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In vitro leukocyte response of three-spined sticklebacks (*Gasterosteus aculeatus*) to helminth parasite antigens

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ABSTRACT

Helminth parasites of teleost fish have evolved strategies to evade and manipulate the immune responses of their hosts. Responsiveness of fish host immunity to helminth antigens may therefore vary depending on the degree of host-parasite counter-adaptation. Generalist parasites, infective for a number of host species, might be unable to adapt optimally to the immune system of a certain host species, while specialist parasites might display high levels of adaptation to a particular host species. The degree of adaptations may further differ between sympatric and allopatric host-parasite combinations. Here, we test these hypotheses by *in vitro* exposure of head kidney leukocytes from three-spined sticklebacks (*Gasterosteus aculeatus*) to antigens from parasites with a broad fish host range (*Diplostomum pseudospathaceum*, *Triaenophorus nodulosus*), a specific fish parasite of cyprinids (*Ligula intestinalis*) and parasites highly specific only to a single fish species as second intermediate host (*Schistocephalus pungitii*, which does not infect *G. aculeatus*, and *Schistocephalus solidus*, infecting *G. aculeatus*). *In vitro* responses of stickleback leukocytes to *S. solidus* antigens from six European populations, with *S. solidus* prevalence from <1% to 66% were tested in a fully crossed experimental design. Leukocyte cultures were analysed by means of flow cytometry and a chemiluminescence assay to quantify respiratory burst activity. We detected decreasing magnitudes of *in vitro* responses to antigens from generalist to specialist parasites and among specialists, from parasites that do not infect *G. aculeatus* to a *G. aculeatus*-infecting species. Generalist parasites seem to maintain their ability to infect different host species at the costs of relatively higher immunogenicity compared to specialist parasites. In a comparison of sympatric and allopatric combinations of stickleback leukocytes and antigens from *S. solidus*, magnitudes of *in vitro* responses were dependent on the prevalence of the parasite in the population of origin, rather than on sympatry. Antigens from Norwegian (prevalence 30–50%) and Spanish (40–66%) *S. solidus* induced generally higher *in vitro* responses compared to *S. solidus* from two German (<1%) populations. Likewise, leukocytes from stickleback populations with a high *S. solidus* prevalence showed higher *in vitro* responses to *S. solidus* antigens compared to populations with low *S. solidus* prevalence. This suggests a rather low degree of local adaptation in *S. solidus* populations, which might be due to high gene flow among populations because of their extremely mobile final hosts, fish-eating birds.

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1. Introduction

Helminths are frequent parasites of natural fish populations, but interactions of helminths with the piscine immune system are under-investigated. This might be attributed to the fact that helminth infections in aquaculture are often relatively easy to control (*e.g.* by control of invertebrate intermediate hosts) and rarely have prominent commercial impact [1]. However, in their natural habitat, parasites often drastically reduce host fitness and thus pose strong selection pressures on their hosts, which therefore have evolved powerful counter-measures to control infection [2]. The success of helminth parasites largely depends on their ability to evade and/or manipulate the generally efficient immune system of their fish hosts [3,4]. The evolutionary arms race of host-parasite counter adaptations (often described as Red Queen Dynamics) promote parasite virulence and infection success on the one hand [5–7], but host immunocompetence and prevention of infection on the other [8–11]. In cyprinids and salmonids, activation of granulocytes is considered to be an important part of the immune defence against parasitic helminths [12–16]. In sticklebacks, traits of cellular innate immunity, such as respiratory burst activity, were elevated in a population that was adapted to higher parasite infection pressure [9]. Adaptation of stickleback immunocompetence to local parasites is presumably supported by selection for certain MHC class II genotypes [11]. The basis of functional cellular immunity in such differential adaptive situations is not well investigated to date in teleost fish.

1.1. Parasites with a broad and a narrow host range – Diplostomum and Schistocephalus

Helminths are experts in evasion and manipulation of their hosts' immune functions and the respective strategies may depend on the host range. Generalist parasites might not be able to adapt their antigenicity (antigenic surface) for the immune system of a certain host species and might instead use other immune evasion strategies. An example is the trematode *Diplostomum pseudospathaceum*, which infects the immunological inert eye lens of various freshwater fish species. After penetrating the skin (or gills) of their fish host, the tissue migrating larval stage (diplostomulum) of *D. pseudospathaceum* finds its way along the blood vessels to the eye lenses. Antigens from such a generalist parasite might trigger stronger immune responses compared to antigens from specialist parasites, such as the cestode *Schistocephalus solidus*. The adult stage of the tapeworm *S. solidus* reproduces in the gut of warm-blooded vertebrates, most often fish-eating birds. Eggs are released in the faeces of the final host. A first, free-swimming larval stage (coracidium) hatches in water, and develops to the second larval stage (procercoid) after ingestion by a cyclopoid copepod. The third larval stage (plerocercoid) develops in the body cavity of the obligatory and specific second intermediate host: the three-spined stickleback (*Gasterosteus aculeatus*). Besides the immune system, the aggressive environment of the stickleback's stomach may prevent infection [17,18], but once in the body cavity of the three-spined stickleback host, clearance of *S. solidus* plerocercoids is rare [19]. Experimental transfer of *S. solidus* plerocercoids to fish species other than three-spined sticklebacks lead to rapid death of the larvae [20,21], underlining the specific adaptation of *S. solidus* to three-spined sticklebacks, but suggesting that the immune system of fish is in principle able to clear *S. solidus* infections. In three-spined sticklebacks, established plerocercoids of *S. solidus* take all the resources that the parasite needs from the host and grow to up to 20–30% (w/w) of their host's body weight [19], thereby reducing the fitness of the hosts and resulting in decreased or even absent reproduction [22–26].

1.2. Host-parasite local mutual adaption

A specialist like *S. solidus* might even have optimised its surface antigens (immune evasion) for a frequently infected local host population. If this is the case, immunity of sympatric hosts might have a weaker response to the parasites' antigens compared to immunity of allopatric hosts. We thus hypothesize that antigenicity (strength of *in vitro* leukocyte response) decreases from generalist to specialist parasites and among specialists from parasites that do not infect *G. aculeatus* to *G. aculeatus*-infecting parasites, and among *G. aculeatus*-infecting parasites from allopatric to sympatric host-parasite combinations.

Previous studies of local adaptation of teleost fish hosts and their parasites have mainly focussed at infectivity and host mortality, but have rarely included immunological patterns of adaptation [5–7,27–30]. Some of these studies observed local advantages of the (co-evolved) host population. In those studies, hosts were genetically best adapted to the local parasite population and showed inferior performance in preventing infections with non-local parasites of the same species [27,28]. Such situations would disadvantage immigrant hosts, but favour migrating parasites, thus promoting gene flow in the parasites. Other studies failed to detect local adaption in host-parasite systems [29] but a larger third group of studies describes a co-evolutionary local advantage of the parasite population, which became more infective for local compared to non-local hosts of the same species [5–7]. Such a constellation would promote immigrant hosts and disadvantage foreign parasites, which may promote gene flow among hosts. Therefore, parasites may play an important role in the dynamic process of diversification and speciation of their teleost fish hosts and *vice versa* [9].

1.3. The present study

In this study, responses of three-spined stickleback head kidney leukocytes (HKL) to antigens of helminth fish parasites were investigated with an *in vitro* system, enabling large-scale comparisons between parasite species, as well as comparisons across different host populations. Since activation of granulocytes is important in the immune defence of fish against parasitic helminths [12–16] and in sticklebacks cellular innate immunity was elevated in a population with higher parasite infection pressure [9], we quantified the respiratory burst (RB) activity of HKL. We hypothesized that *in vitro* exposure of HKL to parasite antigens might influence leukocyte viability and the frequencies of cellular subsets and therefore analysed numbers of viable HKL and the granulocytes to lymphocytes ratio (G/L ratio) after *in vitro* stimulation.

We investigated HKL *in vitro* responses to antigens from generalist parasites, such as the eye fluke *D. pseudospathaceum* that infects, among other fish species, also three-spined sticklebacks (*G. aculeatus*) [31], and the cestode *Triaenophorus nodulosus*, with several fish species including *G. aculeatus* as second intermediate hosts [32]. Furthermore, antigens from *Ligula intestinalis* were used, a parasite specific to cyprinids as second intermediate hosts, which does not infect *G. aculeatus*. Finally, tapeworm antigens from two highly specialised *Schistocephalus* species, *Schistocephalus pungitii*, specific for nine-spined sticklebacks (*Pungitius pungitius*) and *S. solidus*, specific for *G. aculeatus*, were used (Table 1). From the latter, seven hosts and corresponding parasite populations from across Europe were tested to investigate potential local adaptation in the *G. aculeatus*–*S. solidus* system.

After *in vitro* stimulation, stickleback HKL were analysed by means of flow cytometry to determine the cell viability and the granulocyte to lymphocyte ratio. In addition, the respiratory burst

Table 1
Parasite antigen sources. Naturally- or laboratory-infected hosts originated from different populations: NO (lake 'Skogseidvatnet', Norway), SC3 ('Loch Olabhat', Scotland), GPS (lake 'Grosser Plöner See', Germany), NST (lagoon 'Neustädter Binnenwasser', Germany), IBB (brook 'Ibbenbürener Aa', Germany), SP (channel near 'Xinzo de Limia', Spain), LBT (lake 'Lebrader Teich', Germany), MGS (lake 'Müggelsee', Germany).* Total pool weight.

