

Habitat-specific adaptation of immune responses of stickleback (*Gasterosteus aculeatus*) lake and river ecotypes

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Freshwater populations of three-spined sticklebacks (*Gasterosteus aculeatus*) in northern Germany are found as distinct lake and river ecotypes. Adaptation to habitat-specific parasites might influence immune capabilities of stickleback ecotypes. Here, naive laboratory-bred sticklebacks from lake and river populations were exposed reciprocally to parasite environments in a lake and a river habitat. Sticklebacks exposed to lake conditions were infected with higher numbers of parasite species when compared with the river. River sticklebacks in the lake had higher parasite loads than lake sticklebacks in the same habitat. Respiratory burst, granulocyte counts and lymphocyte proliferation of head kidney leucocytes were increased in river sticklebacks exposed to lake when compared with river conditions. Although river sticklebacks exposed to lake conditions showed elevated activation of their immune system, parasites could not be diminished as effectively as by lake sticklebacks in their native habitat. River sticklebacks seem to have reduced their immune-competence potential due to lower parasite diversity in rivers.

Keywords: ecological immunology; *Gasterosteus aculeatus*; ecotypes; parasites; immune response; specific growth rate

1. INTRODUCTION

Three-spined sticklebacks (*Gasterosteus aculeatus*) are a powerful evolutionary model system due to the rapid and repeated phenotypic divergence of freshwater forms from a marine ancestor throughout the Northern Hemisphere (Bell & Foster 1994). For example, a large-bodied benthic form and a smaller, more slender limnetic form are present in coastal lakes of British Columbia, Canada (McPhail 1984). Gene flow between stickleback ecotypes (or ecomorphs) is limited even in close neighbouring populations of the different ecotypes (Reusch *et al.* 2001; Hendry & Taylor 2004). Strong assortative mating may explain the limited gene flow, and the reproductive isolation between stickleback ecotypes is thought to reflect ecological speciation (McKinnon *et al.* 2004; Olafsdottir *et al.* 2006; Vines & Schluter 2006). This ecological speciation is driven by environmental factors (e.g. climate, resources) and interaction with other species (e.g. resource competition, predation; Schluter 2001; Vamosi & Schluter 2004). Interaction with habitat-specific pathogens (e.g. parasites) and the underlying immunological capabilities of ecotype hosts has yet to be fully considered as a potential factor in the divergence of species.

Freshwater stickleback populations in northern Germany are divided into lake and river ecotypes. Genetic divergence between local lake and river ecotypes is higher than that between populations of the same ecotype from separate drainage systems (Reusch *et al.* 2001). Apparently, the different ecotypes do not mate in the wild although

sexually mature lake and river sticklebacks will mate when placed together in a tank in the laboratory (Rauch *et al.* 2006a). Hybridization of the two ecotypes would be possible in the wild, as lakes in the region are connected by rivers, permitting free movement between habitat types.

It is suggested that adaptation to habitat-specific pathogens (e.g. parasites) is an advantage that might contribute to the formation of a mating barrier between the two stickleback ecotypes. Three-spined sticklebacks are exposed to a diverse range of parasites, and single fish can be infected by numerous different parasite species (Chappell 1969; Wootton 1976; Zander *et al.* 1999; Kalbe *et al.* 2002; Wegner *et al.* 2003a) and different genotypes of the same species (Rauch *et al.* 2005). Species diversity of abundant parasites in the study region is far greater in lakes when compared with river habitats (Kalbe *et al.* 2002). Laboratory infections of lake and river sticklebacks with a typical lake parasite, the eye fluke *Diplostomum pseudospathaceum*, showed that lake sticklebacks were more resistant to the infection, demonstrating habitat (parasite)-specific adaptation in the immune response of stickleback ecotypes (Kalbe & Kurtz 2006).

Diversity of major histocompatibility complex (MHC) genes could play a role in the variation in infection resistance between the stickleback ecotypes. Investigations on the influence of MHC genes on parasite load are summarized and reviewed by Apanius *et al.* (1997). In laboratory parasite infections, sticklebacks with intermediate numbers (5–6) of MHC class II beta alleles showed lower infection rates than sticklebacks with high (9) or low (3) numbers of alleles (Wegner *et al.* 2003b), indicating that intermediate (optimal) MHC diversity conveys resistance better than high MHC diversity. When

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investigating the role of MHC class II beta in F2 hybrids of lake and river sticklebacks after field exposure, Rauch *et al.* (2006a) did not find that MHC genotype influenced resistance to parasite infections. In contrast, genomic background, independent of MHC genotype, explained a significant percentage of the variation in parasite load (Rauch *et al.* 2006a). In a laboratory experiment, it has been shown that fishes can mount a genotype-specific defence which works independently from the MHC-based adaptive immune system (Rauch *et al.* 2006b). In the present study, we aimed to identify functional immune parameters that might be activated differentially in the two ecotypes. Here, only pure family lines from lake and river sticklebacks were investigated, as we were not only interested in MHC-related immune defence, but also in other defence components such as innate immunity.

The effects of host–parasite interaction can vary between parasite species (Combes 2001; Hoole *et al.* 2003). Characteristics of the immune defence of the host may depend on infection route, target organ and pathogenicity of the invading parasite species (Buchmann *et al.* 2001; Tully & Nolan 2002; Roberts *et al.* 2005; Wiegertjes *et al.* 2005; Reite & Evensen 2006). A major part of the innate immune defence of fish hosts against macro parasites is the activation of granulocytes (Whyte *et al.* 1989; Nie & Hoole 2000; Kurtz *et al.* 2004; Scharsack *et al.* 2004). Production of oxygen radicals is a key function of activated granulocytes (Verburg-van Kemenade *et al.* 1996; Serada *et al.* 2005). 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 of the specific immune response of fishes (Rijkers *et al.* 1980; Le Morvan-Rocher *et al.* 1995). Lymphocyte proliferation is used as a measure for activation of the specific immune system against parasites in fish hosts (Hamers & Goerlich 1996; Nie *et al.* 1996; Scharsack *et al.* 2000).

