the water as small larvae (cercaria) by its snail host (*Lymnaea stagnalis*). The cercariae penetrate the fish actively through the skin and then migrate into the eye lenses of the host.

The fish that were assigned to be infected (the optimal males in group 1 and 2, the sub-optimal males in group 3) were exposed twice to the three parasite species with similar infection doses to ensure an elevation in the adaptive immune response. The first exposure took place 17 weeks, the second 4 weeks before the experiment. Each fish was offered copepods infected with a total number of 4 larvae (first exposure) and 6 larvae (second exposure) of the respective nematode and additionally exposed to 20 cercaria of *D. pseudospathaceum* (both exposures). The fish that were assigned to be non-infected were treated in the same way but were offered the number of uninfected copepods the infected partner got.

MHC classIIb determination

MHC determination of male and female sticklebacks was performed using single-strand conformation polymorphism (SSCP) as described in Wegner *et al.* (2003a). For each fish the number of MHC alleles was counted following Reusch *et al.* (2001). Mean allele number of the males was 5.89, which was also true within the groups optimal and non-optimal with respect to the female, whereas mean allele number of the females was 6.33. Males were assigned to be optimal or non-optimal with respect to the female as described in Milinski *et al.* (2005). From now on we will use the term 'optimal' MHC for the best fitting

male according to the females' MHC class II B genotype and 'non-optimal' for all other males.

Experimental design

The top view of the setup is presented in Figure 14. We created an experimental setup with a box for the female which could be placed in front of the aquaria of two males without disturbing them. Between the female test tank and the males' aquaria a removable opaque plate was installed. The handling of this plate did not disturb the fish in most of the tests. In the closed position the plate hindered the female to see the two males and their aquaria for the olfactory test.

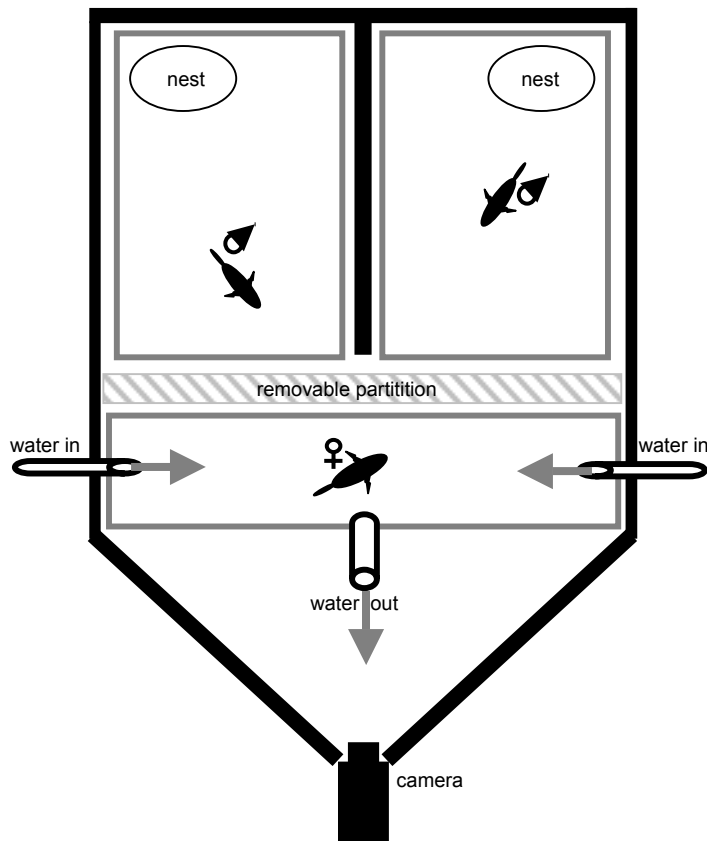


Figure 14: Draft of the experimental setup from top view with the female compartment and the two males' aquaria. An opaque partition could be added or removed between female and male aquaria, depending on which kind of test was performed. Water inflow was on either side, water outflow in the lower middle of the female aquarium. Connected to the inflow hoses were small tubes connected to bottles with male water. The behaviour of the female was recorded with a video camera placed opposite to the male aquaria which enabled also the observation of the males' activity towards her.

If a disturbance was recognised, the measurement of the time was only started when the male's started courtship and the female normal swimming behaviour again. To analyse the female's preference for a distinct male (with visual, olfactory or combined cues) the time it spent in front of each male was measured with respect to the following parameters: Time was only recorded if the female appeared to be calm and undisturbed. Furthermore, the female had to be facing the male (if visual cues were available) or the water inlet (if olfactory cues were available). As an exception to this, time was also measured if the female was within 5 cm of the walls bearing the inlets. Finally, time was never recorded if the fish was in the middlemost area (i.e. 3 cm around the centre of the set-up). The analysis of all films was performed by an independent person (R. Sommerfeld), without knowledge of the used male-male combinations.

On each side of the female aquarium an inlet strainer was installed. We used a peristaltic precision pump (ISMATEC) to keep up a flow through system with constant water supply and outflow. A second pump was used to add ~100 ml/min of male water equally on either side of the female tank. For the male water supply 1 L was taken directly from above the nest of the male instantly before the experiment (Aeschlimann *et al.* 2003) and added on the respective side of the female tank. After a 5 minute break with tap-water supply, the sides of the male water were switched to control for side preference. For later analysis the mean time of the two olfactory tests was used. For the visual test, the plate was gently lifted resulting in visibility of the whole male aquaria including the nests and the males themselves. In this test, pure tap-water was added on both sides of the female tank. The combined test is conforming to the visual test but with additionally male water supply on either side of the female tank as described for the olfactory test. This test is probably the closest to the natural choice situation in the field with availability of visual and olfactory cues.

The three male-male combinations we tested combined certain MHC background with respect to the female and infection status: (1): optimal-infected versus optimal non-infected, (2): optimal-infected versus not-optimal-non infected and (3): optimal non-infected versus non-optimal infected one. Test (1) was used to test whether female sticklebacks can distinguish between infected

and non-infected males if their MHC background is similar. If so, they should choose the non-infected male since it should be in better condition and therefore the better father. In test (2) we expect the females to choose the optimal male if infection status plays no role for her choice, or the non-infected male if the actual infection is more important than the MHC. According to results from previous studies (Wegner *et al.* 2003a, b, 2004) we expect the combination of test (3) to be the one most probably occurring in nature with the optimal male having lower parasite load, e.g. no parasites. Females should prefer the optimal non-infected male in this situation.

The female and the two available males were siblings from the same family, assuming their genetic background to be similar. Each triplet consisted of one female that was used only once during the experiment and a pair of males. Only three females were used with a second, different male pair in a distinct spawning cycle. For each male a digital photograph was taken directly after the test and mean redness of the throat colouration was analysed for a certain region. This region was chosen from the tip of the snout to the ankle of the mouth, to the edge of the gill-lid and back on the other side. All pictures were measured twice, on two consecutive days in the R/RGB 8-bit colour model (Frischknecht 1993). The marking of the area as well as the values for the red colouration were highly repeatable: 95,8% for the marked area and 99,9% for the mean colouration.

Fish Dissection

Fish were dissected 4-6 weeks after the last experiment. The dissection was performed as described in Kalbe *et al.* (2002). *C. lacustris* larvae were counted by squeezing the total gut of the fish between two glass plates and *D. pseudospathaceum* larvae were counted in the dissected eye-lenses of the fish, under the dissection microscope respectively. *A. crassus* larvae were counted in the swim bladder on a slide under the microscope. For the test of female preference, we determined whether an exposed fish was infected with at least one of the parasites, which was the case for all of them. We recorded condition index, HIS and GSI for each male. For the HSI, liver weight per total fish weight

was calculated and GSI was calculated as weight of the testes per total fish weight. For the body condition we used the formula by Bolger & Conolly (1989).

Statistical analyses

We measured the time the female spent in front of each male and calculated preference indices from the time (P_m) the female spent in front the male as proportion on total time (P_t) spent choosing (R . Sommerfeld, P_m/P_t). Then we decided for each male pair which one was the redder, had the higher HSI, GSI and condition, which one was infected and which one was optimal. With matched-pairs, two-tailed t -tests we then analysed whether the female spent more time in front of one male according to those traits within certain test situations. Due to a lack of normal distribution, data of GSI was log transformed and redness was Box-Cox transformed (Sokal & Rohlf 1997). The varying sample sizes are due to the fact that some females did not see both sides of the setup which was a pre-requisite for the analysis and that we could not get all trait measurements for each male. For details of the sample size of each test see table 2.

We first analysed the mean female preference for all females of the three test situations: test 1: both males optimal, one infected, one non-infected, test 2: optimal infected vs. suboptimal non-infected and test 3: optimal non-infected vs. non-optimal infected. In a second step, a possible preference for optimality was calculated from the groups 2 and 3, in which one male was optimal and the other was non-optimal. In the last step, the possible preference for the infected male as well as the correlations for the different mal traits was calculated using exclusively the males of test 1, in which both males were optimal in respect to the female and the only difference between them was the infection of one of them.