Parasite species	Host species	Population	Natural-/laboratory infection	Pooled individuals	Weight (mg)	Used for experiment	Parasitizes *G. aculeatus*
S. solidus	*G. aculeatus*	NO	Lab. inf.	6	85 ± 30	3	+
S. solidus	*G. aculeatus*	SC3	Lab. inf.	10	168 ± 32	3	+
S. solidus	*G. aculeatus*	GPS	Lab. inf.	7	987*	3	+
S. solidus	*G. aculeatus*	NST	Lab. inf.	10	2267*	1, 3	+
S. solidus	*G. aculeatus*	IBB	Lab. inf.	19	116 ± 37	3	+
S. solidus	*G. aculeatus*	IBB	Nat. inf.	11	94 ± 32	2	+
S. solidus	*G. aculeatus*	SP	Lab. inf.	12	178 ± 48	3	+
S. pungitii	*P. pungitius*	LBT	Nat. inf.	7	1333*	1	
L. intestinalis	*R. rutilus*	MGS	Nat. inf.	6	4200 ± 1140	1	
T. nodulosus	*P. fluviatilis*	MGS	Nat. inf.	41	8.5 ± 4.6	1	+
D. pseudospathaceum	*L. stagnalis*	GPS	Nat. inf.	–	–	1, 2	+

activity of *in vitro* stimulated HKL was analysed in a zymosan-induced chemiluminescence assay.

In a first experimental set, *in vitro* responses of HKL from a single stickleback population to antigens of the five parasite species were tested (experiment 1: helminth species). In a second set of experiments, we compared *in vitro* responses of HKL derived from sticklebacks of seven different populations to *S. solidus* and *D. pseudospathaceum* antigens from a single origin each (experiment 2: host origins). With a third experimental set up, HKL from six stickleback populations were exposed in a fully crossed design to *S. solidus* antigens derived from the same habitats, to test if stickleback HKL responses differ between sympatric and allopatric host-parasite combinations (experiment 3: sympatric/allopatric combinations).

2. Materials and methods

2.1. Experimental sticklebacks

We took advantage of the availability of seven three-spined stickleback (*G. aculeatus*) populations from across Europe, within the 'stickleback cluster' of the DFG priority programme 1399 'Host-Parasite Coevolution'.

The majority of sticklebacks used for head kidney leukocyte (HKL) isolation were laboratory-raised, first generation offspring of wild caught individuals. Parental sticklebacks originated from seven European populations, a lake in the West of Norway ('Skogseidvatnet', NO), lakes on the Scottish island North Uist ('Loch Sandary', SC1 and 'Loch Scadavay', SC2), an inland lake and a brackish lagoon of the Baltic Sea in Northern Germany ('Grosser Plöner See', GPS and 'Neustädter Binnenwasser', NST), a brook in Western Germany ('Ibbenbürener Aa', IBB) and a drainage channel system in the Northwest of Spain near 'Xinzo de Limia' (SP). Investigated populations were sampled with support of local co-operators, which were holding necessary licences and helped to obtain sampling permits from the local authorities. Live sticklebacks were transported according to EU legislation for non-commercial and solely scientifically used material.

Stickleback offspring were bred at the IEB Münster, Germany, except for the Scottish (SC1, SC2) populations (bread at the IEZ Bonn, Germany) and a German (GPS) population (bread at the MPI Plön, Germany). Wild caught sticklebacks of the Scottish (SC1, SC2), a German (IBB) and the Spanish (SP) population were used in experiment 2 (host origins) and second generation offspring of a German (NST) population in experiment 1 (parasite species) and 3 (sympatric/allopatric combinations). Upon arrival (SC1, SC2, GPS individuals), and at least two weeks before experimentation (NO, NST, IBB, SP individuals), sticklebacks were kept in 125 L glass tanks separated by populations (origins) at the IEB Münster. All tanks were connected to a water recirculation system which provided aerated and filtered water at 18–20 °C. Sticklebacks were kept at 15/9 h light/dark cycles and fed daily *ad libitum* with frozen red mosquito larvae and dry food (TetraMin, Tetra). Sticklebacks were maintained and treated in accordance with the local animal welfare authorities and the EU Directive 2010/63/EU for animal experiments.

S. solidus prevalence in the stickleback populations used in the present study were under constant surveillance for several years (at least 3 years), except for the Scottish populations (1 year). Prevalence ranged from <1% (NST, GPS; Kalbe, pers. comm.) over 3–5% (IBB; personal observation), 0–10% (SC1; Rahn, pers. comm.) 0–11% (SC3; Rahn, pers. comm.), 10% (SC2 [33]), 30–50% (NO; Kalbe, pers. comm.) to 40–66% (SP; personal observation [34],).

2.2. Experimental parasites

For *in vitro* stimulation of stickleback head kidney leukocytes (HKL), antigen preparations of four cestode species and a trematode species were used (Table 1). *S. solidus* plerocercoids originated from the stickleback populations used for HKL isolation and were partly provided by the members of the 'stickleback cluster' (see 2.1 Experimental sticklebacks). Scottish *S. solidus* originated from sticklebacks of a third Scottish lake ('Loch Olabhat', SC3), about 7 km away from 'Loch Sandary' (SC1) and 10 km from 'Loch Scadavay' (SC2). For antigen preparation, *S. solidus* plerocercoids were grown in sympatric host-parasite combinations in laboratory-raised and -infected three-spined sticklebacks. Only in experiment 2 (host origins) *S. solidus* antigens were prepared from naturally infected sticklebacks from a German (IBB) population.

Plerocercoids of a second *Schistocephalus* species (*S. pungitii*), were collected from wild caught nine-spined stickleback (*P. pungitius*), from lake 'Lebrader Teich' (LBT) about 6 km away from lake 'Grosser Plöner See' (GPS). Plerocercoids of *L. intestinalis* were collected from roach (*Rutilus rutilus*) caught in the lake 'Müggelsee' (MGS) close to Berlin, Germany. Plerocercoids of the pike tapeworm, *T. nodulosus* were collected from twenty European perches (*Perca fluviatilis*) that originated also from the lake 'Müggelsee' (MGS). Cercariae of *D. pseudospathaceum* were isolated from ten infected snails (*Lymnaea stagnalis*) from the lake 'Grosser Plöner See' (GPS) as described in Hibbeler et al. [35].

2.3. Antigen preparations

Parasite antigens were prepared from pools of at least six individuals (Table 1). Antigen preparations were kept on ice during preparation and phosphate-buffered saline (PBS, pH 7.4,

Calbiochem 524650) was used at 4 °C. Plerocercoids of *S. solidus*, *S. pungitii*, *L. intestinalis* and *T. nodulosus* were collected from fish body cavities under sterile conditions, and weighed. Plerocercoids were washed and frozen with PBS at −20 °C (*T. nodulosus*: 0.25 g wet weight mL^{-1}, all other species: 0.5 g wet weight mL^{-1}). After thawing, teguments of the cestodes *S. solidus*, *S. pungitii* and *L. intestinalis* were detached from the worm body by vortexing. Tegument antigen preparations were decanted and worm bodies were washed with PBS to remove remaining tegument fragments. The remaining worm bodies (in case of *L. intestinalis* sections of worm bodies) were homogenised manually in a 1.5 mL tube with a pestle and adjusted with PBS to a concentration of 0.25 g original wet weight mL^{-1}. Tegument and body antigen preparations were sonicated (Sonoplus 2070, Bandelin, Germany) for 120 s (duty cycle 10%, power 60%) on ice. Solid material was removed by centrifugation (600 × *g*, 4 °C, 10 s). Instead of centrifugation, antigen preparation from the tegument of *S. solidus* from naturally infected sticklebacks of a German (IBB) population used in experiment 2 (host origins) was 0.45 μm filtered. The protein content of each preparation was determined with a Bradford assay and adjusted to 400 mg L^{-1} (protein fraction) with Leibovitz 15 medium (PAA E15-020) with 10% (v/v) distilled water and 10 mmol L^{-1} HEPES buffer (Lonza 17–737) and stored at −80 °C. Antigens of *T. nodulosus* were prepared as whole body preparation as described above, without detaching the tegument beforehand. The antigen preparation from *D. pseudospathaceum* was prepared as described by Hibbeler et al. [35], diluted and stored as described above.

Fish protein preparations were produced from muscle tissue and pooled from six individual laboratory-raised sticklebacks (*G. aculeatus*), originating from a German (NST) population and three individual roaches (*R. rutilus*) originating from a pond at the IEB Münster, Germany, following the protocol for parasite body antigens as described above.

Each antigen/protein preparation was controlled microscopically for sterility after incubation of 25 μL subsamples in wells of a 96-well half-area flat bottom microtitre plate (Greiner Bio-One) at 20 °C in a water vapour saturated atmosphere with 3% CO_2 for 4 days.

