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ORIGINAL PAPER

Innate versus adaptive immunity in sticklebacks: evidence for trade-offs from a selection experiment

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Abstract In vertebrates, the immune system consists of two arms of different characteristics: the innate and the acquired immune response. Parasites that are only shortly exposed to the immune system are most efficiently attacked by fast, constitutive innate immune mechanisms. Here, we experimentally selected within four fish families for high innate resistance versus susceptibility of three-spined sticklebacks (*Gasterosteus aculeatus*) against infection with the eye-fluke (*Diplostomum pseudospathacaeum*), a parasite whose metacercariae are protected from the immune system within the eye lens. We predicted that in families with high susceptibility, the adaptive immune system would be upregulated when challenged with infection. In accordance, we found that MHC class IIB expression is increased by approximately 50% in those lines selected for higher parasite load (i.e. low innate response). This suggests extensive genetic correlations between innate and adaptive immune system and/or crosstalk between both lines of defense. An efficient, specific innate immune response might reduce overall activation of the immune system and potentially alleviate associated effects of immunopathology.

Keywords Major histocompatibility complex (MHC) · Ecological immunity · Innate resistance · Adaptive immunity · Gene expression · Three-spined stickleback (*Gasterosteus aculeatus*) · Parasites · Artificial selection

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Introduction

In vertebrates immune responses can be partitioned into innate and acquired (adaptive) responses. Innate responses are fast, because the underlying effector molecules (e.g. oxygen radicals) are constitutively expressed awaiting the infective agent (Janeway and Medzhitov 2002). These fast mechanisms are responsible for the clearance of the majority of infections (Parham 2003). While some innate immune mechanisms can show specificity in their response (Schmid-Hempel and Ebert 2003), the majority are, however, relatively unspecific and can result in damage of self-tissue (e.g. oxidative stress, Bogdan et al. 2000). The acquired/adaptive immune response, on the other hand, is characterized by high specificity due to somatic rearrangement of antigen receptors. Mounting of the adaptive response is relatively slow taking approximately 2 weeks in mammals and, depending on temperature, up to several weeks in cold blooded vertebrates, such as fish (Whyte et al. 1997). However, innate and adaptive immune responses are not independent pathways, but mutually depend on one another (Dixon and Stet 2001; Goldstein 2004).

The adaptive immune response not only relies on excitation of the innate immune system, but can on the other hand also regulate innate responses. For example, natural killer cells as a part of the innate immune system can be inhibited by MHC class I molecules, which represent the adaptive immune system (Dixon and Stet 2001). This and other such interactions might cause a trade-off within individuals between allocating resources to either a fast and potentially self-damaging immune response, or one that is highly specific but slow (Sheldon and Verhulst 1996; Schmid-Hempel and Ebert 2003). Such a trade-off might also be condition dependent, regulated by expression and reciprocal effects of either trait. To test a potential relationship between innate and adaptive immune responses we selected three-spined stickleback *Gasterosteus aculeatus* for innate resistance (i.e. first exposure) against the eye fluke *Diplostomum pseudospathacaeum* and measured the adaptive immune response by expression levels of MHC class IIB molecules after selection. We applied bi-directional selection for increased and reduced parasite load after primary exposure within four family backgrounds by full sib matings. The primary immune response against *Diplostomum* in sticklebacks is characterized by specific genotype x genotype interactions (Rauch et al. 2006), with an underlying complex, polygenic genetic trait (Kalbe and Kurtz 2006). Differences in parasite load following primary exposure outweigh the effect of the adaptive immune response, which consistently appeared after multiple exposures (Kalbe and Kurtz 2006). Therefore, the innate immune system seems to be of primary importance for defense against *Diplostomum* infections. Furthermore, *Diplostomum* is highly suited for testing innate versus acquired immunity because migration of cercariae is so fast (less than 24 h from entry to eye lens) that any acquired immune response is far too slow during primary exposure. After this period metacerariae encyst in the eye lens where they are protected from any immune response.