In addition, we correlated the male traits we measured (condition index, GSI, HSI, redness and MHC allele number) for group 1 (both males optimal), to get an idea of how the parasitisation could have influenced them. Furthermore, we wanted to gain information about what kind of messages the females might achieve if they choose for a certain male trait.

Results

All male pairs: preference for non infected irrespective of MHC background

No significant difference in male traits (body condition ($N=48$), HSI ($N=45$), GSI ($N=46$) and redness ($N=41$)) could be detected between the three groups of male pairs we investigated: 1. optimal infected vs. suboptimal non-infected, 2. optimal non-infected vs. non-optimal infected and 3. both males optimal in respect to the female (ANOVA: all $P>0.14$). Therefore, we pooled the data of all females to test whether they chose for the infection status (infected or sham-exposed) of the two male variants irrespective of the MHC background (Table 2: "all males"). All males that were exposed to the three parasites were infected with at least one of them. Maximum numbers of parasites were 2 for *A. crassus* (20%), 10 for *C. lacustris* (100%) and 9 for *D. pseudospathaceum* (45%). Total infection rates for all three parasites taken together ranged from 1% to 40%.

We found no female preference for the infected or the non-infected male in the visual (two-tailed, matched pairs t -test: $DF=25$, $t=-0.3972$, $P=0.6946$), the olfactory (two-tailed, matched pairs t -test: $DF=26$, $t=0.8061$, $P=0.4275$) or the combined choice situation (two-tailed, matched pairs t -test: $DF=25$, $t=0.2218$, $P=0.8263$).

We then analysed the preference of these females for the male traits redness (visual trait), GSI and HIS. In the visual choice situation, the females significantly preferred the redder male of a given male pair (Fig. 15 a), two-tailed, matched pairs t -test: $DF=19$, $t=-2.5890$, $P=0.0180$) without respect to infection status. In the olfactory choice situation preference for any male trait (GSI, HSI, condition index) could be detected (two-tailed, matched pairs t -test: all $P>0.4$). In the choice situation, where the females could see and smell the two males, they tended to prefer the male with the higher HSI (Fig. 15 c), two-tailed, matched pairs t -test: $DF=24$, $t=-1.8747$, $P=0.0731$). Infected males tended to have a lower HSI than non infected males (t -test: $DF=48$, $t=1.847$, $P=0.0710$). No preference for any other investigated trait was found (Table 2).

Male pairs differing in MHC: preference for better fitting MHC

To test whether the females chose a male for its MHC background independently of the infection, we pooled the two groups in which one male was optimal in respect to the female and the other was not optimal (Table 2: “opti vs. nonopti”). We found no preference for the optimal male in the visual choice test (two-tailed, matched pairs t -test: $DF=15$, $t=-1.0183$, $P=0.3247$), whereas, in the olfactory choice test, a significant preference for the optimal male could be detected (two-tailed, matched pairs t -test: $DF=15$, $t=-2.4466$, $P=0.0272$). Testing the preference for GSI, we found females significantly preferring males with smaller gonads (two-tailed, matched pairs t -test: $DF=15$, $t=3.2617$, $P=0.006$)

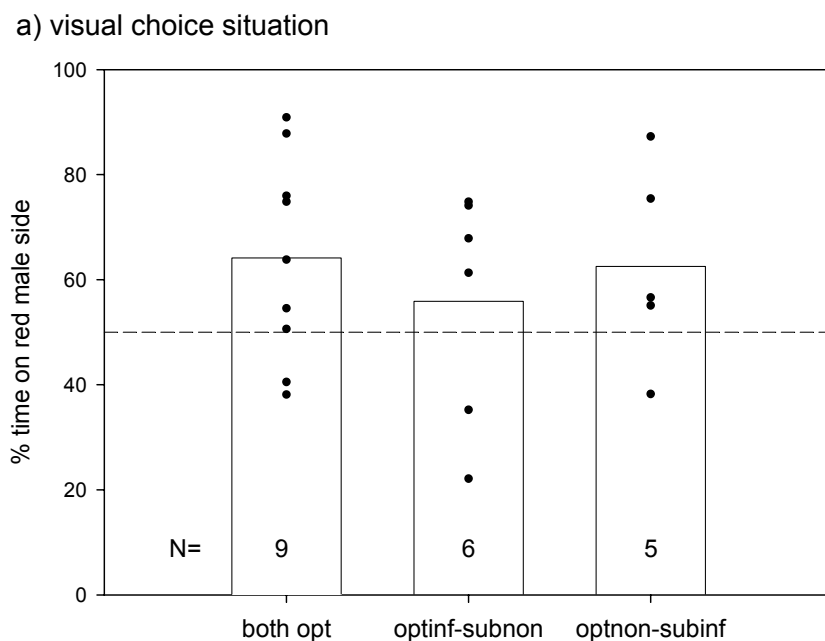
Concerning the other investigated male traits, in the visual test, 8 out of 11 females preferred the redder male of the pair (72.73%, Table 2). The males optimal with respect to the female were not always the redder ones within a pair and we found that the females choose the same male in the visual and in the olfactory choice in only 6 out of 11 cases (54.55%). In the choice situation where the females could see and smell the males, they did neither choose the optimal nor the redder male (two-tailed, matched pairs t -test: optimal: $DF=15$, $t=0.9505$, $P=0.3569$, redness: $DF=10$, $t=1.0064$, $P=0.3380$). In this test situation, the females did not prefer the males for any other of the investigated traits (Table 2).

Both males optimal: preference for non infected

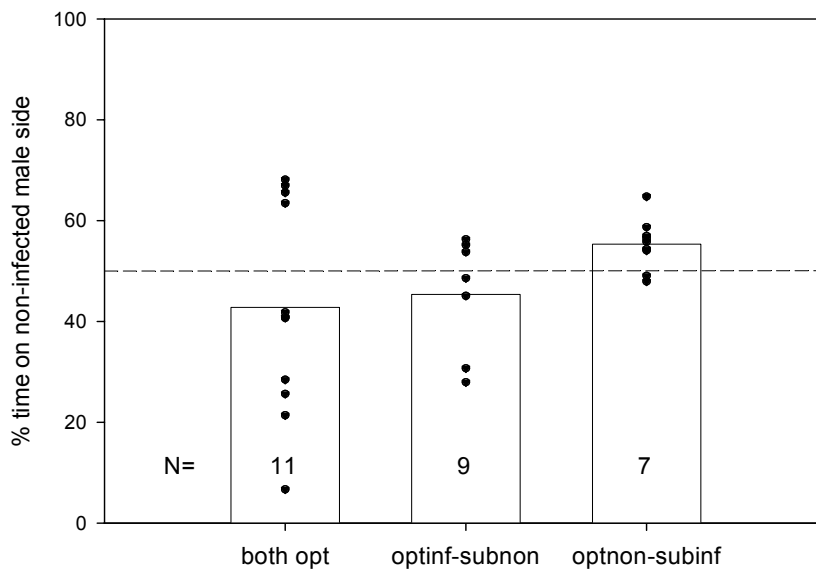
We wanted to know whether the male traits are altered by parasitisation and whether the females can distinguish an infected from a non infected male if they both have the same MHC allele number and are optimal in respect to the female. No significant difference could be detected in any of the investigated traits (redness, HSI, GSI) according to infection (two-tailed t -test: all $P>0.1$). Females did neither prefer the non-infected nor the infected male in the visual, the olfactory or the combined choice situation (two-tailed, matched pairs t -test, all $P>0.2$).