2.4. Leukocyte isolation and in vitro stimulation

Cells and media were kept refrigerated during the preparations. Basic medium was Leibovitz 15 (PAA E15-020) supplemented with 10 mmol L^{-1} HEPES buffer (Lonza 17–737) and 10% (v/v) distilled water to adjust osmotic pressure according to stickleback serum osmolarity (subsequently named L-90). Fish were anesthetized by a blow on the head, decapitated and the body cavities were opened. Head kidneys were removed under sterile conditions and transferred to 40 μm cell strainers (BD Falcon) in petri dishes (3.5 cm Ø) with 1 mL L-90 with heparin (2 × 10^4 IU L^{-1}, Applichem A3004). Single cell suspensions of head kidney leukocytes (HKL) were prepared by forcing the tissues through the strainers with a plunger of a syringe. HKL were washed once with heparinised L-90 (600 × *g*, 4 °C, 5 min), once with L-90 without heparin, and resuspended in culture medium (L-90 with 1 × 10^5 IU L^{-1} penicillin, 100 mg L^{-1} streptomycin (PAA P11-010), 4 mmol L^{-1} L-Glutamin (PAA M11-006), 5% (v/v) foetal bovine serum (PAA A11-103) and 1% (v/v) heat inactivated, pooled carp serum).

Total cell numbers in head kidney isolates were determined by means of flow cytometry (see 2.5 Flow cytometric analysis) and cell suspensions were adjusted to 4 × 10^6 cells mL^{-1} with culture medium. For *in vitro* stimulation, HKL were seeded out in 96-well half-area flat bottom microtitre plates (Greiner Bio-One) at a density of 1 × 10^5 cells $well^{-1}$ in a final volume of 100 μL culture medium $well^{-1}$. From each individual fish, HKL were cultured with medium alone as a negative control and cultured with lipopolysaccharides (LPS, 20 mg L^{-1}, Sigma L7895) and pokeweed mitogen (PWM, 2 mg L^{-1}, Sigma L8777) as positive controls. Protein preparations from fish muscle tissues and bovine serum albumin (BSA, Carl Roth CP77) were used to test potential effects of allogeneic and xenogeneic proteins (protein control, 10 mg L^{-1}). Parasite antigens were added to final concentrations of 10 mg L^{-1} (protein fraction) each. All cultures were incubated for 4 days at 20 °C in a water vapour saturated atmosphere with 3% CO_2 and were controlled microscopically for sterility afterwards.

2.5. Flow cytometric analysis

Subsamples of freshly isolated and cultured head kidney leukocytes (HKL) were analysed by means of flow cytometry (FACSCanto II, BD, USA). Total cell numbers (per sample/culture) were determined with the standard cell dilution assay (SCDA [36],) modified by Scharsack et al. [37]: after *in vitro* culture, HKL culture plates were placed on ice (30 min) to detach adherent cells. Resuspended HKL (5 μL cell suspension per sample of freshly isolated HKL, 25 μL from each culture well) were transferred to individual wells of a 96 well round bottom microtiter plate (BD Falcon). Samples were supplemented with propidium iodide (2 mg L^{-1}, Sigma 81845) and green fluorescent reference particles (4.5 μm, Polysciences 16592-5, 1.5 × 10^4 particles $well^{-1}$ for freshly isolated and 3 × 10^4 particles $well^{-1}$ for cultured HKL) and measured with the automated sampling unit of the flow cytometer. Forward- and side scatter (FSC/SSC) characteristics of up to 1 × 10^4 events in the single cell gate were acquired in linear mode. Fluorescence intensities at 530 nm and 585 nm were measured using log-scale. Flow-cytometric data were analysed with the FacsDiva software (version 6.1.2, BD, USA). Dead cells (propidium iodide positive) and cellular debris (low FSC/SSC characteristics) were excluded from further evaluation. Lymphocyte- and granulocyte populations were identified according to their characteristic FSC/SSC profiles [37]. Cell viability (absolute numbers of viable cells) before and after *in vitro* culturing were calculated according to *N* (viable cells) = events (viable cells) × number (standard beads)/events (standard beads). Granulocyte to lymphocyte ratio (G/L ratio) was calculated according to G/L ratio = number (viable granulocytes in culture)/number (viable lymphocytes in culture).

2.6. Production of reactive oxygen species

The respiratory burst (RB) activity of head kidney leukocytes (HKL) after *in vitro* cultivation was quantified in a lucigenin-enhanced chemiluminescence (CL) assay, modified after Scott & Klesius [38] as described by Kurtz et al. [39]. After removing a subsample for the flow cytometric analysis (25 μL $well^{-1}$, see 2.5 Flow cytometric analysis), 65 μL of cell suspension was transferred from each well of an HKL culture plate to a well of a white CL 96-well plate (Nunc), prefilled with 20 μL of 2.5 g L^{-1} lucigenin (N,N′-dimethyl-9,9′-biacridiniumdinitrate, Sigma M8010) in PBS and 95 μL medium (RPMI-1640, PAA E15-039) supplemented with 10 mmol L^{-1} HEPES buffer (Lonza 17–737) and 10% (v/v) distilled water. CL plates were incubated for 30 min at 20 °C in a water vapour saturated atmosphere with 3% CO_2 to enable lucigenin uptake by the cells. The RB was induced by addition of 20 μL zymosan suspension from *Saccharomyces cerevisiae* (7.5 g L^{-1}, Sigma Z4250) in PBS to every well. Relative luminescence units (RLU) of individual wells were measured for 3 s, in 5 min intervals during 3 h incubation at 20 °C in an Infinite 200 multimode reader (Tecan, Switzerland). For data analyses the area under the kinetic curve (RLU area, integral from t_0 to t_{3h} of kinetic RLU curve) was determined using Magellan 6.5 software (Tecan, Switzerland).

2.7. Statistics

SPSS Statistics software (version 20, IBM, USA) was used for statistical analyses. Normal distributions of data were verified by visual inspection of residual histograms. Since head kidney leukocytes (HKL) from a single stickleback population were used in experiment 1 (helminth species), those data were analysed using a Greenhouse-Geisser corrected repeated measurement analyses of variance (RM ANOVAs). Greenhouse-Geisser corrected two-way RM ANOVAs with antigen treatment as the within-subject factor and the stickleback population as the between-subject factor were used to analyse experimental data with HKL from more than one stickleback population. If significant effects were detected with the ANOVAs, pairwise comparisons with sequential Bonferroni corrected paired-sample *t*-tests [40] were used as *post-hoc* tests. Data of sympatric *G. aculeatus*-*S. solidus* combinations were compared to the mean of the data from the allopatric combinations in a paired-sample *t*-test and compared separately for stimulation with tegument and body antigens. Correlations of *in vitro* leukocyte responses and geographical distances as well as different *S. solidus* prevalences across populations were analysed by sequential Bonferroni corrected Mantel-tests [41]. Mantel-tests were computed using the ade4 statistical package in R (version 3.0.2, R Core Team 2013, Austria) with means of allopatric stimulated host populations and allopatric combined parasite populations. Results of the statistical analysis are summarized in Table 2 of the Supplementary material.

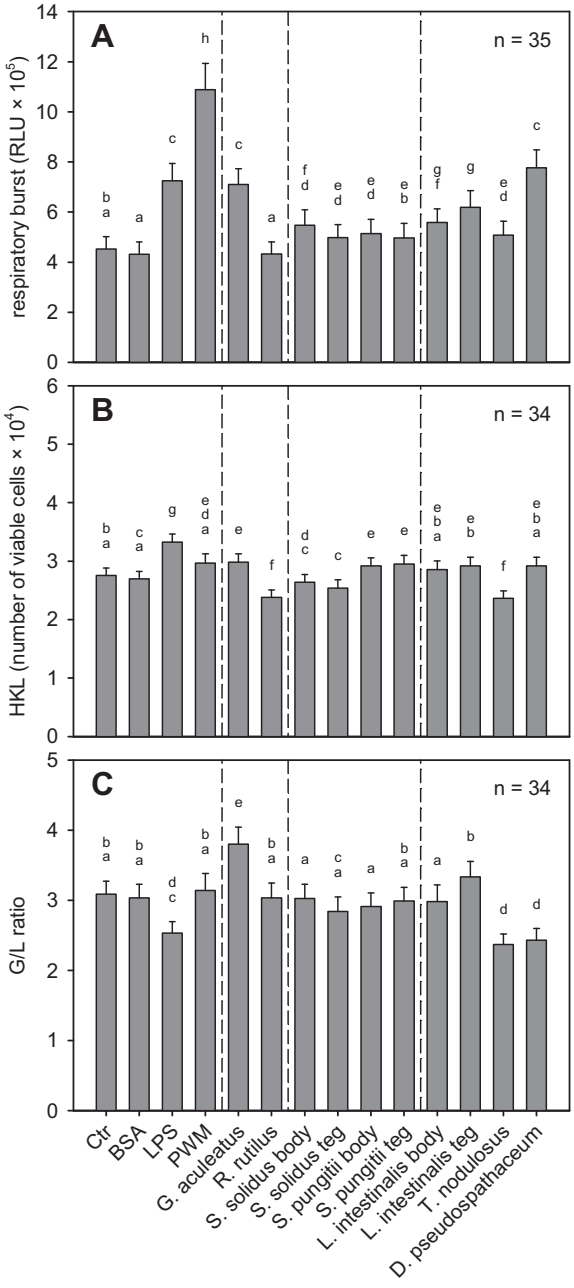

Fig. 1. Experiment 1 (helminth species): *In vitro* responses of stickleback leukocytes to antigens from five helminth parasite species. Respiratory burst activity (A), cell viability (B), and granulocyte to lymphocyte ratio (C) was analysed after 4 days of incubation of head kidney leukocytes (HKL) from laboratory-raised second-generation sticklebacks from a German (NST) population. HKL from individual fish were cultured in medium alone (Ctr), with BSA (10 mg L^{-1}), LPS (20 mg L^{-1}), PWM (2 mg L^{-1}), fish muscle proteins (10 mg L^{-1}) or parasite antigens (10 mg L^{-1}). Parasite antigens were prepared from the tegument (teg), the body without tegument (body), or the whole bodies (*T. nodulosus*, *D. pseudospathaceum*). Different letters above error bars (mean + standard error) indicate significant differences ($p < 0.05$).