In the present study, laboratory-bred parasite-free lake and river sticklebacks were exposed reciprocally to lake and river parasites and parasite infection. After eight weeks of exposure, immune parameters such as lymphocyte proliferation, frequencies of granulocytes and respiratory burst activity were analysed in head kidney leucocytes (HKL). Additionally, growth performance was recorded as specific growth rates (SGRs; Barber 2005). Variation in parasite burden, immune defence and growth performance between the stickleback ecotypes may contribute to our understanding of the evolution of speciation and the maintenance of mating barriers between lake and river stickleback ecotypes.

2. MATERIAL AND METHODS

(a) *Experimental stickleback*

Three-spined sticklebacks were caught from a river (Schwale) and a lake (Vierer See) belonging to different drainage systems in northern Germany in autumn 2002. To obtain sticklebacks with a pure river and a pure lake genotype, respectively, six crosses between wild-caught river and lake sticklebacks were produced. For comparability reasons, with a second experiment studying the role of MHC on parasite load, where the design required a breeding procedure over two generations to produce

hybrid lines containing fish with a distinct river or lake MHC, we also produced a second generation of the pure lines (for results on parasite load of hybrid lines and breeding protocol, see Rauch *et al.* 2006a). After hatching, F2 offspring were grown for seven months in summer conditions at a density of 20 fish per tank, before these fish were used in the experiment.

(b) *Field exposure*

The F2 fish were exposed in cages placed in the river and in the lake for eight weeks in August and September 2004, close to the original capture locations of the parental generation. In the river, cages were placed at intervals of 10 m in mid-channel (approximate width = 2 m and depth = 0.5 m). In the lake, cages were placed approximately 10 m offshore (depth 1 m) at intervals of 10 m along the shoreline. Cages were cylindrical (1 m length and 40 cm diameter, placed horizontally) and covered with 4 mm wire mesh (Rauch *et al.* 2006a).

In total, 120 sticklebacks were exposed, 20 fish from each of the three river and the three lake families. Ten fish from each family were placed in the river and 10 in the lake. To expose fish to different within-river and within-lake conditions, we randomly distributed two fish from each family between two cages, repeated this procedure 10 times and placed 10 cages in the river and 10 cages in the lake. This procedure resulted in some cages containing no fish and other cages containing two fish from a specific family. In addition, we released 8–10 fish of the hybrid lines in each cage. For the present study on the activation of immune cells, hybrid lines were not used although they allowed for the control for an MHC effect, because it was not clear whether other parts of the immune system we were especially interested in, such as the innate immunity, had a river or a lake origin. Prior to release and after catching, we measured the length (total length to the nearest millimetre) and mass (to the nearest milligram) of every fish. Fish growth performance (both in terms of length and weight) was compared between experimental groups using SGRs, corrected for variation in initial size following Barber (2005). Briefly, SGRs were calculated using: $SGR = 100 \times (\ln_{\text{size at end}}) - (\ln_{\text{size at start}})$, where size = length (mm) or weight (mg). Prior to release, each experimental fish was identified using five polymorphic microsatellites to discriminate between them after the experiment. The microsatellites Gac1097, Gac1125, Gac4170, Gac5196 and Gac7033 developed by Largiader *et al.* (1999) were used with the microsatellite analysis protocols in Reusch *et al.* (2001).

(c) *Parasite infection*

Sticklebacks were collected eight weeks after release and at the time of capture had not attained sexual maturity. Size and weight of the fish were measured again. Individual identity was determined with the microsatellite markers. Fish were killed by immersion in an overdose of methane sulphonate (MS 222; 1.5 g l^{-1}) in tap water. All fish were examined for parasite infection. Body surface and all inner organs including eyes and gills were screened for parasites (for details on parasite screening, see Kalbe *et al.* 2002). In total, 15 species (two of which were present only on hybrid lines, not shown) of macro parasites were recorded and counted on individual stickleback, except for *Trichodina* sp. (table 1). For *Trichodina* sp., number of parasites was estimated to be 10, 50 or 100 on the visible parts of the fins. An estimation of *Trichodina* sp. numbers was possible, as fish were not heavily infected and numbers did not exceed 100 individuals per fish. Both

Table 1. Class, species name, prevalence in per cent (P%), mean intensity (MI) of parasites per fish for lake and river sticklebacks after reciprocal exposure to the two habitats. (Note: in the lake exposure, abundance of *Diplostomum* sp. per fish was significantly higher in river stickleback. * $p < 0.0031$, significant difference between lake and river sticklebacks in the same habitat exposure with Bonferroni correction for 16 tests; ** , temporary ectoparasite, see text.)