As found for all females, the females of this group also tended to prefer the redder male of a pair in the visual choice situation (Fig. 15 b), two-tailed,

matched pairs t -test: $DF=8$, $t=-2.1735$, $P=0.0615$, results of preference tests summarized in Table 2 as “both optimal”). However, in the olfactory choice situation, the females had no possibility to choose the better fitting male due to the MHC background because both males had optimal MHC with respect to the female. The only male trait found to be correlated negatively with the MHC allele number within this group of males was the GSI (linear regression: $P=0.0200$) and the females significantly preferred the males with the higher GSI in the olfactory choice situation (two-tailed, matched pairs t -test: $DF=9$, $t=-2.3848$, $P=0.0409$). In the test where the females could see and smell the males, 8 out of 10 females preferred the male with the higher HSI (Fig. 15 c), two-tailed, matched pairs t -test: $DF=9$, $t=-0.9093$, $P=0.3897$).



b) olfactory choice situation



c) combined choice situation

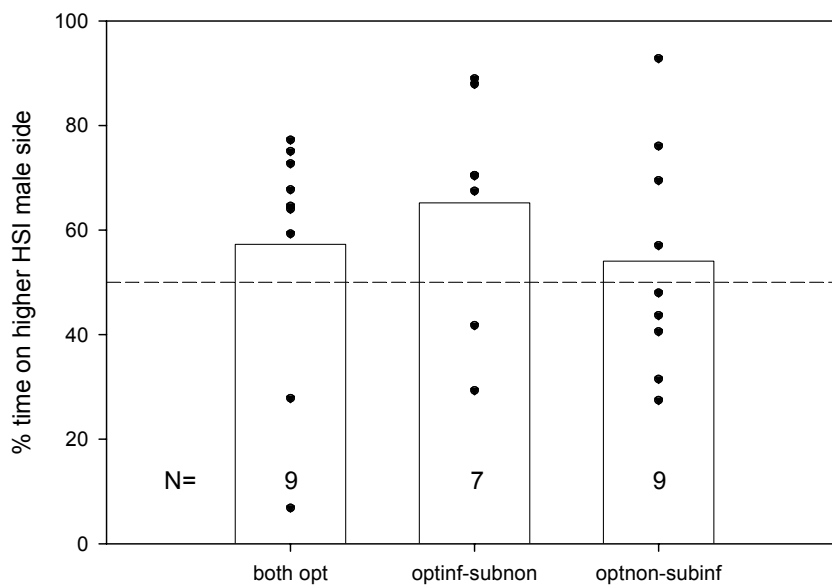


Figure 15: Mean time (%) the female spent in front of the a) redder male in the visual choice situation, b) optimal fitting male in the olfactory choice situation, c) male with the higher HSI in the combined choice situation (visual and olfactory test at the same time). For each of the three tested male-male combinations the data points for the individual females are shown. The columns represent the three male subgroups of the analyses: both males optimal with respect to the female, one male infected the other one non-infected (both optimal), one male optimal and infected versus non-optimal non-infected (optinf-subnon), one male optimal non-infected versus suboptimal infected (optnon-subinf). Dotted horizontal lines indicate the no-choice situation with 50% time spent in front of either male.

Table 2: Summary of *P* values the two-tailed, matched pairs *t*-tests for female preferences for infection (infected male vs. non-infected male), optimality (optimal male vs. non-optimal male with respect to the female), redness (red male vs. dull male), GSI (high GSI male vs. low GSI male) and HSI (high HSI male vs. low HSI male) in the visual, olfactory and combined choice situation.

choice test	male combination	N	infection	N	optimality	N	redness	N	HSI	N	GSI
visual	both optimal	9	0.537	-	9	0.062	9	0.386	9	0.736	
	all males	26	0.694	-	20	0.018	25	0.236	24	0.962	
	opti-nonopti	16	0.385	16	0.324	11	0.171	16	0.438	15	0.825
olfactoric	both optimal	10	0.329	-	-	10	0.290	10	0.041		
	all males	27	0.428	-	-	21	0.479	25	0.598		
	opti-nonopti	16	0.696	16	0.027	-	16	0.453	15	0.006	
combined	both optimal	9	0.783	-	9	0.917	9	0.390	9	0.714	
	all males	26	0.826	-	20	0.447	25	0.073	24	0.421	
	opti-nonopti	16	0.788	16	0.357	11	0.338	16	0.125	15	0.176

Correlations of traits and preferences

To analyse whether those traits the females chose for in the test situation with two MHC similar, optimal males, they were correlated with each other. We calculated multivariate correlations including body condition, GSI, redness and MHC allele number. HSI was significantly, negatively correlated with GSI (Fig. 16, $r=-0.5875$, $N=16$, $P=0.0167$) with significantly lower values for infected than non-infected males (two-tailed *t*-test: $DF=12.06$, $t=2.379$ $P=0.0347$).

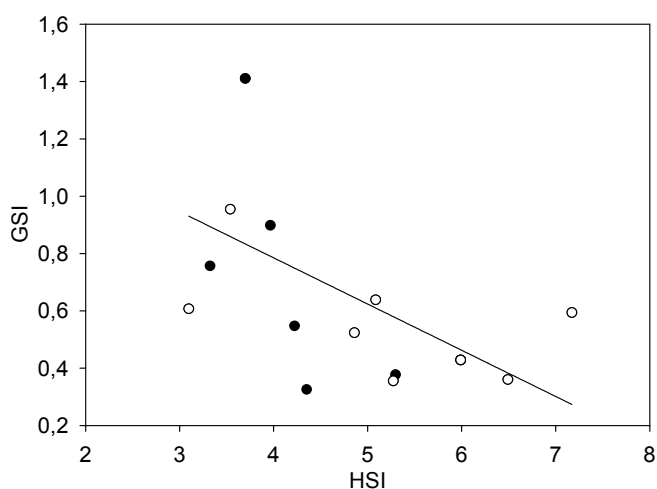


Figure 16: Relationship between hepatosomatic index (HSI) and gonad index (GSI) in the group with both males optimal with respect to the female for infected (filled dots) and non-infected (open dots) males. Data points represent individuals.

HSI was significantly affected by condition index, GSI and infection (ANOVA: $F_{4,11}=7.1611$, $P=0.0043$, effect of condition: $F_{1,11}=8.2590$, $P=0.0152$, effect of GSI: $F_{1,11}=15.2972$, $P=0.0024$, effect of infection: $F_{1,11}=9543$, $P=0.0328$, interaction GSI x infection: $F_{1,11}=3.0621$, $P=0.1079$). Body condition, but not HSI, showed a strong association with redness, independently of the parasitisation of the male (Fig. 17, ANOVA: $F_{3,14}=4.5964$, $P=0.0193$, effect of condition: $F_{1,14}=12.1936$, $P=0.0036$).

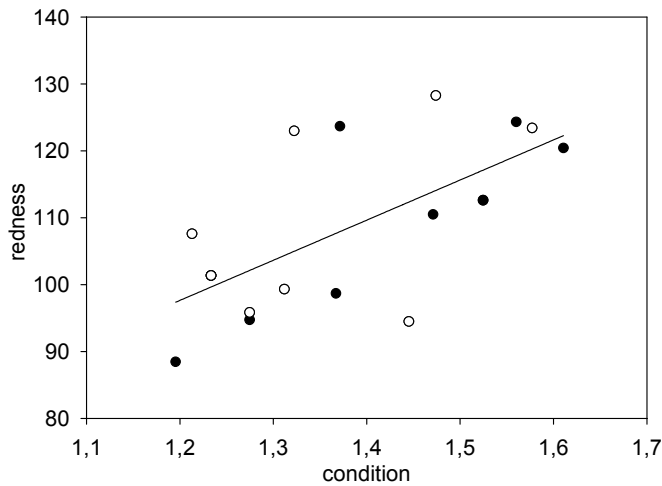


Figure 17: Relationship between condition and redness in the group with both males optimal with respect to the female for infected (filled dots) and non-infected (open dots) males. Data points represent individuals.

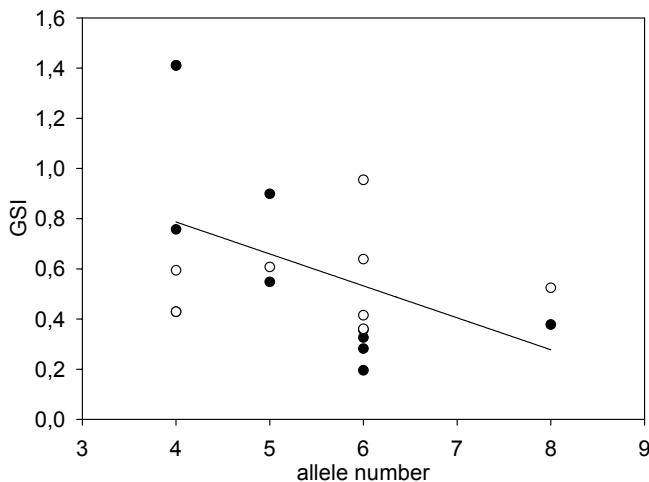


Figure 18: Relationship between MHC allele number and GSI in the group with both males optimal in respect to the female for infected (filled dots) and non-infected (open dots) males. Data points represent individuals.

The GSI was significantly correlated with the absolute MHC allele number of the males and the interaction of infection and MHC allele number (Fig. 18, ANOVA: $F_{4,14}=4.1593$, $P=0.0200$, effect of MHC allele number: $F_{1,14}=7.3341$, $P=0.0170$, effect of infection X MHC allele number: $F_{1,14}=7.9214$, $P=0.0138$), while infection alone and body condition showed no significant association (effect of infection: $F_{1,14}=0.0359$, $P=0.8525$, effect of condition: $F_{1,14}=2.5418$, $P=0.1332$).