3. Results

3.1. Controls

Medium controls (leukocytes in medium alone) and positive controls (LPS, PWM) were included in each experiment. Head kidney leukocytes (HKL) from positive controls showed increased respiratory burst (RB) activity (Figs. 1A, 2A and 3A, Fig. 5A Supplementary material) compared to medium controls, whereas the cell viability was only higher with LPS (Figs. 1B, 2B and 3B, Fig. 5B Supplementary material). The granulocyte to lymphocyte ratio (G/L ratio) was reduced by the addition of LPS (Figs. 1C, 2C and 3C, Fig. 5C Supplementary material). In experiment 1 (helminth species) and experiment 2 (host origins) a protein control (BSA) was included. The RB activity, the cell viability, and the G/L ratio did not differ between HKL from protein and medium controls (Figs. 1 and 2). Therefore the protein control was not used in experiment 3 (sympatric/allopatric combinations). Proteins from *G. aculeatus* and *R. rutilus* served as additional protein controls in experiment 1 (helminth species). Xenogeneic proteins from *R. rutilus* merely decreased the cell viability (Fig. 1B), but did not change the respiratory burst activity (Fig. 1A) and G/L ratio (Fig. 1C) compared to medium controls. Allogeneic stickleback proteins increased the RB activity of HKL to the level of LPS stimulation (Fig. 1A) and the cell viability was elevated compared to medium controls (Fig. 1B). The G/L ratio was more prominently influenced by allogeneic proteins as by the positive controls or parasite antigen stimulations (Fig 1C). The strong *in vitro* responses to allogeneic proteins might be explained by a transplant rejection response mediated by major histocompatibility complex (MHC) cell surface antigens of the same species, whereas xenogeneic MHC antigens were not recognised. Similar observations were made with mixed cultures of rat lymphocytes, which were fully reactive to MHC alloantigens, but displayed no detectable primary reactivity to surface antigens from xenogeneic mammalian cells [42].

3.2. Experiment 1 (helminth species): in vitro responses of stickleback head kidney leukocytes to antigens from five helminth parasite species

To investigate reactions of three-spined stickleback (*G. aculeatus*) head kidney leukocytes (HKL) to different parasite species, HKL of sticklebacks from a German (NST) population were stimulated *in vitro* for 4 days with antigens from four cestode and one trematode species.

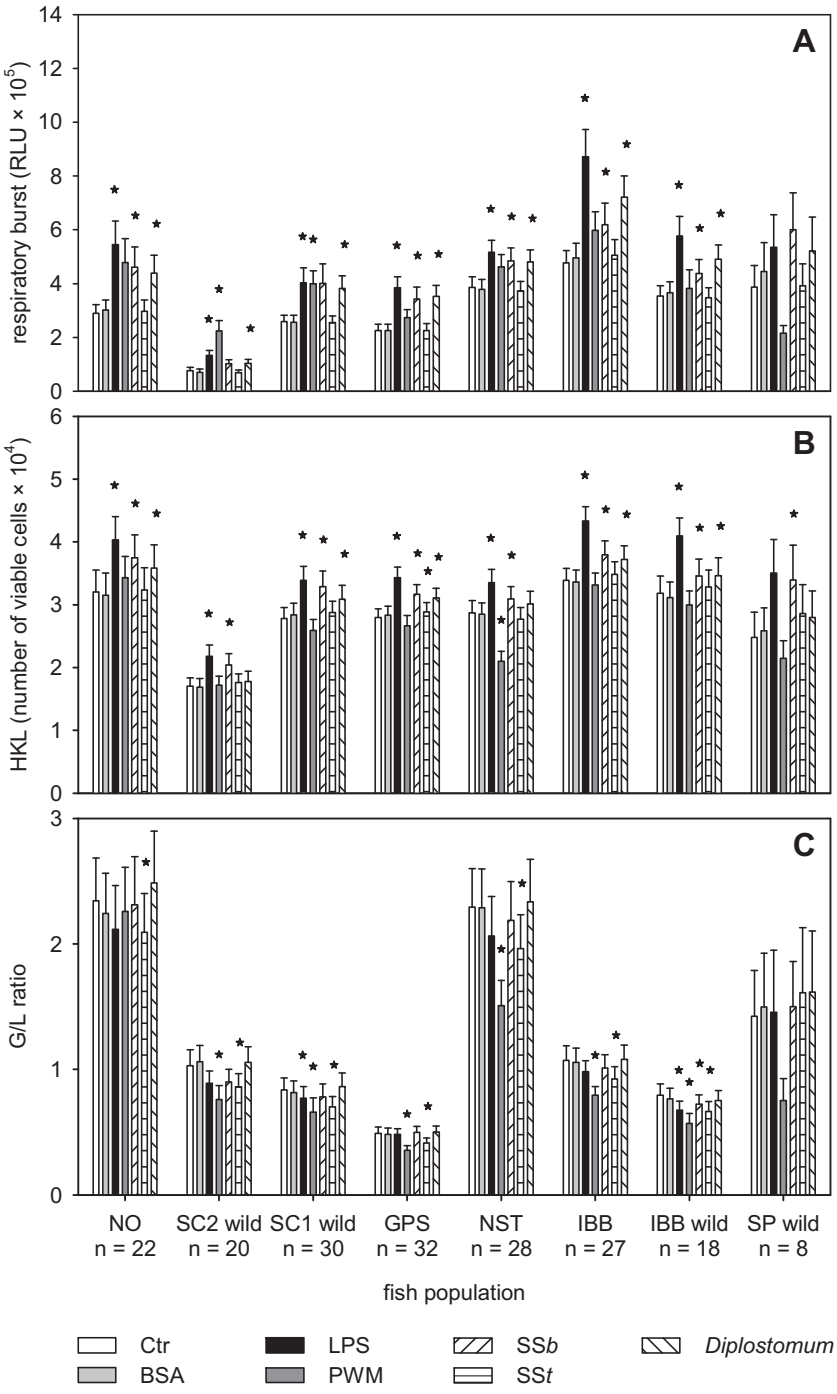

Fig. 2. Experiment 2 (host origins): *In vitro* responses of head kidney leukocytes from seven stickleback populations to parasite antigens. Respiratory burst activity (A), cell viability (B), and granulocyte to lymphocyte ratio (C) was analysed after 4 days of incubation of head kidney leukocytes (HKL) from laboratory-raised and wild caught (wild) sticklebacks from Norway (NO), Scotland (SC1, SC2), Germany (GPS, NST, IBB) and Spain (SP). HKL were cultured in medium alone (Ctr), with BSA (10 mg L^{-1}), LPS (20 mg L^{-1}), PWM (2 mg L^{-1}), or parasite antigens (10 mg L^{-1}). *S. solidus* from a German (IBB) population were used to prepare antigens from the tegument (SS*t*, 0.45 μm filtered) and the body without tegument (SS*b*). *D. pseudospathaceum* antigens were prepared from full bodies (*Diplostomum*). Means + standard errors are given and asterisks indicate significant differences ($p < 0.05$) to corresponding medium controls.

Incubation of HKL with antigens from the specialist, *G. aculeatus*-infecting cestode *S. solidus* significantly elevated the respiratory burst (RB) activity of HKL. Comparable RB activities were observed with antigens of the close relative of *S. solidus*, *S. pungitii*, which specifically infects nine-spined sticklebacks (*P. pungitius*) (Fig. 1A). In contrast, the cell viabilities were decreased by *S. solidus* but increased by *S. pungitii* antigens compared to medium controls (Fig. 1B). The granulocyte to lymphocyte (G/L) ratios were not affected by *S. solidus* and *S. pungitii* antigens (Fig. 1C).