Nevertheless, the individual major histocompatibility complex (MHC) genotype, one of the key determinants of adaptive immunity, is also important for defense against *Diplostomum* infections when infections occur over several weeks—at least in the context of an optimal number of MHC class IIB sequence variants (Wegner et al. 2003). An optimal intermediate MHC diversity is associated with decreased levels of the innate oxidative burst response in mixed tapeworm and microsporidean

infections (Kurtz et al. 2004). Expression of MHC molecules is influenced by the MHC genotype (Wegner et al. 2006) and is correlated with oxidative stress probably caused by activation of the immune system (Kurtz et al. 2006). This not only demonstrates how tightly the parts of the adaptive immune system are interwoven with innate responses, but also shows the suitability of MHC class II expression levels as a molecular marker for measuring the activation of costly immune mechanisms.

We investigated whether altering the innate defense by artificial selection shows any effect on the other major system in the immune response. Accordingly, we selected based on infection rates right after primary exposure, i.e. an innate immune response. Our within-family selection procedure separates residual genetic variance present within families. If such variation is present, we would hypothesize that those selection lines with a more potent, selected innate response would down-regulate expression of parts of the adaptive immune system, i.e. MHC class IIB, and vice versa.

Materials and methods

Fish families and breeding design

Founding fish originated from a large, outbred population, Grosser Plöner See, Northern Germany. F1 fish were lab-bred by random single-pair matings. After spawning eggs were removed from the nest and kept in an aerated glass jar until hatching. Fry was then transferred to flow-through aquaria (16 l) in densities of ≈30 fish per tank. Out of these families four were picked for selection, which differed significantly from each other in terms of innate resistance against *Diplostomum* infection determined in an initial infection screen (see below). From each of these families ten males and ten females were picked randomly for selection. These fish were then exposed and non-invasively screened as described below. Within each family the two males and females with highest as well as lowest parasite load were chosen for breeding of the F2. Breeding of F2 fish proceeded in June/July 2003. We assayed a total of 16 inbred families. To control for possible effects of inbreeding, we also produced two outbred clutches by randomly choosing males and females from different families, which either had low or high parasite load. In each exposure round one inbred family of each family background and direction of selection (i.e. eight families in total) and two outbred families (one upwards selected, and one downwards selected) were exposed and afterwards screened for MHC expression. In the two rounds of exposure a total of 20 families was used. Fish were kept under identical conditions during the experimental procedure. Lab conditions were 18°C water and air temperature at a 16/8 h light–dark cycle.

Parasites exposure and screening

For exposure we used cercariae from five *Lymnaea stagnalis* snails, which is the first intermediate host in the *Diplostomum pseudospathacaeum* life cycle. By using five different snails we created a genotypically diverse infection, because different snails are rarely infected with the same *Diplostomum* clone (Rauch et al. 2005). Snails used for the initial screen differed from those used for the exposure of parents and F2 fish,

but used the same snails for both parents and offspring during the selection procedure. F2 fish were exposed in two rounds of 10 families (8 inbred + 2 outbred) each, on the 8th December 2003 and 18th of January 2004, respectively. On day of exposure cercariae were isolated from each snail and mixed to equal contributions. Ten fish were then exposed individually in a small 1l tank with 20 cercariae as exposure dose for 4–5 days until screening. The long exposure time should ensure that each cercariae was likely to have encountered the fish. After the initial screen, however, we found only relatively low infection rates (Fig. 1). Therefore, we increased the exposure dose to 30 cercariae in the exposure of parents and selected F2 families. Since metacercariae inhabit the eye lens of the fish, infection rate can be assessed non-invasively by inspection under a stereo lens (Leica MZ5, 6.3–50× magnification). To do so, we put each fish in a petri dish under the microscope. We carefully turned the fish's eyeball with blunt tweezers and adjusted the light source accordingly until we could see into the lens and count the metacercariae.