Discussion

The results of our study show that female sticklebacks use different male traits within their mate choice. In visual choice situations redness seems to be the most important trait for females to choose for, irrespective of the infection or MHC allele number of the males. In olfactoric choice situations with the same male pairs, females based their preference on the MHC allele number, without respect to infection. However, in the combined choice situation a combination of both, olfactory and visually traits might be used, which could be reflected by the HSI of the males.

Visual choice

When only visual signals are available, females chose for the red colouration which, in previous studies was found to reflect the parasite load of a given male (Milinski & Bakker 1990). However, in our experiment we found no difference in redness between infected and non-infected males (*t*-test: $DF=43$, $t=-1.059$, $P=0.2955$). Redness is meant to be costly because it increases risk of predation (Endler 1980, Bakker *et al.* 1997, van der Veen 2005) and therefore is meant to be a reliable signal. In other species carotenoids-based colouration also acted as a cue for female mate choice (Houde & Torio 1992, Hill 1993). This might be due to the costs of resource allocation if the carotenoids are additionally used as scavengers for free oxygen radicals that emerge from immune defence (Hill 1999, von Schantz *et al.* 1999). However, we could not detect condition dependent or infection dependent alterations in colouration, although this is described in other studies. This lack might be due to the *ad libitum* feeding conditions in the laboratory (Candolin 2003) and does not necessarily underline the unreliable signal hypothesis. If a cue evolved for instance by Fisherian selection (Pomiankowski & Iwasa 1991), it might be used for mate choice for some time, but if the choice becomes too costly (e.g. inspecting time), females might switch to other signals that might be less frequent and therefore e.g. faster to evaluate. However, if female preference changes the old, cheap cues might still persist especially if they are not costly (Møller & Pomiankowski 1993).

If cues are true handicaps that are strongly condition dependent, females will use them as reliable traits, even if the choice is costly (Andersson 1986, Iwasa *et al.* 1991, Houle & Kondrashov 2002). Colour measurements on wild caught sticklebacks of the same population showed fish with a better body condition to be redder (Krobbach, unpublished data) an effect which could be masked in laboratory bred fish (Candolin 2001). It is known that sticklebacks are able to change the red colouration quickly (pers. observation) and it is maybe a reliable trait only under certain environmental conditions. Females might therefore choose for redness only if no other (e.g. olfactory) traits are available. The colouration is the only visual trait we investigated because it is meant to be the most important one (Künzler & Bakker 2001). However, in our experiment it is not possible to disentangle red colouration from other visual traits that are possibly used for the females' mating decision. The zig-zag courtship dance of the males (Bronseth & Folstad 1997) as well as pectoral spine asymmetry (Mazzi *et al.* 2003) or nest quality (Barber 2001a) could, among others also play a role in sticklebacks visual mate choice. For the females the visual choice was limited since they could not inspect the males or their nests directly, which might also play a role for their mating decision (Barber *et al.* 2001a).

Olfactory choice

In the olfactory choice situation, the females did not prefer the non-infected male of a given pair. This is in contrast to other species like mice (Kavalier & Colwell 1995). In our experiments this might have two reasons: 1. sticklebacks seem not to be able to detect parasitisation olfactory, either via modification of the males' excretory-secretory products during infection or the direct smell of the parasite or 2. the males in our experiment might have had such a good body condition due to high food supply that they did not suffer from the infection. The latter is supported by the comparison of conditional traits of infected vs. non-infected males which did not differ in any of the investigated traits (HSI, GSI, condition index).

In the choice situations where one male was optimal with respect to the female and the other was not, the females chose the optimal one. The MHC dependent choice in sticklebacks was first described by Reusch *et al.* (2001)

and is also known from other species like mice (Potts *et al.* 1991, Ehmann & Scott 2002). It enables the female to choose a male with good genes that promises good parasite resistance for the offspring (Wegner *et al.* 2003a). However, we detected also olfactory preference for GSI, surprisingly for high GSI in test situations with both males optimal. Although we prevented sight between the males and therefore excluded this possibility, competition might play a more important role to choose a mating partner if both are optimal. Due to the MHC alleles it would make no difference with which male the female spawns, but in the nest of the better competitor, the eggs could be more secure with respect to predation. In contrast, Females preferred males with lower GSI if one male was optimal and the other non-optimal. In this combination, optimal males are the ones with low GSI (11 out of 15 male pairs) and for the females the genetic background, e.g. the MHC allele number might be more important than the GSI which might strongly depend on the environmental conditions.

Combined choice

If visual and olfactory signals were available at the same time, the females were not consistent in their choice. They neither preferred the redder nor the optimal male. Instead, in this combined choice situation, females preferred males with a higher HSI, a measurement for the body condition of the males. Although possible, the HSI alone might not be a trait the females choose directly on (e.g. via odour), but since secondary sexual traits are known to reflect condition, females might be able to estimate a male's condition by the combination of visual and olfactory cues. In addition, they might use one trait after the other to reduce the total costs of the choice due to longer inspection time (Candolin 2003).

In particular, the female chose the males with the higher HSI, which also have the lowest GSI. Large gonads are common in species in which sperm competition is high (Hosken & Ward 2001) and also male sticklebacks do invest more in sperm if there are possible rivals around (Zbinden 2004). Since the individual males in our experiment could not see any other males, competition can be assumed to be low and males might have invested less into reproduction. Males are able to fertilise many eggs with low cost for the

production of sperm and therefore, in a situation without competition, we do not expect them to heavily invest into sperm production.

However, we find a negative correlation of MHC allele number with the GSI and this indicates that males with low MHC allele numbers have larger gonad, reflecting higher investment into sperm. This could be due to the fact that males with low MHC numbers have to activate the innate immunity, which is not directly related to the MHC (Kurtz *et al.* 2006), to fight the parasite infection. This part of the vertebrate immune system is more costly (Moret & Siva-Jothy 2003) than the MHC related adaptive immunity. Those fish should therefore invest more into actual reproduction, because they might suffer so much that on a later point in time reproduction is not possible any more (Poizat *et al.* 1999). However, it might also be that the males with a low MHC allele number are generally of bad quality. Interestingly, we did not find a correlation of HSI and MHC allele number, although we would expect a low condition for low numbers in this case. Taken together this fits the multiple messages theory with HSI and GSI acting as indicators for different aspects of the males' condition.

If females manage to combine several visual and olfactory traits, they might get information about condition via the HSI and about the MHC allele number via the GSI at the same time. Using multiple ornaments they can therefore get a more reliable overall picture from the male.

Summary

Although different cues might indicate different or similar aspects of male condition (multiple messages) they might be used together to get a more reliable picture, which is also more difficult to cheat (redundant signals). Under non-restricted conditions as in the laboratory, redness for example might be less reliable due to resource availability. Resource allocation in this case might be more likely than under field conditions. In choice situations where the female achieves exclusively visual or olfactory traits it might go for a trait that only reflects one part of the total condition and is therefore not shown by the overall better male. We conclude that the different hypotheses for the use of multiple ornaments are not mutually exclusive in the three-spined stickleback. Our

results indicate that under different environmental conditions the informative effect of different ornaments might change and that females should include multiple messages into their mating decision to get a reliable overall measure of the males' condition.

Chapter IV

Enclosure experiment



Female mating decisions of three-spined sticklebacks: A field study

Abstract

Many laboratory as well as field experiments have been performed on mating behaviour and female preferences in three-spined sticklebacks (*Gasterosteus aculeatus*). While work in the laboratory focuses more on the detection and proof of possible traits and preferences, field studies survey the overall reproductive success. In total, more studies were performed under controlled laboratory conditions, while only few field experiments are available until today. However, both methods also have disadvantages which we wanted to minimise by the use of controlled enclosure cages in the field.

With a comprehensive enclosure experiment, we investigated reproductive success of males and females according to their immuno-genetic background, carrying a certain MHC class IIb variant number. MHC alleles play a major role for adaptive immune reaction and therefore individual parasite resistance. According to results from previous studies we expected females to spawn with males promising optimal parasite resistance for the offspring, e.g. around 5 MHC alleles. However, we could not find more eggs from male-female combinations of optimal MHC than of such with more or less variants. Furthermore, from 25 clutches we collected, eggs were assigned to come from chosen or sneaked fertilizations, to detect a possible cost of sneaking for the female. The according MHC variant number did not differ between these three categories, hence sneaking seems to cause no loss of benefit for the offspring. Males were caught and dissected at the end of the two weeks experiment. We found that males with an intermediate number of 5 or 6 MHC variants had fertilised the eggs of more females than males with high or low number, which might be due to their optimal parasite resistance.

Keywords

enclosure experiment, reproductive success, three-spined stickleback, parasite diversity, MHC class IIb

Introduction

Field studies concerning the reproductive success of male and female three-spined stickleback are rare. Most field research was done on DNA fingerprinting to evaluate different reproductive tactics like nesting, sneaking and egg stealing (Rico *et al.* 1991, 1992, Jones *et al.* 1998, Largiader *et al.* 2001). Others focussed on observation and quantification of male aggressive behaviour in the presence of potential sneaking neighbours (Ridgway & McPhail 1987, LeComber *et al.* 2003) or on population comparison (Ishikawa & Mori 2000). However, to our knowledge only single field studies are available until today that incorporate both, estimation of female mate choice for certain male traits and parenthood analysis (Goldschmidt & Bakker 1990, Bakker & Mundwiler 1994).