Antigens from the cestode *L. intestinalis*, which does not infect *G. aculeatus*, stimulated the RB activity of HKL to a higher extent than antigens from the *Schistocephalus* species (Fig. 1A). HKL stimulated with *L. intestinalis* antigens did not differ significantly in

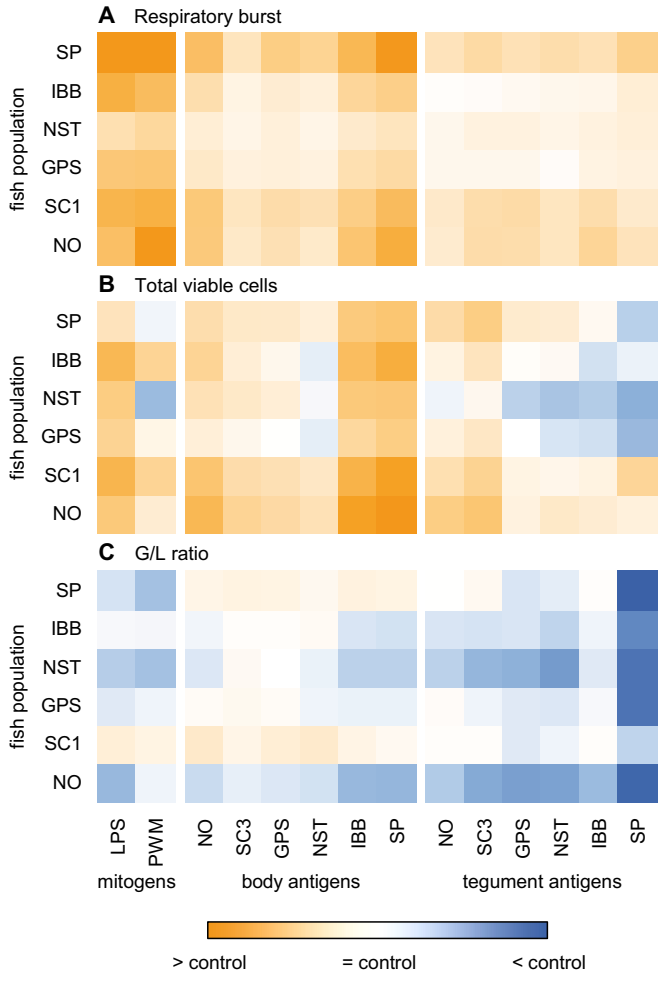

Fig. 3. Experiment 3 (sympatric/allopatric combinations): *In vitro* responses of leukocytes from six stickleback populations to *S. solidus* antigens from identical populations in a fully crossed design. Respiratory burst activity (A), cell viability (B), and granulocyte to lymphocyte ratio (C) after 4 days of incubation of head kidney leukocytes (HKL) from laboratory-raised sticklebacks from Norway (NO, $n = 19$), Scotland (SC1, $n = 24$), Germany (GPS, $n = 31–32$; NST, $n = 28$; IBB, $n = 29$) and Spain (SP, $n = 23$). HKL were cultured in medium alone (Ctr), with LPS (20 mg L^{-1}), PWM (2 mg L^{-1}), or *S. solidus* tegument or body antigens (10 mg L^{-1}) from the corresponding populations. Means of stimulated cultures were normalised to corresponding medium controls (see also Fig. 5, Supplementary material).

their G/L ratios from medium controls, but G/L ratios were significantly higher with tegument antigens compared to body antigens (Fig. 1C).

Stimulation with antigens of the pike-tapeworm *T. nodulosus*, which besides other fish species also infects the three-spined stickleback as a second intermediate host [43], led to elevated RB activity of HKL comparable to the *Schistocephalus* stimulations (Fig. 1A). Antigens of *T. nodulosus* strongly decreased the cell viability (Fig. 1B) and the G/L ratio (Fig. 1C). Antigens from the eye fluke *D. pseudospathaceum*, which also infects *G. aculeatus*, induced the strongest RB activity of HKL across all parasite species (Fig. 1A) and reduced the G/L ratio comparable to stimulation with LPS or *T. nodulosus* antigens (Fig. 1C).

3.3. Experiment 2 (host origins): in vitro responses of head kidney leukocytes from seven stickleback populations to parasite antigens

To compare the *in vitro* responses of three-spined stickleback (*G. aculeatus*) head kidney leukocytes (HKL) from different populations, HKL were cultured with *S. solidus* and *D. pseudospathaceum* antigens.

Parasite antigens from the body of *S. solidus* (specialist, infective for *G. aculeatus*) and those from *D. pseudospathaceum* (generalist, infective for *G. aculeatus*) significantly increased the respiratory burst (RB) activities of HKL from the investigated populations, except for HKL from the Spanish (SP) population and HKL from the Scottish (SC1, SC2) populations that were stimulated with *S. solidus* body antigens (Fig. 2A). In contrast to experiment 1 (helminth species, Fig. 1A), *S. solidus* antigens from the tegument did not increase the RB activity of HKL, which might be explained by removal of particles by the 0.45 μm filtration of the antigen preparation used in the present experiment.

The cell viability varied between stickleback populations and was lowest in one of the Scottish (SC2) populations. Incubation with *S. solidus* body antigens and those of *D. pseudospathaceum*, usually resulted in higher cell viabilities than in medium controls, except for HKL from one Scottish (SC2) population, a German (NST) population and the Spanish (SP) population after stimulation with *D. pseudospathaceum* antigens. With *S. solidus* antigens from the tegument, higher cell viability was observed with HKL of sticklebacks from a German (GPS) population only compared to medium controls (Fig. 2B).

The parasite antigens that stimulated the RB activity and the viability of HKL (*S. solidus* body antigens and those of *D. pseudospathaceum*) did not affect the granulocyte to lymphocyte (G/L) ratios. Instead, stimulation with antigens from the tegument of *S. solidus* resulted in a significant decrease of G/L ratios except for HKL of the Spanish (SP) population (Fig. 2C). The *in vitro* responsiveness of stickleback HKL was relatively similar across populations overall, with the exception of the Spanish (SP) population, where only the cell viability deviated significantly from the corresponding control after stimulation with *S. solidus* body antigens (Fig. 2B). The Scottish (SC2) population was notable because the viability of cells (Fig. 2B) and correspondingly the respiratory burst activity after the culture (Fig. 2A), was lower compared to the other populations. The G/L ratios varied across populations to a higher extent than across treatments within populations. A similar pattern of variation across populations was already present in the fresh HKL isolates before *in vitro* treatments (*e.g.* NO, NST – high; SC1, SC2, GPS – low; data not shown), thus was not an effect of the cell culture.

3.4. Experiment 3 (sympatric/allopatric combinations): in vitro responses of head kidney leukocytes from different stickleback populations to sympatric and allopatric Schistocephalus solidus antigens

Three-spined stickleback (*G. aculeatus*) head kidney leukocytes (HKL) and *S. solidus* antigens from six populations (origins) across Europe with varying *S. solidus* prevalence (see 2.1 Experimental sticklebacks) were cultured in sympatric and allopatric *in vitro* combinations in a fully crossed experimental design.

The stimulation with *S. solidus* antigens significantly increased respiratory burst (RB) activity in the majority of treatments. Among parasite populations, the highest RB activity was induced by antigens from the body of the Norwegian (NO), German (IBB) and Spanish (SP) *S. solidus*. This was consistent with all six stickleback origins tested here (Figs. 3A and Fig. 5A Supplementary material). Mantel-tests revealed that across allopatric combinations of both hosts and parasites, the RB activity was positively correlated with the parasite prevalence matrix, but not with the geographical distance matrix.

Similarly to RB activity, HKL stimulation with *S. solidus* antigens from the body of the Norwegian (NO), a German (IBB) and the

Spanish (SP) populations resulted in the highest viability of HKL. Highest HKL viability within the tegument antigen treatment was observed for the Norwegian (NO) and the Scottish (SC3) *S. solidus* populations (Figs. 3B and Fig. 5B Supplementary material). Granulocyte to lymphocyte (G/L) ratios were lowest in cultures with HKL from the Spanish (SP) population (Fig. 5C Supplementary material). Stimulation with *S. solidus* antigens altered the G/L ratio to a minor extent, but overall every stickleback populations' stimulation with tegument antigens of Spanish (SP) parasites significantly reduced the G/L ratio (Figs. 3C and Fig. 5C Supplementary material).

Significant differences between sympatric and allopatric host-parasite combinations were only detected in the cell viability of HKL cultures with *S. solidus* tegument antigens. In comparison to sympatric combinations, the cell viability was increased in HKL cultures that were stimulated with allopatric *S. solidus* antigens (Fig. 4).

4. Discussion

4.1. Parasite species induce different immune reactions in stickleback HKL cultures

In the present study, we investigated *in vitro* responses of head kidney leukocytes (HKL) from three-spined sticklebacks (*G. aculeatus*) to antigens from helminth parasite species, which partly infect *G. aculeatus* naturally. Among the parasite species tested here, antigens from *D. pseudospathaceum*, which has a relatively broad range of fish host species, induced the strongest *in vitro* responses of stickleback HKL, followed by antigens from *L. intestinalis* (specific for cyprinids) and the two *Schistocephalus* species; *S. pungitii*, specific for the nine-spined stickleback (*P. pungitius*) and *S. solidus*, specific for *G. aculeatus*.

Cestodes often suppress (manipulate) the immune reactions of their hosts, which minimize harmful effects on themselves but may also reduce pathological effects on the hosts [44]. *L. intestinalis* and the *Schistocephalus* species gain most of their final weight in the body cavity of their second intermediate fish hosts, and are confronted with the host's immunity for months, or even years. In contrast, larvae of digenean *Diplostomum* are confronted with the immune system of their fish host only for a short term (<24 h), during the migration from the skin to the immunological inert eye lenses [45,46]. In the present study, the *in vitro* respiratory burst (RB) activity of HKL was highest upon stimulation with *D. pseudospathaceum* antigens and consecutively lower with *L. intestinalis*, *S. pungitii* and *S. solidus* antigens. This suggests that maintenance of low immunogenicity requires specific adaptation to the host's immune system, which is achievable for specialist parasites, such as *S. solidus*, but not for generalists, such as *D. pseudospathaceum*. However, antigens from digenean parasites, such as *D. pseudospathaceum*, might generally be less adapted to hide from a host's immune response and therefore might induce higher *in vitro* responses.