class	parasite species	transmission	infective stage	lake exposure			river exposure					
				lake stickleback (n=25)			lake stickleback (n=22)			river stickleback (n=28)		
				P (%)	MI	P (%)	P (%)	MI	P (%)	P (%)	MI	P (%)
Ciliata	<i>Trichodina</i> sp.	direct	—	68	24.71	70	28.13	5	10	4	10	
Monogenea	<i>Gyrodactylus</i> sp.	direct	—	—	—	—	—	68	6.13	79	6.45	
Digenea	<i>Diplostomum</i> sp.	active	cercariae	100	9.96*	100	36.87*	—	—	—	—	
	<i>Echinostomum</i> sp.	active	cercariae	16	1	26	1.17	—	—	—	—	
	<i>Cyathocotyle prussica</i>	active	cercariae	8	1	4	3	—	—	—	—	
	<i>Tylocephalus clavata</i>	active	cercariae	8	3	13	5	—	—	—	—	
	<i>Proteocephalus filicollis</i>	trophic	larvae in copepod	4	1	4	1	41	2.67	11	2	
Acanthocephala	<i>Vaipora campylancristota</i>	trophic	larvae in copepod	24	1.33	22	2.40	—	—	—	—	
	<i>Acanthocephalus lucii</i>	trophic	cystacanth in isopod	—	—	4	1	—	—	—	—	
Nematoda	<i>Camallanus lacustris</i>	trophic	larvae in copepod	40	1.50	9	1	—	—	—	—	
	<i>Anguillicola crassus</i>	trophic	larvae in copepod	8	1	13	1	—	—	4	1	
	<i>Raphidascaris acus</i>	trophic	larvae in various invertebrates	—	—	—	—	5	2	36	1.40	
Crustacea	<i>Argulus foliaceus</i> **	direct	—	—	—	52.17	1.58	—	—	—	—	

Trichodina sp. and *Gyrodactylus* sp. can reproduce on the fish. Therefore, numbers found on the fish may not reflect the actual infection intensity in the wild. For every stickleback, a relative parasite load averaged over all parasite species present in the respective habitat was calculated. In both the habitats, maximal number of parasites per fish from each species was recorded. Number of individual parasites from one species found on a fish was divided by the maximal number of the respective parasite species found in the habitat. Fish not infected by a parasite species that was present in the habitat scored a zero for this species. The parasite load of individual fish was calculated as the average of relative numbers of each parasite species present in the respective habitat.

(d) Isolation of head kidney leucocytes

For immunological assays, leucocytes were isolated from the head kidney of sticklebacks. All steps for leucocyte preparation were performed on ice and only refrigerated media and cooled centrifuges were used. Cell suspensions from head kidneys were prepared by forcing the tissues through a 40 µm nylon screen (BD-Falcon, USA). Isolated HKL were washed twice (4°C, 10 min 550×g) with RPMI 1640 diluted with 10% (v/v) distilled water (R-90) and resuspended in a final volume of 1 ml R-90 (Scharsack *et al.* 2004).

(e) Flow cytometric analysis of head kidney leucocytes

Total cell numbers in HKL isolates were determined with the standard cell dilution assay (SCDA, Pechhold *et al.* 1994) in a modified form (Scharsack *et al.* 2004): washed cells (25 µl) were transferred to individual flow cytometer tubes; 3×10^4 green fluorescent standard particles (4 µm, Polyscience, USA) and propidium iodide (2 mg l⁻¹, Sigma Aldrich) were added to each tube. FSC/SSC characteristics of at least 10 000 events were acquired in linear mode; fluorescence intensities at wavelengths of 530 and 585 nm were acquired at log scale with a flow cytometer (FACSCalibur, Becton and Dickinson, USA). Flow cytometric data were analysed with the CELLQUEST PRO v. 4.02 software for acquisition and analysis. Cellular debris with low FSC characteristics was excluded from further evaluation. Standard particles (green fluorescence positive) were discriminated from viable HKL (propidium iodide negative, green fluorescence negative). Total numbers of cells in individual samples were calculated according to: $N[\text{vital cells}] = \text{events}[\text{vital cells}] \times \text{number}[\text{standard beads}] / \text{events}[\text{standard beads}]$. Total cell counts were needed to adjust concentrations of individual HKL suspensions to 1.25×10^6 cells per ml, to have comparable cell numbers for the subsequent respiratory burst assay.

Additionally, flow cytometric measurements of freshly isolated HKL were used to determine proportions of granulocytes (FSC/SSC^{high}) and lymphocytes (FSC/SSC^{low}) in individual HKL samples (Scharsack *et al.* 2004).

(f) Respiratory burst activity of head kidney leucocytes

As one of the most important effector mechanisms of cell-mediated innate immunity, the respiratory burst activity of HKL was quantified in a lucigenin-enhanced chemiluminescence (CL) assay modified after Scott & Klesius (1981), as described in Kurtz *et al.* (2004). In white 96 well flat-bottomed microtitre plates, 160 µl of cell suspension (2×10^5 HKL per well) were added to 20 µl lucigenin solution (2.5 g l⁻¹ PBS). Plates were incubated for 30 min at 18°C to

allow uptake of lucigenin by the cells. Phagocytosis and production of reactive oxygen species (ROS) was initiated by the addition of 20 µl zymosan suspension (7.5 g l⁻¹ PBS) and measured for 3 h at 18–20°C. The CL assay was measured using a microtitre plate luminometer (Berthold, Germany). Relative luminescence (RLU) was evaluated for each sample using the WINGLOW software. Maximum of respiratory burst activity (RLU s⁻¹, given in figure 3) calculated by the WINGLOW software represents the peak of the activity curve recorded during the 3 h of measurement.