In sticklebacks, mate choice was mainly studied in laboratory experiments with a focus on female preference for one or only few male traits (reviewed in Jennions & Petrie 2000, but see Künzler & Bakker 2001). Under field conditions, females might use multiple traits for their mating decision to get a better overall picture of the mating partners' condition (reviewed in Candolin 2003). The choice can be depend on the status of the female itself (Bakker *et al.* 1985), the status of the male (Moore & Moore 1988) and the environment including e.g. parasites or predators (Kennedy *et al.* 1987, Wedekind 1994, Milinski & Bakker 1990, Forsgren 1992, Rowe 1994, Godin & Briggs 1996).

To get an idea of the traits that are used by the females, restricted laboratory experiments are necessary. They investigate single aspects of female choice under controlled laboratory conditions and exclude side effects like, e.g. competition or predation using wild caught or laboratory bred fish, which might be selected for special traits. Furthermore, computer animated males can be tested with real females which helps to focus even more on the one aspect of interest (Künzler & Bakker 1998, 2001). However, it is important

to investigate the applicability of these results from laboratory experiments in the natural habitat of the fish.

In contrast to laboratory studies, field observation or correlative studies on natural populations investigate animals within their natural habitat with all restrictions of abiotic and biotic factors e.g. food, light conditions, competition and predation. This gives a picture of how females choose and how successful the resulting offspring is (Kraak *et al.* 1999). Only few studies investigated the impact of male stickleback traits on reproductive success. Bakker & Mundwiler (1994) found males being redder during egg collection to receive more eggs. However, contrary to laboratory experiments, females face the choice between several males and the interaction between individuals in the field which is important to include direct, tactile contact of males and females that might influence mate choice (Rowland 1999). In this context also male-male interaction might influence the female fitness via egg-stealing or sneaking (Largiarder *et al.* 2001, LeComber *et al.* 2003). Still, we have no knowledge about life-history, genetic background or competitive ability of animals investigated in the field. A good possibility to combine the advantages of laboratory experiments and field observations and reduce their disadvantages is the use of outdoor enclosures (Sargent 1982). With controlled stocking, experiments are performed under natural conditions according to the question of the study. All fish can interact freely and compete for resources like food, territories and mating partners. Enclosure experiments are therefore the best compromise to test findings from laboratory experiments under semi-natural conditions in the wild. Thereby, one can estimate reproductive success of each individual according to male traits and condition.

Females gain fitness benefits via their mate choice which can enlarge the quantity or quality of their offspring (Clutton-Brock & Vincent 1991). Thus, males often exhibit secondary sexual traits that carry reliable information about their ability to provide direct and indirect benefits (Andersson 1994). Possible direct benefits include e.g. parental care or nuptial gifts (Reynolds & Gross 1990, Kirkpatrick & Ryan 1991), whereas indirect benefits are heritable, genetic advantages for the offspring (Zahavi 1975), e.g. parasite resistance (Hamilton & Zuk 1982). Females are able to judge a male for the traits it exhibits and

distinguish an adequate mating partner. In three-spined stickleback (*Gasterosteus aculeatus*), females are known to use multiple traits in mate choice (McLennan 2003). In this species, exclusively the males care for the offspring. Hence, for the female the costs of mating an inferior father would be high since fitness might be reduced. The male builds a nest, courts a female to spawn the eggs, fertilises the eggs and cares for the offspring until it hatches (Wootton 1976). The structure of the nest (Barber *et al.* 2001a) as well as the parental care (Kraak *et al.* 1999, Bakker & Mundwiler 1999) could influence the quality of the offspring directly. Courtship cues, including zigzag dance and red breeding colouration might reflect the condition and genetic background of the male (Bakker 1993). To obtain indirect, genetic benefits females estimate the genetic quality of the male via these traits. The red breeding colouration or symmetry of pectoral spines are only two examples of visual traits that were found to be important in laboratory experiments (Bakker & Milinski 1991, Mazzi *et al.* 2003). The males' red throat colouration reflects parasite load, with redder males having less parasites (Milinski & Bakker 1990, Frischknecht 1993) and is also a measure for male aggressiveness (Candolin 1999). However, female sticklebacks prefer males not only for visual, but also for olfactoric cues. Aeschlimann *et al.* (2003) showed female preferences for males with certain MHC class IIb allele number which they calculate by a self-referential process. Females choose males leading to an intermediate number of 5-6 MHC variants for their offspring (Aeschlimann *et al.* 2003, Reusch *et al.* 2001). Fish with an intermediate number of MHC alleles have a fitness advantage, because they are less likely to get infected under experimental conditions as well as in the wild (Wegner *et al.* 2003b, a) and suffer less from the infection (Kurtz *et al.* 2004).

In addition to egg collection from several females that have chosen a certain male as a mating partner, males can enlarge their reproductive output by sneaking into neighbouring nests (van den Assem 1967, Mori 1995). For sneaking, male sticklebacks are able to change their colouration to a cryptic female-like or more light (Frommen, pers. communication) colouration to approach a foreign nest in which a female had spawned her eggs shortly before. The sneaker swims through the nest, sometimes even before the nest

owner himself (Zbinden *et al.* 2003), and fertilizes the eggs. Another possibility for males to cheat females is the egg stealing behaviour. Females prefer to spawn into nests that already contain eggs (Ridley & Rechten 1981) and therefore males can enlarge the attractiveness of their nest by stealing eggs from foreign nests and bring them into the own one (e.g. Goldschmidt & Bakker 1990). Little is known about frequency of sneaking and egg stealing in the wild (Jones *et al.* 1998, Largiader *et al.* 2001) but both behaviours were observed in the population of sticklebacks we used for our experiment (pers. observation). We determined eggs to result from sneaked, stolen and chosen male-female combinations and included it into our analysis.

We stocked the enclosures with wild caught males and females of selected MHC class IIb genetic background and sampled nests and male sticklebacks after two weeks. We performed microsatellite-based parenthood analysis and determined the amount of sneaked and chosen fertilizations to incorporate this information into the analysis of female choice. In conjunction with male breeding colouration, morphological characteristics and parasite load we analysed the relative importance and interaction of MHC-related cues and other sexual traits on mate choice of sticklebacks in a natural breeding habitat.

Methods

The enclosures

We used six outdoor cages (No. 1- 6) installed in the shallow water of the lake shore of the Grosse Plöner See in Schleswig-Holstein, Germany. The metal cages were 3x3 m in size with 1 m in height and were anchored in the ground and sealed to the water body of the lake with a base of sandbags and gravel. The meshes were 5 mm wide, thus small particles and most invertebrates (intermediate hosts to various parasite species), zooplankton and algae could pass through while predators and other fish were kept outside. In the middle of each cage a pole was installed to fix nylon net as cover against airborne predators, like sea gulls or herons. Wooden wave breakers were installed in 10 m distance around the whole setup in direction to the lakeside.

Fish and experimental setup

Young of the year three-spined sticklebacks (*Gasterosteus aculeatus*) were caught at the shoreline of the lake Grosser Plöner See on 1st of November 2002 in the location where later the enclosures were installed. The fish were housed in the lab in 190 L tanks at 6°C and a daily light period of 10 hours. In April 2003, for 191 fish a small part of one of the first dorsal spines of each fish was clipped for MHC class IIb and gender genotyping (see below). This is shown not to harm the fish (Kraak *et al.* 1997). Subsequently, the fish were kept individually in 8 L tanks.

Six groups of experimental fish were composed each containing two females with four, six and eight different MHC class IIb variants each, resulting in six females per group. To each of these groups either six or twelve males selected for the same three MHC diversity classes were added in order to control for potential density effects on mate choice. Unfortunately, we lacked a sufficient number of fish with the required MHC variants, so that we substituted the missing by such with three, five or seven (instead of four, six or eight, respectively) MHC variants.

All experimental fish were weighed (to the nearest 0.1 mg), the total length was measured (to the nearest mm) and the number of eye flukes

catching (genus *Diplostomum*) they carried from before the in the eye lenses was counted *in vivo* under a dissection microscope. After all groups were completed they were kept in 190 L tanks and consecutively transferred to spring (3 weeks at 12°C, 12/12 h light/dark period) and summer conditions (18°C, 14/10 h light/dark periods) until early June 2003. Two weeks later (13th of June 2003) the groups were released into the enclosures in that particular area of the lake where they had been caught seven months before.