MHC expression

To measure MHC class IIB expression we first extracted total RNA from gill tissue. After a time period long enough to guarantee the onset of an adaptive immune response (i.e. >6 weeks), we randomly picked 80 fish out of the exposed F2 generation for RNA extraction on 4th April 2004. The choice of fish was not biased towards a specific round or treatment and therefore represented a random subsample. Two gill arches were transferred to tissue lysis buffer containing 1% β-mercaptoethanol immediately after killing the fish and were homogenized using a Retsch ball mill. RNA was then purified using the NucleoSpin II RNA extraction kit (Macherey-Nagel) including on-column DNA digestion. One microgram of RNA was then used for reverse transcription using anchored oligo dT primers and the

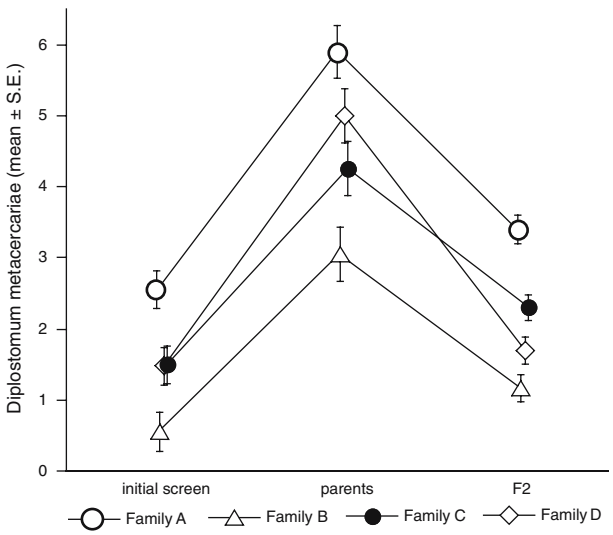

Fig. 1 Parasite load of *Diplostomum* metacercariae for each family during the three infections (F2 rounds pooled). A different set of snails was used to isolate *Diplostomum* cercariae during the initial screen

Omniscript RT kit (Qiagen). Template concentration of MHC class IIB transcripts was determined using relative quantification against the housekeeping gene β actin using the $\Delta\Delta$CT method (Livak and Schmittgen 2001). A detailed description of the expression assay can be found in Wegner et al. (2006).

In parallel the eye balls of the sacrificed fish were removed to enable accurate counting of metacercariae in F2 fish. Mean fish size per family ranged from 41.16 ± 1.17 to 51.26 ± 2.28 mm at time of dissection.

Statistical analysis

All analyses were conducted in JMP 5.1 (SAS institute). To assess the success of our selection procedure, we used a full factorial repeated measures ANOVA with direction of selection as repeated measurement and family background and round as additional factors. Additionally, we also used a paired *t* test using pooled parasite load estimates of selection lines within family. There were no significant differences between the two exposure rounds or any interaction term containing round. Therefore we pooled the data of the two rounds into a single analysis.

To estimate the heritable genetic component we used parent–offspring regression. By nature of our selection regime parasite load of mothers correlated to that of fathers. Therefore, we used the mid-parent value as a regressor for the mean parasite load in the offspring. Infection success decreased between exposure of parents and F2 fish, possibly caused by ageing sporocysts in the snail. To obtain reliable slope estimates for the regression we used standardized values, which were adjusted to the mean of the respective exposure round. For standardization we subtracted the mean of each round of exposure from each value and divided the difference by the standard deviation to express each measurement in standard deviate units (Sokal and Rohlf 1995).

The MHC expression differs strongly between families (Wegner et al. 2006). In the analysis of MHC expression we accounted for this by using a two-way ANOVA with direction of selection and family background in a full factorial model.

Results

Among family variation in parasite load

In all rounds of exposure, we found significant differences among families. Family A was always most susceptible and family B most resistant against *Diplostomum* infection (Fig. 1, univariate test with degrees of freedom adjusted to size of the smallest group, family effect: $F_{3,37} = 33.066$, $P < 0.001$). There were also strong differences among the different exposure rounds (Fig. 1, univariate test with degrees of freedom adjusted to size of the smallest group, family effect: $F_{2,37} = 89.382$, $P < 0.001$), which were partly caused by the increase in exposure dose between the initial screen and artificial selection but might also reflect ageing sporocysts during the timespan between parent exposure and offspring exposure. Despite of this, the overall pattern of family resistance did not change during the course of the exposures as shown by the non-significant interaction effect ($F_{6,37} = 1.396$, $P = 0.242$).