(g) Cell cycle analysis

As a parameter for activation of the adaptive immune system, we determined the relative number of lymphocytes in the G_{2-M} phase of the cell cycle after DNA labelling with propidium iodide by means of flow cytometry. During the cell cycle in the G₀₋₁ phase, cells have a single set of chromosomes and a constant content of total DNA. Cells starting to proliferate enter the S (synthesis) phase characterized by increasing amounts of DNA per cell. In the G_{2-M} phase, cells have completed DNA synthesis, are endowed with a double set of chromosomes (DNA) and start to divide. Accordingly, proliferating cells in the G_{2-M} phase can be distinguished from G₀₋₁ and S phase cells by their higher DNA content. For cell cycle analysis, HKL were fixed with ethanol (100 µl cell suspension as described above in 900 µl ice cold ethanol 98%) and stored at 4°C. Before measurement, cells were centrifuged (550×g, 10 min, 4°C) and supernatant ethanol was removed. Cells were resuspended with RNAse (500 mg l⁻¹ PBS) and incubated for 10 min at room temperature to remove background labelling of RNA. Propidium iodide (Sigma Aldrich) was added to a final concentration of 7.5 mg l⁻¹ and cells were incubated again for 10 min at room temperature. Individual samples were measured for 3 min or up to 30 000 events with a Becton Dickinson FACSCalibur flow cytometer. Red fluorescence (propidium iodide) was measured in linear mode. Data were evaluated with the CELLQUEST PRO v. 4.02 software. Cellular debris (low scatter characteristics) and aggregated cells (high scatter characteristics) were subtracted from further evaluation. Doublet cells were subtracted from single cells as described in Wersto *et al.* (2001). Lymphocytes were identified according to their characteristic FSC/SSC profile. Frequencies of lymphocytes in G₀₋₁, S and G_{2-M} phases were acquired by DNA content analysis of red fluorescence intensity (propidium iodide labelling) of single cells from the lymphocyte gate.

(h) Data analysis

The effect of habitat of exposure and origin on length and weight increase, parasite species number, parasite load, oxidative burst activity, proportion of granulocytes and lymphocytes, and proportion of proliferating lymphocytes were tested with ANOVA with the fixed factors habitat and origin and their interaction and family effect nested within origin. Family effect was controlled for in this design, but family effect statistics are not shown, as drawing conclusions from the nested factor statistics are not recommended in such a split-plot design (Zar 1999). Cage effect was not included in the full model as some family cage combinations were absent. Cage effects were tested separately in a one-way ANOVA for river and lake cages. Wherever necessary, data were log, square-root or Box-Cox transformed in order to normalize non-normally distributed data.

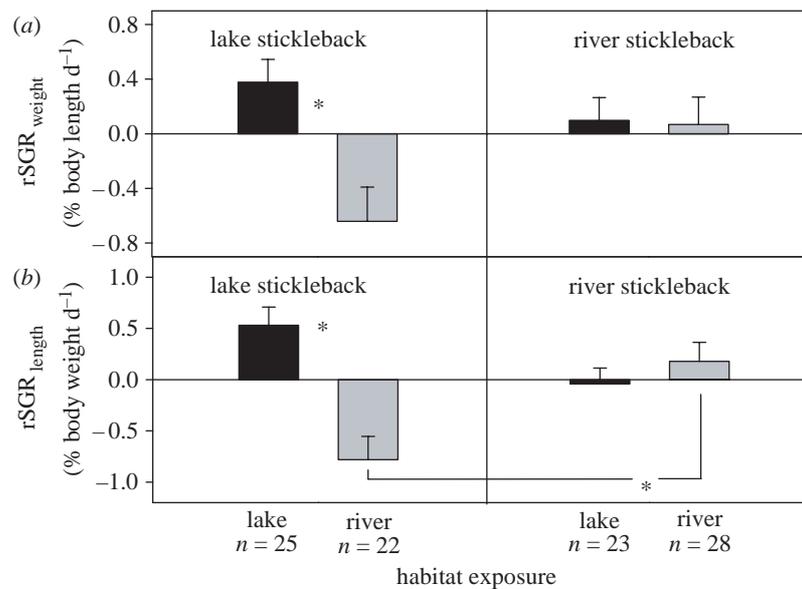


Figure 1. Differences in relative growth performance (rSGR) in sticklebacks in terms of (a) weight and (b) length (mean + s.e.). Lake sticklebacks had significantly reduced growth when exposed to river conditions when compared with lake conditions, in terms of both length and weight (* $p < 0.0125$, t -test, p value Bonferroni corrected for multiple tests).

To identify significant differences in infection intensities for each parasite species in lake and river sticklebacks under each experimental condition, we used student's t -tests with Bonferroni correction for multiple testing (p value set to 0.0031 for 16 tests, table 1). Effects of habitat of exposure and origin on fish length/weight increase, number of parasite species per fish, parasite load, proportion of granulocytes, respiratory burst activity and proportion of proliferating lymphocytes were tested in a *post hoc* test using student's t -tests, with Bonferroni correction for multiple testing (p value set to 0.0125 for four tests).