Catching

After fourteen days in the enclosure, males were caught and photographed digitally for later standardized analysis of the red breeding colouration (see below), measured and weighed. All recovered males were dissected within two days after capture including screening for macro-parasites and weighing of organs (gonads, kidney, liver, spleen). Condition indices were calculated for each fish following Bolger & Connolly (1989) with the exponent $b=3.314$), using weight and length measurements from catching day. All other body indices were calculated by dividing the weight of the organ by the total weight of the fish (liver, spleen, gonads).

For each fish the number of following parasites was counted: trematodes: *Diplostomum spec*, *Apatemon cobitidis*, *Cyathocotyle prussica*, *Echinochasmus spec* and *Phyllodistomum folium*, cestodes: *Valipora campylancristrota*, *Proteocephalus filicollis*, nematodes: *Anguillicola crassus*, *Camallanus lacustris*, *Contracaecum sp.*, and others: *Acantocephalus sp.*, parasitic glochidia (parasitic stages of freshwater bivalves) and *Gyrodactylus gasterostei*.

Individual parasite diversity was calculated: $\frac{n_s}{sd(N_s)} * \frac{1}{sd(N_s)} * 10$, with n_s being the number of parasites of a certain species s and $sd(N_s)$ the standard deviation of all parasites of that species within the population (Kalbe *et al.* 2002). Absolute parasite load was calculated using the formula $\sum_s \frac{n_s}{N_s}$ with n_s being the number of individuals of a parasite species s and N_s the absolute number of this parasite within the population (Rauch *et al.* 2005).

Colouration

For each male a picture was taken from its throat using a digital camera (Olympus E20-p) with 36mm macro objective. Closing time of the lens was 1/60 sec, aperture 7 and illumination came from 4 cold lights with 3300 K colour temperature according to daylight illumination. Alignment was performed at 3700 K automatically. The red colouration of a marked area on the throat of each fish (Fig. 19) was measured in IP Lab (Version 3.6.2 for Mac OS 9.2.2, Scanalytics, Inc.) as mean intensity. The photographs of all fish were measured twice, in random order on two consecutive days. This method was highly repeatable: 95.9 % for the marked area as well as 99.98 % for the mean colouration of each fish.

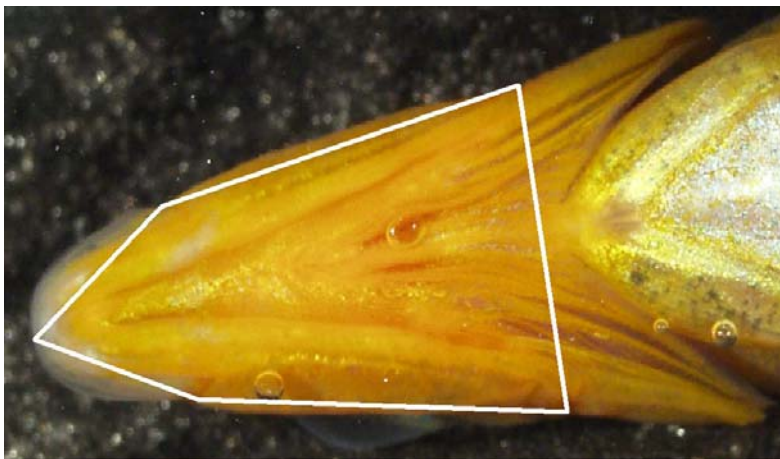


Figure 19: Digital photograph of the throat of a stickleback showing the corner-points of the marked region used for measurement of red courtship colouration.

Molecular methods & analyses

DNA was extracted from the spine clipping material using DNA Tissue Kit (Invitex), following the manufacturers instructions. The gender of all fish was then determined by amplification of a sex specific marker described in Griffiths *et al.* (2000). In males two products get amplified, one of 370bp in length and one of 600bp, while females just show the 600 bp product. MHC class IIb typing was performed with Single-Stranded conformation polymorphism (SSCP) (Binz *et al.* 2001, Reusch *et al.* 2001). By using two primer combinations, we amplified a 124 bp long stretch from the highly polymorphic exon 2 including the peptide binding region (PBR, Binz *et al.* 2001). PCR products were separated by capillary electrophoresis using native polymers on an ABI 3100 capillary

sequencer (Applied Biosystems, Weiterstadt, Germany, Wegner 2004a) and MHC class IIb variants were counted as described (Reusch *et al.* 2001, Wegner *et al.* 2003a, b).

Parenthood analysis

We collected all nests from the enclosures and picked 48 eggs from each nest for parentage analysis using nine polymorphic microsatellite loci. Genetic fingerprints were determined by capillary electrophoresis for all eggs and the possible parental fish from the according enclosure. The most likely parents were assigned to each egg by using the software Cervus Version 2.0 (Tristan Marshall, 1998-2001). The best fitting parent for every egg (95-80% probability, at least 8 similar loci) was chosen and complemented with the next best fitting fish from the other gender.

According to parenthood analysis each egg was assigned to one of the following categories: chosen, sneaked and stolen. Males that fertilized the majority of the eggs in a nest were assigned to be owner of this nest and resulting eggs were assumed to come from females that have chosen this male. Eggs were assigned to be fertilized by a sneaker if in one nest the same female had eggs fertilized by the nest owner and another male. Eggs were assigned to be stolen from other nests if they were not fertilized by the nest owner and no eggs of the same female were found in the same nest.

Data analysis

The optimal MHC class IIb allele number associated with the lowest parasite load for the experimental fish was calculated as the curve-minimum from the quadratic regression of the actual residual parasite load we found (Figure 20).

The main question of the experiment was whether females try to optimise the MHC genotype of their offspring via mate choice. To answer this question we calculated for each male within an enclosure the mean MHC variant number of potentially resulting offspring with every female from the same enclosure (Milinski *et al.* 2005). The male-female combination closest to the optimum of 5.9 is predicted to be preferred by the female because they promise best fitness

for the offspring due to parasite resistance. We counted how many eggs were actually fertilised by such predicted males. Then we counted the eggs for each of the three categories (chosen, sneaked, stolen) and determined the difference of their MHC number to the optimum. Low distance values represent good fitting mating partners and high values represent less fitting partners. We finally compared the distance to the optimum for each of the categories. Not only MHC class IIb variants (olfactory) seem to play a role for the female mating preference, but also visual traits are used. We counted the eggs that were fertilized by the reddest male of the enclosure to consider female choice for visual traits as well.

To exclude difference between the enclosures that might occur due to microhabitat variance, we calculated residuals with the factor 'enclosure' for parasite diversity, parasite number, redness, gonad index, condition index and hepatosomatic index.

Statistical tests were performed using JMP Professional 5.0.1a (SAS Institute Inc. 2002 for Windows) on a level of significance of $P < 0.05$. All tests were two-tailed.

Results

After two weeks of experiment, 25 nests were collected with 20 of them bearing eggs from one or more females. In total, 960 eggs were prepared for microsatellite analysis. In enclosure 3 and 4 we discovered two young pike (*Esox lucius*) that might have preyed upon the fish as well as the nests and since predation was not subject to our experimental design, we excluded those enclosures from further analysis. For the determination of parenthood we took all fish for which genetic data were available (e.g. all fish that were used for stocking) into account, resulting in 535 (55.73%) eggs with 25 males and 21 females as possible parents, which could be clearly determined. Resulting from these numbers, we had 114 possible male-female combinations, of which we detected eggs for 70 of them (61.4%). The large deficit of analysed eggs was due to major problems during DNA extraction because of the low developmental stage of some eggs.

Males

Of the 54 males that were released into the enclosures 25 were caught again after the two weeks of experiment. The missing males could not be caught with the gentle method we use to prevent major disturbance for the photographing that directly followed the catching. All males were dissected, typed for their MHC class IIb and the resulting data was used for the analysis of male traits. Only the 18 males caught from enclosures 1, 2, 5 and 6 the data for all measured traits (redness, parasite load & diversity, condition index, hepatosomatic index, gonad index) were analysed. According to the MHC class II variant number and parasite diversity we found males with 5 or 6 alleles having the lowest parasite diversity although the quadratic fit was not significant due to low number of individuals (figure 20, polynomial regression: residual parasite diversity, $N=18$, $r^2=0.094$, $F_{2,15}=1.886$, $p=0.085$). From the minimum of the fitted curve, we calculated an optimal MHC variant number of 5.9 for the males in our experiment.