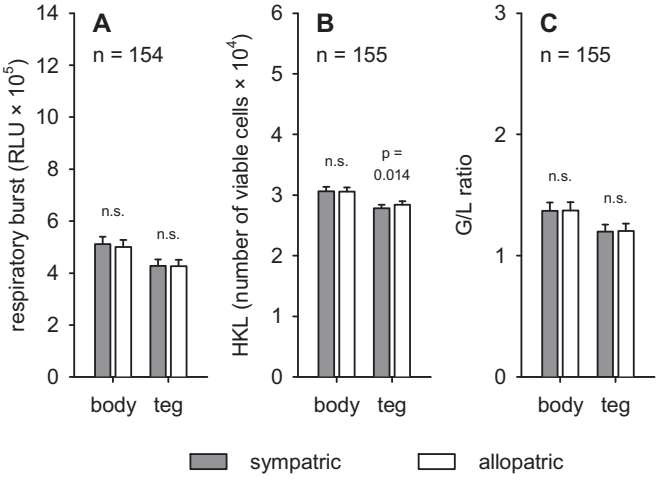

Fig. 4. Experiment 3 (sympatric/allopatric combinations): Comparison of *in vitro* leukocyte responses to antigens from sympatric and allopatric *S. solidus* parasites. Respiratory burst activity (A), cell viability (B), and granulocyte to lymphocyte ratio (C) of the *in vitro* test with head kidney leukocytes (HKL) from six stickleback and *S. solidus* antigens from the identical populations (Fig. 3) were compared for differences between sympatric and allopatric host-parasite combinations (mean + standard error). Antigen preparations from the tegument (teg) and the body without tegument (body) were treated separately (n. s. – not significant).

Although the tapeworm *T. nodulosus* is highly specific to pike as final hosts, it parasitizes approximately 72 fish species [43] and also the three-spined stickleback as a second intermediate host [32]. In the present study, stimulation of stickleback HKL with whole body antigens of *T. nodulosus* resulted in the lowest overall cell viability compared to other parasite antigens tested here. This was predominantly due to low granulocyte viability (data not shown). Nevertheless, *T. nodulosus* antigens induced an RB activity per culture, which was comparable to *Schistocephalus* antigens, presumably a consequence of higher activity of individual granulocytes.

T. nodulosus plerocercoids are encapsulated in cysts in the liver tissue of their intermediate fish hosts [43,47,48]. In burbots (*Lota lota*) and perches (*P. fluviatilis*), encapsulation of *T. nodulosus* plerocercoids was followed by parasite degeneration, whereas Arctic charrs (*Salvelinus alpinus*) also encapsulated, but failed to degenerate *T. nodulosus* larvae [43]. Degeneration of encapsulated *T. nodulosus* is presumably facilitated by granulocytes. In the present *in vitro* study, granulocytes from sticklebacks were activated upon exposure to *T. nodulosus* antigens, but decreased prominently in viability. The strong activation of granulocytes by *T. nodulosus* antigens, might have exhausted granulocyte viability during the culture, but *in vivo*, *T. nodulosus* might reduce the viability of granulocytes immigrating the cysts to avoid damage by their RB activity.

The *Schistocephalus* species *S. solidus* and *S. pungitii*, although closely related and able to hybridize in the lab [49] are specific for either the three-spined stickleback or the nine-spined stickleback (*P. pungitius*). Infection of nine-spined sticklebacks with *S. solidus* infected copepods failed [21], and transplantation of *S. solidus* plerocercoids to *P. pungitius* stopped parasite growth and ultimately leads to death while homo-transplants of both *S. solidus* and *S. pungitii* plerocercoids survived [20,21]. Despite the high *in vivo* specificity of *Schistocephalus* parasites for their stickleback hosts, *in vitro* responses of HKL from three-spined sticklebacks to antigens of the two species were relatively similar. Only cell viability was elevated in cultures with *S. pungitii* antigens, while it remained at the level of protein controls in cultures with *S. solidus* antigens. Lower response levels of HKL to *S. solidus* antigens might be indicative of the higher degree of adaption of *S. solidus* to the immune system of the three-spined stickleback. Absence of differential RB activities of HKL to antigens from the two *Schistocephalus* species might depend on their close relatedness and similarity in immune evasion strategies.

Taken together, antigens from the two generalist parasites, *D. pseudospathaceum* and *T. nodulosus*, and the cestode *L. intestinalis,* which is specialised for cyprinids, induced relatively strong *in vitro* responses. This is presumably a cost of the ability to infect a high variety of hosts, respectively, the lack of adaptation to the stickleback's immune system by *L. intestinalis*. *Vice versa*, antigens from stickleback specific plerocercoids (*Schistocephalus*) excited lower *in vitro* activation, which is likely a sign of specific adaptation to the host's immune repertoire.

4.2. Comparison of parasite tegument and body antigens

Generally lower *in vitro* HKL responses were observed with *Schistocephalus* tegument antigens when comparing antigen preparations from the tegument and the body. By contrast, antigens from the tegument of *L. intestinalis* induced higher *in vitro* responses of stickleback HKL, compared to body antigens, indicating that surface antigens in particular are decisive for the strength of the host's immune response. These findings support the assumption that *S. solidus* tegument antigenicity is best adapted to its specific host, since it induced only low immune activity of *G. aculeatus* HKL.

4.3. Influence of host populations

In experiment 2 (host origins), we investigated variations of the *in vitro* responses of HKL from different stickleback populations. It is known that assortative mating limits gene flow, even between closely neighbouring three-spined stickleback populations [50–55] resulting in a number of different stickleback ecotypes [10,50,56,57] with varying parasite susceptibility [9,10]. Consequently we expected to detect differences across populations in responses of HKL to *in vitro* stimulation with parasite antigens.

In the present study, HKL from a Scottish (SC2) population responded with lower respiratory burst (RB) activity and cell viability compared to HKL from the other stickleback populations. The wild caught Scottish (SC2) sticklebacks used in experiment 2 (host origins), might have been close to their natural age limit (Rahn, pers. comm.) and low *in vitro* responsiveness might be attributed to immuno-senescence [58]. Generally RB activity and cell viability of HKL from the different stickleback populations responded in a similar pattern to the *in vitro* stimulation. This was also the case in a comparison of wild caught and laboratory-raised individuals of a German (IBB) population. The granulocyte to lymphocyte (G/L) ratio exhibited prominent cross population differences and was elevated in the Norwegian (NO) and a German (NST) population and lowest in another German (GPS) population. However, a similar pattern of differences in the G/L ratios across populations was observed in fresh HKL isolates before the *in vitro* culture (data not shown) and might be a sign of adaptations of immunocompetence to varying habitat conditions rather than a consequence of *in vitro* stimulation.

4.4. Sympatric and allopatric host-parasite combinations

In experiment 3 (sympatric/allopatric combinations), HKL from six stickleback populations across Europe were exposed to *S. solidus* antigens derived from the same six habitats in a fully crossed experimental design, to test if stickleback HKL respond differentially to antigens from sympatric and allopatric parasites. The sticklebacks used for this experiment were of similar ages as opposed to those used in experiment 2 (host origins). This might explain why differences in HKL responses between stickleback populations were less abundant than in the prior experiment.

We hypothesized that *in vitro* responses of HKL would be higher to antigens from allopatric parasites compared to sympatric parasites, which was significant in the cell viability after stimulation with *S. solidus* tegument antigens. This might be attributed to the fact that parasites are under selection pressure to adapt their 'antigenic' surface for their respective host population, while body antigens are 'hidden' from host immunity anyways. Differential co-evolution across host-parasite population pairs can only occur if gene flow between host and parasite populations is prevented. Genetically separated populations of *G. aculeatus* are well documented, and investigations on the relationships of *S. solidus* populations indicate that genetic divergence between populations from Alaska, Oregon and Wales is lower than that of their three-spined stickleback hosts [59]. The lack of strong differences between HKL responses to sympatric and allopatric *S. solidus* antigens might be explained by low or absent divergent co-evolution of different host-parasite population pairs. This might be caused by higher dispersal rates of *S. solidus* genotypes with its mobile final hosts, fish eating birds, compared to relatively low dispersal rates of the stickleback intermediate hosts.

4.5. The effect of parasite prevalence

S. solidus infects *G. aculeatus* populations with varying prevalence from <1% (Kalbe, pers. comm.) to up to 79% [60]. Parasites like *S. solidus* and *L. intestinalis* manipulate the behaviour of their intermediate fish hosts [61,62]. This results in higher host predation rates [63–65], which consequently diminishes host population sizes [60,66]. If the predator is the parasite's definite host, it will thereby accelerate its life cycle completion rate, which may increase parasite population size and parasite-to-host biomass ratio [60]. Such dynamics may fluctuate, but often stabilise in a certain range, depending on the habitat specific host-parasite interactions and successful parasite transmissions.

In vitro, HKL from stickleback populations with the highest *S. solidus* prevalence (Norway, NO; Spain, SP) increased their respiratory burst (RB) activity and cell viability more prominently after stimulation with *S. solidus* antigens compared to HKL from two German (GPS, NST) populations with the lowest prevalence. This suggests that HKL of sticklebacks are genetically predisposed to react more strongly to *S. solidus* in populations with high prevalence of the parasite. Conversely, *S. solidus* antigens from populations with high *S. solidus* prevalence (NO, SP) induced higher *in vitro* responses of HKL, compared to parasite antigens from populations with low *S. solidus* prevalence (GPS, NST). Thus, parasite virulence, or at least antigenicity, seems to increase in populations with high parasite prevalence.