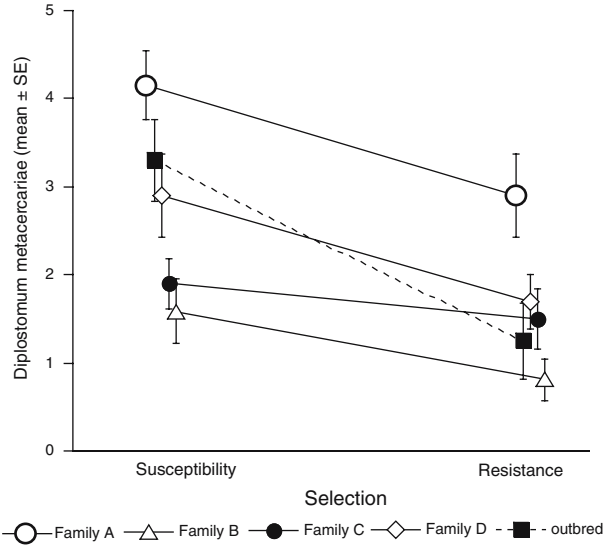

Fig. 2 Parasite load of *Diplostomum* metacercariae for the *upwards* and *downwards* selected lines within each inbred family and the outbred families. The two rounds of selection were pooled to obtain a single value for each direction of selection within families

Table 1 *Diplostomum pseudospathacaeum* infection in selected families

Source	*df* (numerator, denominator)	*F*	*P*
Family	**4, 80**	**6.792**	**<0.0001**
Round	1, 80	2.738	0.1018
Round*Family	4, 80	1.745	0.1482
Selection	**1, 80**	**13.789**	**0.0002**
Selection*Family	4, 80	1.194	0.3181
Selection*Round	1, 80	0.3173	0.5178
Selection*Family*Round	4, 80	1.0871	0.3686

Repeated measures ANOVA with direction of selection as measurement repeated within round and family background. Significant terms are printed bold

Success of selection experiment

By selectively pairing resistant and susceptible individuals within families we could produce differences between the paired selection lines (Fig. 2, Table 1). As the non-significant interaction term (family × selection in Table 1) suggests, these differences were consistent within all family backgrounds resulting in lower parasite load in downwards and increased parasite load in upwards selected lines. This difference was also significant in a paired *t* test using family background as statistical unit ($t = -4.119$, $N = 5$, $P = 0.015$). After adjusting for differences in infection rate between parent and offspring infections by using standardized parasite loads, we found a significant correlation between phenotypic values of the mid-parent and the resulting offspring (Fig. 3, $R^2 = 0.551$, $F_{1,18} = 22.094$, $P < 0.001$). The broad sense heritability estimate h^2 derived from the slope of the regression was 0.420, indicating a medium to high heritability of the trait. Explanatory value of mother-offspring regression was

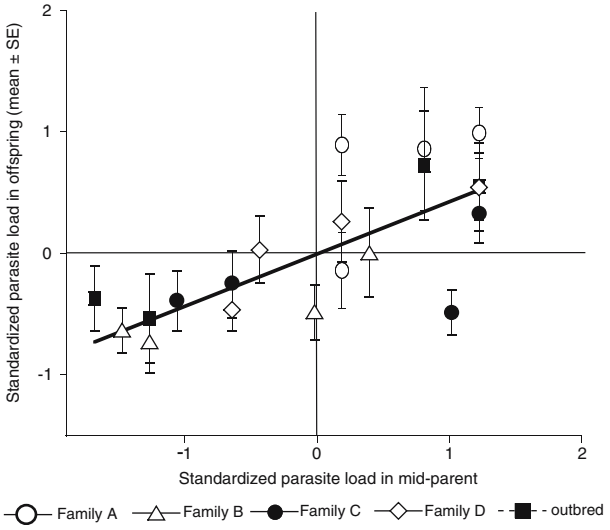

Fig. 3 Mid-parent offspring regression of parasite loads in F1 parents and F2 offspring. Standardized values within each round of infection were used because of systematic changes in infection rates during the course of the experiment. For standardization we substracted the mean of each round of exposure from each value and divided the difference by the standard deviation to express each measurement in standard deviation units

marginally higher than the combined mid parent ($R^2 = 0.592$, $F_{1,18} = 26.090$, $P < 0.001$) and father–offspring regression was lower but still significant ($R^2 = 0.429$, $F_{1,18} = 13.511$, $P = 0.002$). This along with a steeper slope of the mother–offspring regression (0.435) compared to the father–offspring regression (0.371) indicates the influence of maternal effects, which was however small when compared to genetic effects. To avoid confounding effects of correlations of phenotypic values of fathers and mothers we also calculated h^2 via realized selection. The result was equally large ($h^2 = 0.34$) demonstrating the robustness of our heritability estimate.