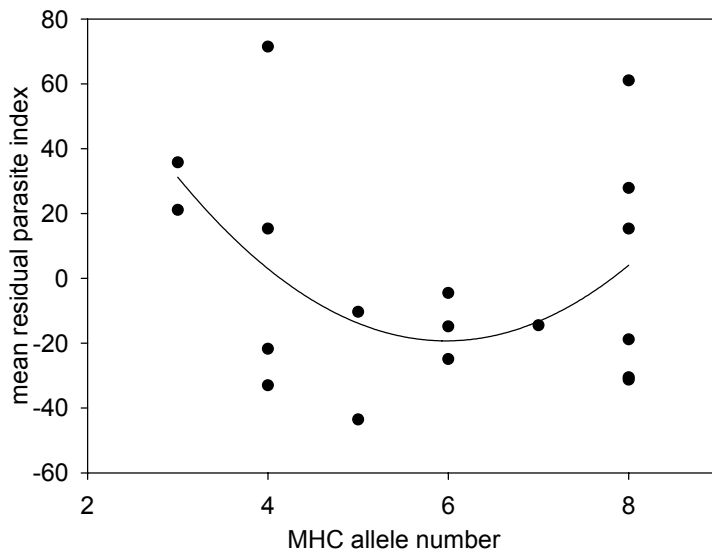


Figure 20: Relationship of residual parasite diversity and number of MHC class IIB variants in the 13 dissected males from enclosure 1, 2, 5 and 6. The quadratic regression fitted best with a minimum of 5.899 which reflects the variant number for optimal parasite resistance ($\text{parasitization} = -17,09 - 0,86 * N_{\text{variant}} + 6,59 (N_{\text{variant}} - 5,83)^2$).

For the absolute parasite load we could not detect such a quadratic relationship with certain allele numbers (polynomial regression: residual parasite load, $N=18$, $r^2=-0.090$, $F_{2,15}=0.2977$, $P=0.46$). However, males with optimal allele number tended to have a higher condition factor than fish with low or high numbers (Fig. 21, two-tailed t -test: $F_{1,15}=-3.1785$, $P=0.095$).

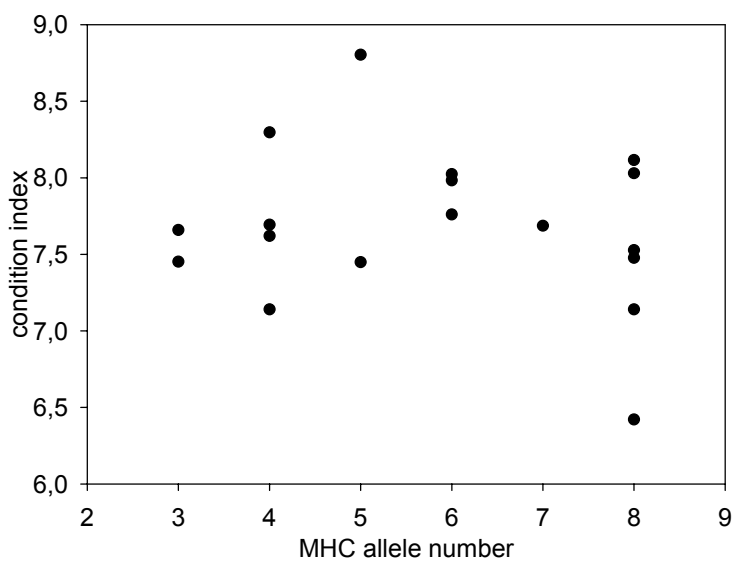


Figure 21: Relationship of condition index and MHC variant number.

According to the redness, males with an optimal MHC allele number were not the ones with the reddest throat colouration compared to those with more or less alleles (Fig. 22, two-tailed t -test: $F_{1,15}=0.0253$, $P=0.8758$). We found a negative correlation between colouration and MHC variant number (linear regression: $N=17$, $r^2=0.4109$, $F=10.4605$, $P=0.0056$). Males with lowest variant number were redder than the ones with the highest number (ANOVA: $F_{2,14}=4.3266$, $P=0.0344$, post-hoc test significant).

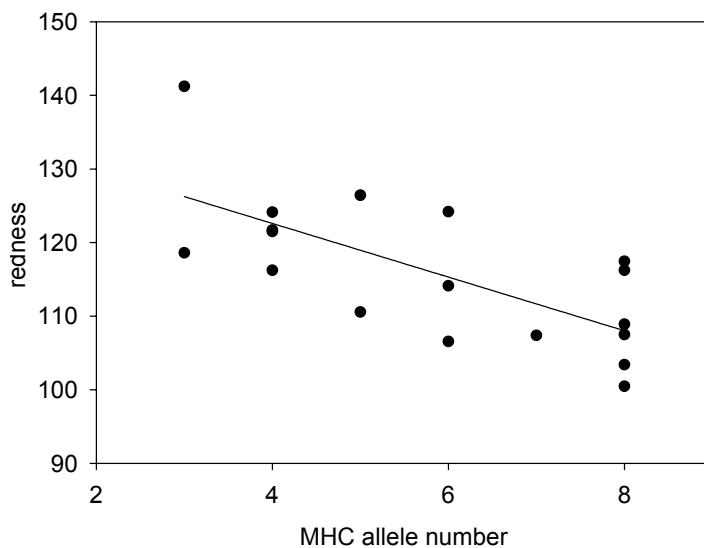


Figure 22: Relation of the mean redness of the males and their MHC variant number with the fitted linear regression (residual redness = $18,60 - 3,19 N_{variant}$). Given are the means of two independent analyses of digital photographs per individual as sum of red pixel.

The males with optimal variant number also fertilized the eggs of more females (two-tailed t -test: $F_{1,16}=6.0706$, $P=0.0255$) although we did not find more nests for them (Pearson: $r^2=0.1052$, $N=18$, $P=0.1144$). Interestingly, we found no difference in any morphological trait between males with an own nest and those without (condition index, hepatosomatic index, gonad index, two-sided t -test: $N=17$, all $p>0.2$) and also not for parasite diversity, parasite number or redness (two-sided t -test: $N=18$, all $P>0.18$).

Mate choice

For the nests we collected, 454 eggs were assigned to be chosen, 41 to be sneaked and 40 to be stolen. We expected females to choose males with which the resulting offspring would be closest to the MHC optimum of the

population. Only 2 females had most eggs fertilized by the predicted nesting male. The mean (\pm std err) combined allele number from male and female was 4.92 (\pm 0.20) for chosen, 4.57 (\pm 0.31) for sneaked and 4.49 (\pm 0.31) for stolen, resulting in differences to the calculated optimal allele number of -0.18 (\pm 0.23) for chosen, -0.47 (\pm 0.40) for sneaked and -0.50 (\pm 0.33) for stolen. For the females a mean egg number of 21.62 (\pm 2.92) was detected for chosen combinations, whereas a mean of 4.56 (\pm 4.45) eggs was fertilized by a sneaker and 4.44 (\pm 4.45) eggs were stolen by neighbouring males. The difference between the numbers of eggs for the chosen to the two other categories was significant (ANOVA: $F_{2,36}=7.956$, $P=0.0014$, post-hoc test significant). However, we did not find eggs fertilized by chosen males to be closer to the expected optimal MHC variant number and possible costs of sneaking can therefore not be analysed.

Due to the other male traits we investigated, e.g. redness, we determined 8 females having spawned their eggs with the reddest male of those we measured. For 7 egg clutches we could not define one trait that the females might have chosen for.

Discussion

The six large outdoor enclosures we used in our experiment provide a good method to investigate mating behaviour and reproduction of three-spined sticklebacks. In each of the cages, males established territories and built nest to collect egg clutches from several females. According to results from previous mate choice experiments in the laboratory (Reusch *et al.* 2001, Aeschlimann *et al.* 2003) we selected the fish stocking for the MHC class IIb variant number. If females choose for the MHC variants via male odour cues, we would have expected to find most eggs from male-female combinations that provide 5-6 variants for the offspring. We found more eggs of optimal male-female combinations than of non-optimal combinations and females spawned more often with an optimal fitting male. However, from visual mate choice experiments in the laboratory it is known that females choose males for traits like red breeding colouration (Bakker & Milinski 1991, Milinski & Bakker 1992, Bakker 1993, Bakker & Mundwiler 1994). Indeed, 8 females did spawn their eggs with the reddest male of the certain enclosure. Redness was negatively correlated with MHC variant number and therefore females in some cases seem to choose mainly because of visual cues even though males were available that would fit better in accordance to the MHC. This result might be influenced by the parasite load and diversity, because reddest males proved to have highest parasite load, but only intermediate parasite diversity. For the females this would mean that parasite load is not as important as low parasite diversity. They therefore choose for optimal MHC with good body condition, but only intermediate redness and parasite load. It is, however, surprising that high parasite load and intermediate parasite diversity are found in the reddest males. It was shown that redness decreases with high parasitisation (Milinski & Bakker 1990, Folstad *et al.* 1994), but this might not be the case in the natural environment. The reddest males might invest all available resources into the actual reproduction, which is underlined by the low body condition we find for these males. Due to their low MHC variant number they might have to fight parasites to a higher degree with the innate immunity, which is known to be much more costly (Kurtz 2003). For these males it could be the only chance to

be fast and collect as many eggs as possible within the first breeding cycle, since later in season they might not have enough resources left to perform a second breeding cycle. In other populations males perform only one breeding cycle (Largiarder *et al.* 2001) but this might be different in our population. This might be a terminal investment strategy (Clutton-Brock 1984), because the probability to die after the first breeding cycle is higher for parasitised males with low MHC variant number.