Taken together, both, responsiveness of stickleback leukocytes to *S. solidus* antigens, as well as immunogenicity of parasite antigens (virulence) increase with the parasite prevalence in populations.

5. Conclusions

The present study demonstrates that immunogenicity of helminth antigens is highest in generalist parasite species that are infective for different fish species. Lowest immunogenicity was observed with antigens from the highly specialised parasite *S. solidus* with leukocytes from its specific second intermediate host. Investigations of six stickleback and corresponding *S. solidus* origins in sympatric and allopatric combinations revealed that high prevalence of the parasites rather than sympatric interaction primes leukocyte responsiveness and immunogenicity of the parasite antigens.

Nevertheless, differences in the *in vitro* responses between populations with high and low *S. solidus* prevalence indicate that local adaptation does occur, but specific sympatric adaptations might be overruled by quantitative effects (high/low *S. solidus* prevalence). Selection pressure by high frequency (likelihood) of infections might be combined with the necessity to interact with a relatively high number of parasite genotypes, which counter-selects specialisation for certain parasite genotypes, as would be expected in specific sympatric adaptations.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2013.10.019.

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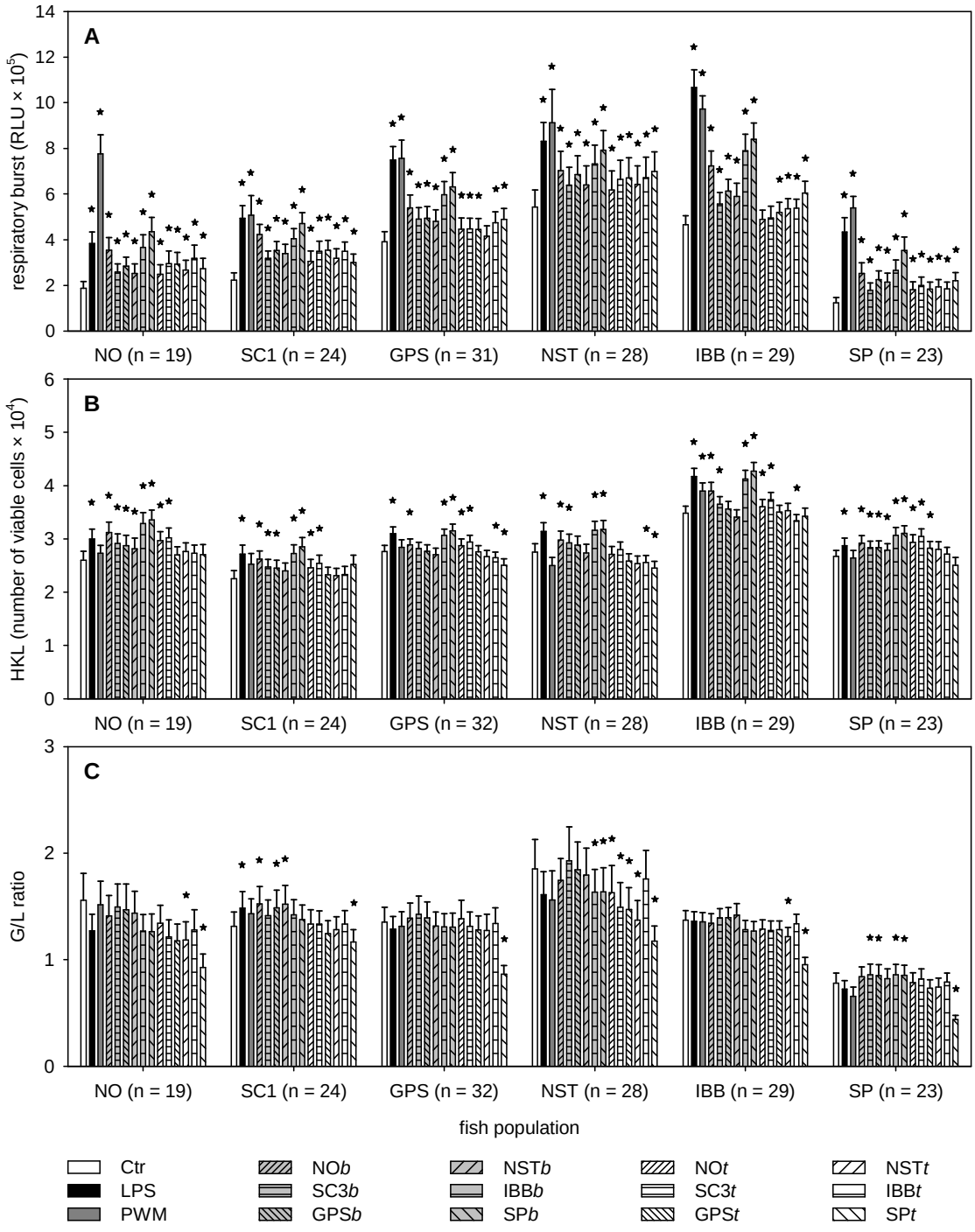

Fig. 5, Franke *et al*. (supplementary material)

Table 2, Franke *et al.* (supplementary material)

Fig. 1A: helminth species - respiratory burst activity
RM ANOVA, *n* fish = 35, $p < 0.001$

Ctr - BSA	0.58	LPS - *S.p.t*	<0.001	*R.r.* - *D.p.*	<0.001
Ctr - LPS	<0.001	LPS - *L.i.b*	<0.001	*S.s.b* - *S.s.t*	0.094
Ctr - PWM	<0.001	LPS - *L.i.t*	0.002	*S.s.b* - *S.p.b*	0.104
Ctr - *G.a.*	<0.001	LPS - *T.n.*	<0.001	*S.s.b* - *L.i.t*	0.027
Ctr - *R.r.*	0.152	LPS - *D.p.*	0.108	*S.s.b* - *L.i.b*	1
Ctr - *S.s.b*	<0.001	PWM - *G.a.*	<0.001	*S.s.b* - *L.i.t*	0.006
Ctr - *S.s.t*	0.018	PWM - *R.r.*	<0.001	*S.s.b* - *T.n.*	0.101
Ctr - *S.p.b*	0.002	PWM - *S.s.b*	<0.001	*S.s.b* - *D.p.*	<0.001
Ctr - *S.p.t*	0.095	PWM - *S.s.t*	<0.001	*S.s.t* - *S.p.b*	1
Ctr - *L.i.b*	<0.001	PWM - *S.p.b*	<0.001	*S.s.t* - *S.p.t*	1
Ctr - *L.i.t*	<0.001	PWM - *S.p.t*	<0.001	*S.s.t* - *L.i.b*	0.005
Ctr - *T.n.*	0.005	PWM - *L.i.b*	<0.001	*S.s.t* - *L.i.t*	<0.001
Ctr - *D.p.*	<0.001	PWM - *L.i.t*	<0.001	*S.s.t* - *T.n.*	1
BSA - LPS	<0.001	PWM - *T.n.*	<0.001	*S.s.t* - *D.p.*	<0.001
BSA - PWM	<0.001	PWM - *D.p.*	<0.001	*S.p.b* - *S.p.t*	0.872
BSA - *G.a.*	<0.001	*G.a.* - *R.r.*	<0.001	*S.p.b* - *L.i.b*	0.002
BSA - *R.r.*	0.95	*G.a.* - *S.s.b*	<0.001	*S.p.b* - *L.i.t*	<0.001
BSA - *S.s.b*	<0.001	*G.a.* - *S.s.t*	<0.001	*S.p.b* - *T.n.*	1
BSA - *S.s.t*	0.001	*G.a.* - *S.p.b*	<0.001	*S.p.b* - *D.p.*	<0.001
BSA - *S.p.b*	<0.001	*G.a.* - *S.p.t*	<0.001	*S.p.t* - *L.i.b*	0.002
BSA - *S.p.t*	0.004	*G.a.* - *L.i.b*	<0.001	*S.p.t* - *L.i.t*	<0.001
BSA - *L.i.b*	<0.001	*G.a.* - *L.i.t*	0.009	*S.p.t* - *T.n.*	1
BSA - *L.i.t*	<0.001	*G.a.* - *T.n.*	<0.001	*S.p.t* - *D.p.*	<0.001
BSA - *T.n.*	0.002	*G.a.* - *D.p.*	0.173	*L.i.b* - *L.i.t*	0.059
BSA - *D.p.*	<0.001	*R.r.* - *S.s.b*	<0.001	*L.i.b* - *T.n.*	0.002
LPS - PWM	<0.001	*R.r.* - *S.s.t*	0.001	*L.i.b* - *D.p.*	<0.001
LPS - *G.a.*	1	*R.r.* - *S.p.b*	<0.001	*L.i.t* - *T.n.*	<0.001
LPS - *R.r.*	<0.001	*R.r.* - *S.p.t*	0.028	*L.i.t* - *D.p.*	<0.001
LPS - *S.s.b*	<0.001	*R.r.* - *L.i.b*	<0.001	*T.n.* - *D.p.*	<0.001
LPS - *S.s.t*	<0.001	*R.r.* - *L.i.t*	<0.001		
LPS - *S.p.b*	<0.001	*R.r.* - *T.n.*	0.001		

Fig. 1B: helminth species - number of viable cells
RM ANOVA, *n* fish = 34, $p < 0.001$