3. RESULTS

(a) Fish condition and growth performance

Of the 120 fish originally exposed to experimental conditions, 98 were recovered—10 fish disappeared (probably died or escaped) from the river cages while 12 fish were similarly unaccounted for in the lake. Fish grew in terms of both length and weight during exposure to experimental conditions, but there was significant variation in growth performance between experimental groups (figure 1). Since stickleback SGR was negatively correlated with individual size at the beginning of the experiment (length: $r = -0.56$, $n = 98$, $p < 0.001$; weight: $r = -0.63$, $n = 98$, $p < 0.001$), we followed Barber (2005) and regressed SGR on initial body size to provide residual SGR values (rSGR) and to correct growth rates for initial body size. As might be expected by the close correlation between length and weight in the sticklebacks ($r = 0.95$, $n = 98$, $p < 0.001$), variation in $rSGR_{weight}$ generally follows that of $rSGR_{length}$ (figure 1a,b). For both weight (figure 1a) and length (figure 1b), a significant effect of habitat ($rSGR_{weight}$: $F_{1,90} = 7.01$, $p = 0.009$; $rSGR_{length}$: $F_{1,90} = 9.07$, $p = 0.003$) but not of stickleback origin ($rSGR_{weight}$: $F_{1,4} = 1.0$, $p = 0.38$; $rSGR_{length}$: $F_{1,4} = 0.97$, $p = 0.38$) was observed. The interaction term between habitat and origin was significant ($rSGR_{weight}$: $F_{1,90} = 7.77$, $p = 0.006$; $rSGR_{length}$: $F_{1,90} = 17.7$, $p = 0.0001$). Lake sticklebacks grew significantly better in their natural environment when compared with river conditions

($rSGR_{weight}$: $p = 0.0012$; $rSGR_{length}$: $p < 0.0001$). Interestingly, we did not detect a significant difference in the growth of river sticklebacks in the two habitats ($rSGR_{weight}$: $p = 0.91$; $rSGR_{length}$: $p = 0.38$). In the river exposure, lake sticklebacks grew less than river sticklebacks ($rSGR_{length}$: $p = 0.0019$). However, this was not significant in terms of weight according to the Bonferroni corrected threshold of significance ($p = 0.0125$) ($rSGR_{weight}$: $p = 0.03$). In the lake exposure, no difference in mean $rSGR_{weight}$ between lake and river sticklebacks was observed ($rSGR_{weight}$: $p = 0.24$). As a trend, mean $rSGR_{length}$ tended to be lower in river fish in lake conditions relative to lake fish ($rSGR_{length}$: $p = 0.02$, not significant with Bonferroni corrected $p = 0.0125$).

(b) Parasite infections

Sticklebacks exposed to the lake habitat were infected with a higher number of parasite species than those exposed to the river habitat (figure 2a; ANOVA: habitat: $F_{1,90} = 70.31$, $p < 0.0001$; origin: $F_{1,4} = 1.21$, $p = 0.3326$; habitat \times origin interaction: $F_{1,90} = 0.30$, $p = 0.5851$). Sticklebacks in the lake habitat were infected with (mean \pm s.e.) 2.96 ± 0.20 parasite species and those in the river habitat with 1.26 ± 0.01 parasite species. *Post hoc* comparisons showed that both lake and river sticklebacks had a higher number of parasite species in the lake than in the river (Bonferroni corrected $p = 0.0125$; figure 2a). For every individual fish, a relative parasite load averaged over all parasite species present in the respective habitat was calculated (figure 2b). Between habitats, relative parasite load was not significantly different, but lake and river origin of sticklebacks had a significant influence on parasite load (ANOVA: habitat: $F_{1,90} = 2.37$, $p = 0.1269$; origin: $F_{1,4} = 7.82$, $p = 0.0484$; habitat \times origin interaction: $F_{1,90} = 2.63$, $p = 0.1082$). In the lake exposure, sticklebacks with river origin showed a significantly higher parasite load when compared with lake sticklebacks in this habitat ($p < 0.0125$, *post hoc t*-test). No significant difference in parasite load was detected between lake and river sticklebacks exposed to the river habitat (figure 2b).

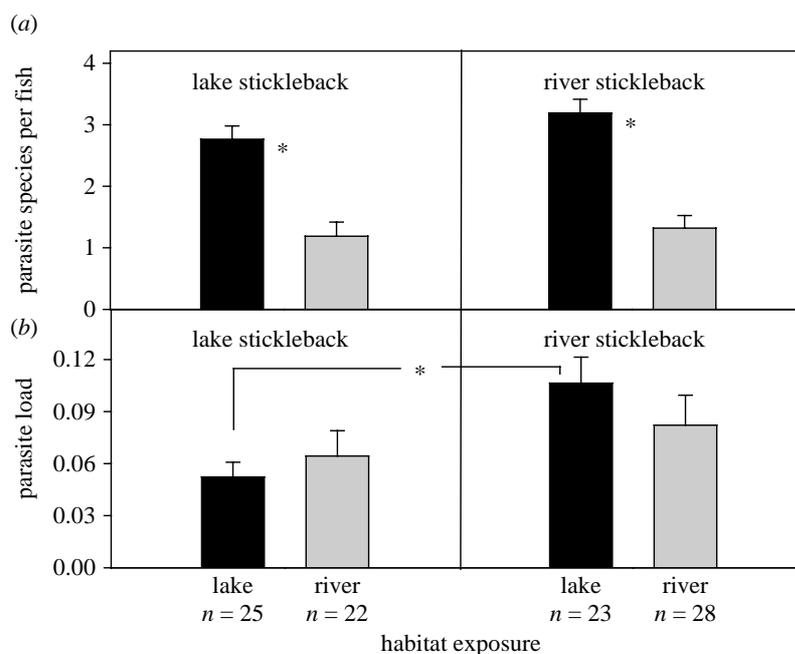


Figure 2. Parasite infections. (a) Number of parasite species per fish (mean \pm s.e.). Sticklebacks exposed in the lake had a higher average number of parasite species than sticklebacks in the river. (b) Relative parasite load per fish. In the lake exposure, river sticklebacks had higher parasite loads ($*p < 0.0125$, t -test, p value Bonferroni corrected for multiple tests).