MHC expression in selection lines

After demonstrating that our experimental selection was successful, we were interested in the effect of altered innate resistance on components of the adaptive immune system, i.e. transcription of MHC class IIB genes. We found a significant increase of MHC expression in those lines selected for increased parasite load, indicating lower innate immune defense (Table 2) corresponding to 50% higher levels of average MHC expression (Fig. 4). Again, as with effects of selection, the within-line effect was smaller when compared to the between-line effect, suggesting extensive effects of genetic background (Table 2).

Table 2 ANOVA table for MHC class IIB expression in 10 families from five family backgrounds

Source	*df*	SS	*F*	*P*
Family background	3	22.116	6.427	**<0.001**
Direction of selection	1	7.756	6.762	**0.012**
Family background*Direction of selection	3	2.819	0.819	0.486
Error	58	66.528		

Within each family bi-directional selection for high and low parasite load was applied. A subset of 80 individuals was used

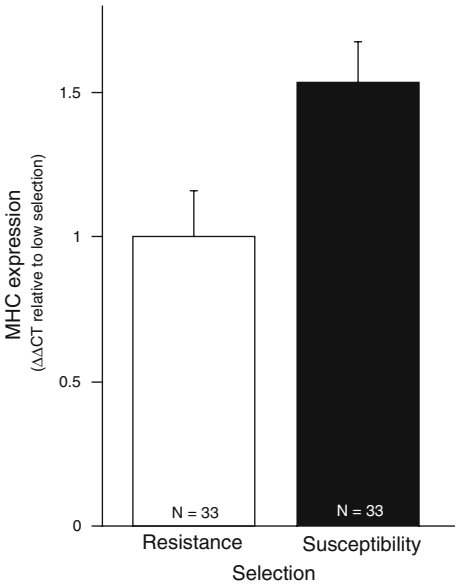

Fig. 4 Average expression of MHC class IIB genes in F2 lines selected for increased or decreased parasite load. Least square means of ΔΔCT values relative to the downwards selected line obtained from the two-way ANOVA including family are shown

Discussion

Theory predicts that reciprocal selection by parasites and counteradaptation by host defence will lead to considerable amounts of genetic variation (Haldane 1949; Hamilton et al. 1990). In this study, innate resistance to primary infection of the eyefluke *Diplostomum pseudospathacaeum* shows such variation among different families of three-spined sticklebacks that were all derived from a single population (Fig. 1). In addition, we found ample genetic variation within families, demonstrated by successful experimental selection (Table 1, Fig. 2). The amount of genetic variation present at the family level differed between families (Fig. 2) showing that genetic variability at relevant loci depends on the family background. Outbred families, in particular, revealed a distinct reaction norm, which was most likely caused by combining different genetic backgrounds. The unimodal distribution of innate resistance against *Diplostomum* infection in a F1 hybrid intercross shows the typical patterns of a polygenic trait (Kalbe and Kurtz 2006). Our data indicate that most loci affecting resistance and susceptibility are also polymorphic within a single population, because selection was successful in all family backgrounds. Within families different alleles can be found at these hypothetical loci with some of them in homozygous form resulting in smaller within-family genetic variability (Fig. 2). The separate mother- and father-offspring regression showed that influence of maternal effects on the offspring phenotype was low. In the breeding design of Kalbe and Kurtz (2006), which balanced resistant and susceptible mothers and fathers, the F1-offspring showed a unimodal distribution further indicating that maternal effects are relatively weak.