During the analysis of paternity for the eggs, we divided them into three categories: chosen, sneaked and stolen. According to further studies of MHC based female mate choice (reviewed in Milinski 2003) we would expect chosen eggs to result from male-female combinations that provide a MHC variant number in the offspring which is closer to the optimum than others. Males are not expected to be choosy, since they can enlarge their fitness by quantity and not quality of the offspring (Birkhead 2000). However, we did not find any difference in the distance to the optimal MHC variant number for any of the three categories. For the stolen eggs the explanation is obvious, since the female had chosen the nest owner, they can be handled as chosen, only that they are reared by a (less fitting) male. The missing difference for the eggs from sneaked male-female combinations remains puzzling. One possible explanation could be the qualitative difference of certain MHC variants that are specific for actually occurring parasites. To disentangle other explanations, further experiments are needed, with severe observation of sneaking behaviour.

After the two weeks of experiment, only half of the males were caught again. In addition, we might not have detected all of the nests in the enclosures. Stickleback nests are built from plant material, that is taken from the surrounding vegetation and they are hard to detect from above. However, our results are the first step in the investigation of stickleback mating behaviour due to MHC variant number under semi-natural conditions. The small number of re-caught males implies some difficulty according the analysis of male traits and their use for the female mating decision. Although we know from laboratory observation that males owning a nest are redder, we could not confirm this with the colouration measurements in our experiment. Nesting males did not differ in redness from non-nesting males, but they fertilised more eggs. Males that care

for a nest are duller than those still courting females and it might be that some of the males already had finished clutch collection and started broodcare. Furthermore, as we can not rule out completely that nests were overlooked, some of the males might be misleadingly assigned as non-nesting. As we stated above, females use not only one, but a mixture of several traits for their mating decision and this might underline the use of olfactory traits, or the use of other traits we could not take into account. We measured the redness of the males, which is assumed to be of major importance, but zigzag dance, symmetry or other possible candidate traits for female choice (Rowland 1995, Bronseth & Folstad 1997, Mazzi *et al.* 2003, Bakker & Mundwiler 1999, Ishikawa & Mori 2000, Barber 2001a) were not taken into account. This might bias the analysis of the female preference.

To improve the results of our study it would be useful to collect the nests of the males not only once after two weeks, but weekly over a longer period. Hence, we could get more information about the life-time reproductive success of males and females according to their MHC variant number. Also, the investigation of certain male strategies, like terminal investment or risk assessment of the single individual could be deepened. Further information could be gained by the analysis of more eggs from one, or total nest contents. We took only 48 eggs per nest and had to face major problems with the DNA extraction of more than half of them. Due to the protein rich egg shell of stickleback eggs and especially the low developmental stage of some of them, we missed information from clutches that were spawned shortly before collection. Not all males might have built a nest instantly and this is another reason to expand the collection period. It would improve the experiment to include more measurements of the males that are known to play a role for the female mating decision. Since the analysis of the zigzag dance could be complicated in field experiments, possible candidates are nest quality, pectoral spine asymmetry or aggression towards other males.

Summary

The comprehensive approach of our study using enclosures under semi-natural field conditions seems to be a good method to investigate the

complexity of female mate choice in sticklebacks. Although our results should be considered carefully they raise some interesting questions about the use of multiple visual and olfactoric ornaments in the field. We could not detect a certain male trait that was correlated with number of eggs fertilized, although redness seems to be a good candidate. Also the role of sneaked fertilizations and stolen eggs might be underestimated and needs further attention for instance to estimate the costs for the females.

Similar enclosure experiments have been conducted using the same cages and location in the lake in 2004 and 2005. Since the analysis of eggs and the according parenthood determination is still in progress we are looking forward to get more results that might shed more light into at least some of the questions we raise.

Conclusion

The maintenance of sex is a widely discussed topic in evolutionary biology (e.g. Trivers 1985, Lively 1992, Ridley 2004, Milinski 2006). One, and probably the most important reason is the advantage of sexually reproducing individuals to adapt more rapidly to environmental changes (Crow & Kimura 1965, but see Maynard Smith 1976). Parasites are a major, and extremely fast changing part of this environment. Therefore, choice for mating partners should include traits reflecting the ability of an individual to cope with parasites. In this thesis, I discussed variability in stickleback male traits that provide information about parasite load e.g. via red throat colouration (Milinski & Baker 1990, Folstad *et al.* 1994) or parasite resistance (MHC) via odour cues (Reusch *et al.* 2001, Aeschlimann *et al.* 2003, Milinski 2003). I show that different parasites can induce various changes in the host's physiology (Chapter I) that might be signalled by sexually selected traits. Also, differences in MHC class I and class II B immuno-genetic background were reflected through the male red breeding colouration, as well as by certain nest characters (Chapter II). Taken together, these might be the prerequisites for female choice of fitter, or more resistant, mating partners.

This thesis shows that visual and olfactory traits have the potential to signal parasite load and resistance, and are used for mate choice (Chapter III). The results suggest both traits to signal complex information. Hence, the usage of a combination of traits seems an ideal strategy to find the best fitting mate and a partner that does not only manage survival in the actual environment, but also entails its genes to the offspring. The enclosure experiment provides us an impression of how mate choice influences reproduction of an individual in the wild (Chapter IV). Nevertheless, further investigations are necessary, especially on potential variation in lifetime reproductive success between males with a different MHC genetic background. Thereby, one could disentangle the effect of parasites on the exhibited male traits and, thus, its influence on female choice. However, the complex interactions of traits, choice and fitness remain puzzling and raise new questions as rapidly as solutions to previous questions are found.

One of these raising questions that might gain even more interest in the future deals with the evolution of the MHC as a component in specific immune defence. The MHC is known to present peptides derived from pathogens to T-cells during the adaptive immune reaction. In a recent paper, Boehm (2006) discussed that this system might have originally evolved for discrimination of genetic individuals in the context of sexual selection, namely to discriminate between potential mating partners. This recognition could be due to the individual diversity of intra-cellular peptides carried to the extra-cellular space. One candidate to be such a carrier is the MHC. MHC-peptide complexes might be detectable in body fluids and thereby used for mate choice, as studies in mice and sticklebacks suggest (Leinders-Zufall *et al.* 2004, Milinski *et al.* 2005). Later in evolution, the presentation of peptides derived from pathogen deletion might have also become important. This could explain the tight linkage between parasite resistance and mate choice.

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wie der Schwatz der Kollegen auf dem Flur...

*Arno Penzias (*1933),
amerikanischer Physiker und Nobelpreisträger 1978*

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Curriculum vitae

CATHRIN KERSTIN KROBBACH, GEB. SKUPCH

GEBOREN AM 08. DEZEMBER 1976

IN SIEGBURG,

VERHEIRATET

DEUTSCH

Ausbildung

- Seit Januar 2003 Promotion in der Arbeitsgruppe Evolutionsökologie am Max-Planck Institut für Limnologie, Plön
- Dezember 2002 Diplom in Biologie mit der Note „gut“ (1,6)
- 2001-2002 Diplomarbeit im Institut für Evolutionsbiologie und Ökologie an der Rheinischen Friedrich-Wilhelms Universität zu Bonn, Thema: „Gibt es weibliche Partnerpräferenzen für Größe und/oder Symmetrie bei computeranimierten Stichlingsmännchen?“, Note: „gut“
- 1996 - 2001 Studium der Biologie mit Abschluss Diplom an der Rheinischen Friedrich-Wilhelms-Universität zu Bonn
- 1987 - 1996 Erwerb der „Allgemeinen Hochschulreife“ mit der Note: „gut“ am St. Adelheid Gymnasium, Bonn-Beuel

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 anderen als die angegebenen Hilfsmittel und Quellen verwendet habe. Die Arbeit hat bisher werde ganz noch zum Teil an anderer Stelle im Rahmen eines Prüfungsverfahrens vorgelegen. Kapitel I wurde mit den unten angegebenen Koautoren bei *Diseases of Aquatic Organisms* zur Publikation eingereicht. Weitere Teile dieser Arbeit sind in Vorbereitung für Veröffentlichungen bei Zeitschriften. Folgende Autoren waren an den einzelnen Kapiteln beteiligt: Kapitel I: M. Kalbe, J. Kurtz und J. P. Scharsack, Kapitel II: Erstautorin: I. Jaeger, Koautoren: S. Griffith, M. Milinski, T. B. H. Reusch, H. Schaschl, Kapitel III: M. Kalbe, Kapitel IV: I. Dankert, M. Kalbe, M. Milinski, T. B. H. Reusch, K. M. Wegner.

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