Mean (\pm s.e.) number of parasites per fish in the lake exposure was higher ($p = 0.0025$) in river sticklebacks (59.17 ± 6.24) than in lake sticklebacks (28.28 ± 6.04) (Bonferroni corrected significance threshold $p = 0.0125$). Mean number of parasites per fish was not different in lake sticklebacks (5.82 ± 2.02) and river sticklebacks (6.18 ± 1.78) exposed to river conditions.

Two parasites (*Gyrodactylus* sp., *Raphidascaris acus*) were found exclusively in sticklebacks exposed to the river habitat (table 1), while three river parasite species were also present in sticklebacks exposed to the lake. Parasite infections of the sticklebacks specified for the single parasite species are summarized in table 1. Highest prevalence (100%) was observed for the eye fluke *Diplostomum* sp. in river and lake sticklebacks exposed to lake conditions. In sticklebacks exposed in the river, we did not detect the presence of *Diplostomum* sp. Infection intensities of *Diplostomum* sp. in the lake exposure were significantly higher in sticklebacks with the river genotype when compared with the lake genotype ($p < 0.0031$; table 1). Fish lice (*Argulus foliaceus*) are temporary ectoparasites and can switch host rapidly, therefore difference between lake and river sticklebacks have to be interpreted with caution. All other parasite species did not show significant differences between river and lake sticklebacks within one exposure habitat. According to Bonferroni correction for multiple testing, the p value for a significant difference with the present data (table 1) was set to $p < 0.0031$ for the 16 tests performed.

(c) Immune response

As a parameter for activation of granulocytes, oxidative burst activity of HKL was analysed. From all tested fish, HKL responded to stimulation with zymosan with elevated respiratory burst activity in a chemoluminescence assay. Sticklebacks in the lake exposure, showed a higher zymosan-induced respiratory burst activity when compared with sticklebacks in the river exposure (ANOVA: habitat:

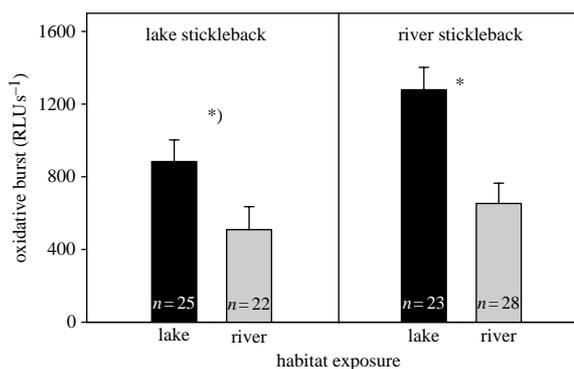


Figure 3. Peak respiratory burst activity of head kidney leucocytes in cultures with zymosan stimulation (mean \pm s.e.). Sticklebacks exposed in the lake had a higher oxidative burst activity than those in the river ($*p < 0.0125$, t -test, p value Bonferroni corrected for multiple tests; *), not significant trend $p = 0.018$).

$F_{1,90} = 24.35$, $p < 0.0001$; origin: $F_{1,4} = 0.45$, $p = 0.5384$; habitat \times origin interaction: $F_{1,90} = 0.01$, $p = 0.9366$; figure 3). River sticklebacks had a higher respiratory burst activity in the lake when compared with the river ($p < 0.0125$, *post hoc t*-test, figure 3). Lake sticklebacks, as a trend ($p = 0.018$) also showed elevated respiratory burst in the lake when compared with the river (not considered statistically significant with Bonferroni corrected $p = 0.0125$). Respiratory burst activity was correlated to per cent granulocytes determined in HKL ($r = 0.272$, $n = 98$, $p = 0.007$). Correspondingly, sticklebacks in the lake exposure had a higher proportion of granulocytes in HKL isolates when compared with those in the river exposure (ANOVA: habitat: $F_{1,90} = 6.60$, $p < 0.0118$; origin: $F_{1,4} = 0.02$, $p = 0.8985$; habitat \times origin interaction: $F_{1,90} = 2.87$, $p = 0.0935$). River sticklebacks had a higher proportion of granulocytes in the lake when compared with the river, but not lake sticklebacks ($p < 0.0125$, *post hoc t*-test, figure 4).

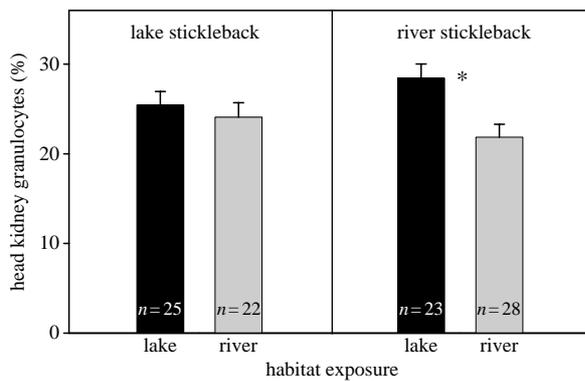


Figure 4. Proportion of granulocytes in head kidney leucocytes (mean + s.e.). River sticklebacks exposed in the lake had a higher proportion of granulocytes in their head kidneys than river sticklebacks in the river (* $p < 0.0125$, *t*-test, *p* value Bonferroni corrected for multiple tests).