Diplostomum metacercariae reside within the eye lens of the fish and impose high fitness costs by interfering with escape (Crowden and Broom 1980; Seppala et al. 2004) as well as foraging capabilities—even at low levels of infection (Owen et al. 1993). Given these high costs for a visual predator along with the medium herita-

bility of this trait (Fig. 3), it is puzzling why selection did not erode genetic variation within the population sampled here. One possible explanation is that strong genotype × genotype interactions maintain polymorphism by negative frequency dependent selection (Dybdahl and Lively 1998). Using the same system Rauch et al. (2006) found strong parasite clone × fish family interactions indicating the presence of such tight coupling of the ''matching alleles'' type (Dybdahl and Storfer 2003). On the other hand, we created a genotypic diverse infection by using cercariae from five different snails. The fact that we still find strong fish family effects might mean that some loci involved in resistance might provide a more general resistance against several parasite genotypes as predicted by ''gene-for-gene'' interactions (Thompson and Burdon 1992). On theoretical grounds host–parasite interactions are a continuum with ''matching alleles'' and ''gene-for-gene'' interactions being extremes on both sides of the spectrum (Agrawal and Lively 2002). Therefore, it is not surprising that different genes involved in resistance might follow either rule of genotype × genotype interaction. The identification of the genes involved in resistance is an interesting goal to pursue and the recent development of genomic tools in sticklebacks, such as linkage maps (Peichel et al. 2001) and the full genomic sequence (http://www.genome.gov) makes it a feasible approach.

One group of genes that is involved in resistance against *Diplostomum* are genes of the MHC class IIB, which are also highly polymorphic (Klein 1986; Bernatchez and Landry 2003). Experimental infection with multiple parasite species, including *Diplostomum pseudospathacaeum*, showed an influence of the number of MHC class IIB sequence variants on the actual infection rate (Wegner et al. 2003), but only after repeated infection. Often even single MHC alleles are associated with resistance against infection (e.g. Briles et al. 1977; Hill et al. 1991; Decamposlima et al. 1993; Godot et al. 2000; Langefors et al. 2001; Grimholt et al. 2003; Harf and Sommer 2005). We also found such associations for sticklebacks infected with *Diplostomum* in a natural population over a time course of 4 years (K.M. Wegner et al. unpublished). MHC genes are, however, not affected by our selection procedure, because they belong to the adaptive arm of the immune system and take effect only via antibodies and prior immunization. Our selection procedure specifically selected for innate immune defence, because only immunologically naïve individuals were used and metacercariae, which reached the eye lens, are protected from adaptive immunity. Since both arms of the immune system are interwoven (Dixon and Stet 2001), it is interesting to investigate the consequences of an efficient innate immune system on the subsequent expression of adaptive immune traits. An antibody mediated adaptive immune response needs to be activated by innate effector molecules (Medzhitov and Janeway 1997). But could a specific innate immune response also prevent the activation of the full repertoire of immunity? We could show that expression of MHC class IIB genes is on average 50% higher in lines selected for increased susceptibility than in the corresponding ones selected for decreased susceptibility (Fig. 4). This increase is approximately twofold higher than the increase following repeated exposure of fish to a suite of three macro-parasites that do challenge the adaptive immune system (Wegner et al. 2006). Such an increase in MHC class IIB expression is particularly relevant because higher MHC expression (i.e. an increased activation of the immune system) was correlated with potential costs in terms of higher levels of oxidative stress (Kurtz et al. 2006). Avoiding such damage by an efficient, i.e. specific, innate response with low levels of immunopathology might reduce overall costs of immunity of the individual.

Diplostomum metacercariae are exposed to the immune system only for a short period of time before they reside and develop in the eye lens of the fish. Therefore, relying on the relatively slow adaptive, MHC-mediated arm of the immune system might not be the best strategy. Rather efficient innate immune mechanism, which are constitutively expressed, are needed to clear infections before encystations (Rauch et al. 2006). The importance of innate immune mechanism was also highlighted by the relative contribution of the adaptive immune response to resistance against *Diplostomum* in sticklebacks. Only after multiple exposures a reduction in infection rate could be observed, which was small compared to the effect of innate immunity (Kalbe and Kurtz 2006). Generally, the health of wild animals, to a great extent depends on its innate immune system (Parham 2003). Therefore, the characterization of immune traits and ultimately genes involved in innate defense against infection and their possible interactions is needed to explain polymorphism and the short-term evolution of the immune system in response to parasites. The degree of specificity observed in the well controlled *Diplostomum*-stickleback system (Rauch et al. 2006) creates the possibility to do so and gain further insight into the nature of parasite mediated-balancing selection.

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