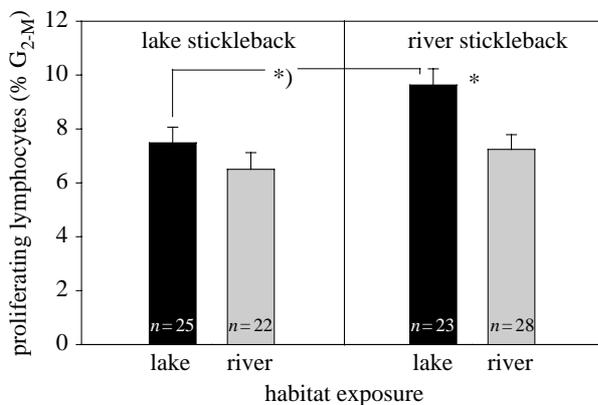


Figure 5. Proliferation of head kidney lymphocytes (mean + s.e.). River sticklebacks exposed in the lake had a higher lymphocyte proliferation rate than river sticklebacks in the river (* $p < 0.0125$, *t*-test, *p* value Bonferroni corrected for multiple tests; *), not significant trend $p = 0.041$).

To analyse the status of the adaptive, specific immune response of exposed stickleback, frequencies of lymphocytes and their proliferation activity was tested by means of flow cytometry. In contrast to oxidative burst activity and percentage of granulocytes, the percentage of lymphocytes in HKL isolates was lower in sticklebacks in the lake habitat than in the river habitat (ANOVA: habitat: $F_{1,90} = 4.82$, $p < 0.0307$; origin: $F_{1,4} = 0.04$, $p = 0.8482$; habitat \times origin interaction: $F_{1,90} = 2.64$, $p = 0.1079$). Proportion of lymphocytes in HKL samples was negatively correlated to proportion of granulocytes ($r = -0.589$, $p < 0.0001$, $n = 98$). Correspondingly, river sticklebacks displayed a trend ($p = 0.0130$, *post hoc t*-test, data not shown) of a lower proportion of lymphocytes in the lake when compared with the river, but not lake sticklebacks.

The proportion of proliferating lymphocytes (lymphocytes in G_{2-M} phase of the cell cycle) was higher in sticklebacks in the lake habitat than in the river habitat (ANOVA: habitat: $F_{1,90} = 8.76$, $p < 0.0039$; origin: $F_{1,4} = 6.79$, $p = 0.0592$; habitat \times origin interaction: $F_{1,90} = 0.56$, $p = 0.4605$). River sticklebacks exposed to lake conditions exhibited a higher proliferation activity than in the river ($p < 0.0125$, *post hoc t*-test, figure 5). In the lake exposure, river sticklebacks displayed a trend of increased lymphocyte proliferation ($p = 0.0413$, *post hoc t*-test, figure 5) when compared with lake stickleback. In general, our results

showed that sticklebacks exposed to the lake habitat, which supports the greater number of parasites species, showed an increased activity in immune parameters. However, habitat effects on immune parameters were most prominent in river sticklebacks exposed to the lake.

(d) Cage effect

The effect of cage was small and did not influence length increase, weight increase, number of parasite species, parasite load, respiratory burst, percentage of granulocytes, percentage of lymphocytes and proportion of proliferating lymphocytes in the river habitat ($p > 0.0125$, *p* value Bonferroni corrected for multiple tests, statistical details not shown). This was also the case for the lake habitat with exception of proportion of proliferating lymphocytes (one-way ANOVA: cage: $F_{9,38} = 3.03$, $p = 0.0080$).

4. DISCUSSION

The results of the present study suggest divergence in adaptation of immune systems to local conditions between populations of different stickleback ecotypes. Patterns of growth, parasite burdens and immune system activity in sticklebacks translocated between lake and river habitats with different levels of parasite diversity are consistent with selection having favoured appropriate shifts in trade-offs between growth and immunity in different habitats. At the end of the exposure experiment, river sticklebacks that naturally experience low parasite burdens had elevated parasite burdens, relative to local lake stickleback when exposed to a high parasite diversity lake habitat. This occurred even though the river sticklebacks appeared to mount a more vigorous immune defence.

All the sticklebacks recovered at the end of the experiment had gained length and weight, but lake sticklebacks exposed to river conditions grew significantly less when compared with lake conditions while river sticklebacks grew equally well in both habitats (figure 1a,b). This might indicate an increased specialization by lake sticklebacks towards prey resources more typically available in lake rather than in river habitats (e.g. zooplankton). On the contrary, river sticklebacks could be more generally adapted to feed on different food resources, e.g. prey from both the water column (drift) and the benthos. In the studies of the evolutionary ecology of sticklebacks, trophic specialization is well described (Schluter 1993, 1995; Rundle *et al.* 2000; Reimchen & Nosil 2001a) and variation in feeding efficiency on particular prey resources is one factor identified in the adaptive radiation of three-spined sticklebacks (Rundle *et al.* 2000).

In the present study, food resources may have been restricted in the enclosure under river conditions and river sticklebacks may have outcompeted the sticklebacks of lake origin, resulting in reduced growth of the latter. In the lake cages, food constraints might have been reduced or even absent. Nevertheless, adaptation to specific food resources might contribute to the apparent reproductive barrier between lake and river sticklebacks. Although the growth of lake sticklebacks was limited in the river exposure, parasite load and immune parameters were not different from river sticklebacks that had been similarly exposed. Limitation in nutritional resources obviously did not result in stress and immunosuppression

of lake sticklebacks exposed to an atypical habitat. Natural (e.g. temperature, photoperiod, salinity) and artificial (e.g. pollution) environmental factors can cause stress in fishes that influences their immune system (Bly *et al.* 1997). Many studies on the influence of environmental stress on immune responses of bony fishes have demonstrated immunosuppression (Clem *et al.* 1990; Rice *et al.* 1996; Bly *et al.* 1997; Jokinen *et al.* 2000; Engelsma *et al.* 2003; Prophete *et al.* 2006). In the present study, the higher parasite burden of river sticklebacks in the lake exposure cannot be explained by immunosuppression, as immune parameters recorded here were elevated in river sticklebacks in the lake exposure when compared with river sticklebacks in their home habitat.

Parasite diversity described in a field study (Kalbe *et al.* 2002) in the two habitat types is reflected in the present exposure experiment, indicating that individual immunological capabilities and not enclosure in cages are causative for different parasite loads of individual sticklebacks. In the lake exposure, river sticklebacks showed significantly higher infections with *Diplostomum* sp. when compared with lake sticklebacks (table 1). In laboratory infections of lake and river sticklebacks with *D. pseudopathacaenum*, river sticklebacks were shown to have a higher susceptibility to the parasite (Kalbe & Kurtz 2006).

Summarizing the results of the parasite infections during the exposure experiment, we have: (i) parasite species richness was higher in the lake (figure 2a), (ii) river sticklebacks in the lake exposure had higher parasite loads when compared with lake sticklebacks in their home habitat (figure 2b; table 1), and (iii) the parasite load of lake and river sticklebacks exposed to river conditions was not different (figure 2b; table 1).

In wild-caught stickleback, it is demonstrated that specialization in pelagic versus benthic feeding results in different infection rates of trophic transmitted parasites, depending on the abundance of intermediate hosts (e.g. Reimchen & Nosil 2001b,c). However, in the present study, difference in parasite load between lake and river sticklebacks in the lake exposure remains significant when only non-trophically transmitted parasites, are tested ($p < 0.0001$), whereas parasite load of only trophically transmitted parasites was not different ($p = 0.84$). This shows that differences in overall parasite load between lake and river sticklebacks in the lake exposure (figure 2) are not due to trophic specialization of lake and river sticklebacks.

The immune system was more active in sticklebacks that underwent exposure to lake rather than river conditions. In river sticklebacks, the respiratory burst activity of HKL was significantly higher in the lake (for lake sticklebacks as a trend $p = 0.018$; figure 3). In naive and *D. pseudopathacaenum*-infected laboratory offspring of lake and river sticklebacks, higher capacities for respiratory burst activity were detected in lake sticklebacks—this was considered as an adaptation to higher parasite abundances in lakes by Kalbe & Kurtz (2006). In the present study, river sticklebacks were exposed to multiple lake parasite species and able to mount a strong respiratory burst response comparable to that of lake sticklebacks ($p = 0.115$).

Granulocytes are an important effector cell type of respiratory burst (Verburg-van Kemenade *et al.* 1996; Serada *et al.* 2005). Accordingly, river sticklebacks

exposed to lake conditions showed elevated proportions of granulocytes in head kidneys when compared with river sticklebacks in their home habitat (figure 4). The proliferation of head kidney lymphocytes of river sticklebacks was also significantly higher in the lake, indicating increased activity of their specific immune system when compared with the situation with exposure to river conditions (figure 5). In summary, the immune parameters we examined here showed clearest differences in river sticklebacks exposed to the two different habitats. Immune parameters were elevated in river sticklebacks in the lake when compared with the river. Lake sticklebacks showed a trend of increased activity respiratory burst activity in the lake.

More parasite species are present in the lake. River sticklebacks in the lake had higher parasite loads than lake sticklebacks and exhibited stronger activation of their immune system than in their native habitat. Thus, although activity of their immune system was high, defence against parasites was not as successful as that displayed by lake sticklebacks in their native habitat. In the river exposure, lake sticklebacks were able to cope with the abundant parasites in a manner comparable to river stickleback.

Wegner *et al.* (2003a) investigated the diversity of MHC genes in distinct lake and river stickleback populations in northern Germany. They found almost twice as many alleles of the MHC class II beta genes in lake sticklebacks when compared with river sticklebacks, suggesting that it is advantageous to maintain a higher diversity of MHC class II in lake habitats with an associated higher diversity of parasite species. In the present study, river sticklebacks exposed to lake conditions invested heavily into immune defence, but still were less successful defending themselves against the abundant parasite fauna than lake sticklebacks. In laboratory parasite infections, sticklebacks with sub-optimal diversity of the MHC class II beta showed elevated respiratory burst activity (Kurtz *et al.* 2004) and elevated expression of MHC class II beta (Kurtz *et al.* 2006), suggesting a compensatory upregulation of innate and adaptive immune mechanisms to compensate deficiencies in MHC diversity.

In the lake exposure, activity of both innate (respiratory burst activity, figure 3) and adaptive immunity (lymphocyte proliferation, figure 5) responses was elevated in river sticklebacks when compared with their home habitat. Both, innate and adaptive immune mechanisms might have been upregulated by river sticklebacks to compensate for limitations in specialized (MHC based) defence mechanisms.

In the present study, two areas of specialization can be defined, which could contribute to the development and maintenance of a pre-zygotic reproductive barrier between lake and river sticklebacks. First is growth potential, which potentially limits the development of lake sticklebacks under river conditions. This may reflect a cost of trophic specialization in the lake ecotype. There is a strong association between individual size and mortality risk in fishes (Sogard 1997), and lake fish may face increased predation risk if they enter river environments. Second is adaptation to habitat-dependent parasite fauna which seemed limited in river sticklebacks, possibly due to the lower parasite diversity in their home habitat. These two areas of specialization may reflect a trade-off between

growth and immunity. River sticklebacks may be better adapted to maintain growth in low-productivity river habitats, but seem to have sacrificed their ability to resist parasites when compared with lake sticklebacks. Our results show that natural enemies may play an important role in the development of divergent selection and reproductive isolation in rapidly diverging taxa.

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