Ctr - BSA	1	LPS - *S.p.t*	<0.001	*R.r.* - *D.p.*	<0.001
Ctr - LPS	<0.001	LPS - *L.i.b*	<0.001	*S.s.b* - *S.s.t*	0.307
Ctr - PWM	1	LPS - *L.i.t*	<0.001	*S.s.b* - *S.p.b*	<0.001
Ctr - *G.a.*	0.016	LPS - *T.n.*	<0.001	*S.s.b* - *L.i.t*	0.001
Ctr - *R.r.*	<0.001	LPS - *D.p.*	<0.001	*S.s.b* - *L.i.b*	<0.001
Ctr - *S.s.b*	0.044	PWM - *G.a.*	1	*S.s.b* - *L.i.t*	<0.001
Ctr - *S.s.t*	0.002	PWM - *R.r.*	<0.001	*S.s.b* - *T.n.*	<0.001
Ctr - *S.p.b*	0.013	PWM - *S.s.b*	0.081	*S.s.b* - *D.p.*	0.002
Ctr - *S.p.t*	0.018	PWM - *S.s.t*	0.003	*S.s.t* - *S.p.b*	<0.001
Ctr - *L.i.b*	0.839	PWM - *S.p.b*	1	*S.s.t* - *S.p.t*	<0.001
Ctr - *L.i.t*	0.227	PWM - *S.p.t*	1	*S.s.t* - *L.i.b*	<0.001
Ctr - *T.n.*	<0.001	PWM - *L.i.b*	1	*S.s.t* - *L.i.t*	<0.001
Ctr - *D.p.*	0.512	PWM - *L.i.t*	1	*S.s.t* - *T.n.*	0.007
BSA - LPS	<0.001	PWM - *T.n.*	<0.001	*S.s.t* - *D.p.*	<0.001
BSA - PWM	0.619	PWM - *D.p.*	1	*S.p.b* - *S.p.t*	1
BSA - *G.a.*	0.011	*G.a.* - *R.r.*	<0.001	*S.p.b* - *L.i.b*	1
BSA - *R.r.*	<0.001	*G.a.* - *S.s.b*	<0.001	*S.p.b* - *L.i.t*	0.975
BSA - *S.s.b*	1	*G.a.* - *S.s.t*	<0.001	*S.p.b* - *T.n.*	<0.001
BSA - *S.s.t*	0.248	*G.a.* - *S.p.b*	1	*S.p.b* - *D.p.*	1
BSA - *S.p.b*	0.001	*G.a.* - *S.p.t*	1	*S.p.t* - *L.i.b*	1
BSA - *S.p.t*	<0.001	*G.a.* - *L.i.b*	0.636	*S.p.t* - *L.i.t*	1
BSA - *L.i.b*	0.231	*G.a.* - *L.i.t*	1	*S.p.t* - *T.n.*	<0.001
BSA - *L.i.t*	0.03	*G.a.* - *T.n.*	<0.001	*S.p.t* - *D.p.*	1
BSA - *T.n.*	<0.001	*G.a.* - *D.p.*	1	*L.i.b* - *L.i.t*	1
BSA - *D.p.*	0.199	*R.r.* - *S.s.b*	<0.001	*L.i.b* - *T.n.*	<0.001
LPS - PWM	0.011	*R.r.* - *S.s.t*	0.009	*L.i.b* - *D.p.*	1
LPS - *G.a.*	<0.001	*R.r.* - *S.p.b*	<0.001	*L.i.t* - *T.n.*	<0.001
LPS - *R.r.*	<0.001	*R.r.* - *S.p.t*	<0.001	*L.i.t* - *D.p.*	1
LPS - *S.s.b*	<0.001	*R.r.* - *L.i.b*	<0.001	*T.n.* - *D.p.*	<0.001
LPS - *S.s.t*	<0.001	*R.r.* - *L.i.t*	<0.001		
LPS - *S.p.b*	<0.001	*R.r.* - *T.n.*	1		

Fig. 1C: helminth species - G/L ratio
RM ANOVA, *n* fish = 34, $p < 0.001$

Ctr - BSA	1	LPS - *S.p.t*	<0.001	*R.r.* - *D.p.*	<0.001
Ctr - LPS	<0.001	LPS - *L.i.b*	0.02	*S.s.b* - *S.s.t*	1
Ctr - PWM	1	LPS - *L.i.t*	<0.001	*S.s.b* - *S.p.b*	0.596
Ctr - *G.a.*	<0.001	LPS - *T.n.*	0.516	*S.s.b* - *L.i.t*	1
Ctr - *R.r.*	1	LPS - *D.p.*	1	*S.s.b* - *L.i.b*	1
Ctr - *S.s.b*	1	PWM - *G.a.*	0.002	*S.s.b* - *L.i.t*	<0.001
Ctr - *S.s.t*	0.067	PWM - *R.r.*	1	*S.s.b* - *T.n.*	<0.001
Ctr - *S.p.b*	0.14	PWM - *S.s.b*	1	*S.s.b* - *D.p.*	<0.001
Ctr - *S.p.t*	1	PWM - *S.s.t*	1	*S.s.t* - *S.p.b*	1
Ctr - *L.i.b*	1	PWM - *S.p.b*	1	*S.s.t* - *S.p.t*	1
Ctr - *L.i.t*	0.324	PWM - *S.p.t*	1	*S.s.t* - *L.i.b*	1
Ctr - *T.n.*	<0.001	PWM - *L.i.b*	1	*S.s.t* - *L.i.t*	0.001
Ctr - *D.p.*	<0.001	PWM - *L.i.t*	1	*S.s.t* - *T.n.*	<0.001
BSA - LPS	<0.001	PWM - *T.n.*	<0.001	*S.s.t* - *D.p.*	0.008
BSA - PWM	1	PWM - *D.p.*	<0.001	*S.p.b* - *S.p.t*	1
BSA - *G.a.*	<0.001	*G.a.* - *R.r.*	<0.001	*S.p.b* - *L.i.b*	1
BSA - *R.r.*	0.988	*G.a.* - *S.s.b*	<0.001	*S.p.b* - *L.i.t*	<0.001
BSA - *S.s.b*	1	*G.a.* - *S.s.t*	<0.001	*S.p.b* - *T.n.*	<0.001
BSA - *S.s.t*	1	*G.a.* - *S.p.b*	<0.001	*S.p.b* - *D.p.*	<0.001
BSA - *S.p.b*	1	*G.a.* - *S.p.t*	<0.001	*S.p.t* - *L.i.b*	1
BSA - *S.p.t*	1	*G.a.* - *L.i.b*	<0.001	*S.p.t* - *L.i.t*	0.18
BSA - *L.i.b*	1	*G.a.* - *L.i.t*	0.002	*S.p.t* - *T.n.*	<0.001
BSA - *L.i.t*	0.063	*G.a.* - *T.n.*	<0.001	*S.p.t* - *D.p.*	<0.001
BSA - *T.n.*	<0.001	*G.a.* - *D.p.*	<0.001	*L.i.b* - *L.i.t*	0.002
BSA - *D.p.*	<0.001	*R.r.* - *S.s.b*	1	*L.i.b* - *T.n.*	<0.001
LPS - PWM	0.004	*R.r.* - *S.s.t*	0.324	*L.i.b* - *D.p.*	<0.001
LPS - *G.a.*	<0.001	*R.r.* - *S.p.b*	1	*L.i.t* - *T.n.*	<0.001
LPS - *R.r.*	0.009	*R.r.* - *S.p.t*	1	*L.i.t* - *D.p.*	<0.001
LPS - *S.s.b*	<0.001	*R.r.* - *L.i.b*	1	*T.n.* - *D.p.*	1
LPS - *S.s.t*	0.332	*R.r.* - *L.i.t*	0.185		
LPS - *S.p.b*	<0.001	*R.r.* - *T.n.*	<0.001		

Fig. 2A: host origins - respiratory burst activity
Two-way RM ANOVA, *n* fish = 185, $p < 0.001$

NO: Ctr - BSA	0.395	GPS: Ctr - BSA	1	IBBwild: Ctr - BSA	0.527
NO: Ctr - LPS	0.004	GPS: Ctr - LPS	<0.001	IBBwild: Ctr - LPS	<0.001
NO: Ctr - PWM	0.089	GPS: Ctr - PWM	0.52	IBBwild: Ctr - PWM	0.669
NO: Ctr - *S.s.b*	0.01	GPS: Ctr - *S.s.b*	<0.001	IBBwild: Ctr - *S.s.b*	0.001
NO: Ctr - *S.s.t*	0.633	GPS: Ctr - *S.s.t*	0.955	IBBwild: Ctr - *S.s.t*	1
NO: Ctr - *D.p.*	0.008	GPS: Ctr - *D.p.*	<0.001	IBBwild: Ctr - *D.p.*	<0.001
SC2wild: Ctr - BSA	0.366	NST: Ctr - BSA	0.556	SPwild: Ctr - BSA	0.185
SC2wild: Ctr - LPS	0.014	NST: Ctr - LPS	<0.001	SPwild: Ctr - LPS	0.151
SC2wild: Ctr - PWM	0.007	NST: Ctr - PWM	0.704	SPwild: Ctr - PWM	0.124
SC2wild: Ctr - *S.s.b*	0.09	NST: Ctr - *S.s.b*	0.002	SPwild: Ctr - *S.s.b*	0.079
SC2wild: Ctr - *S.s.t*	0.581	NST: Ctr - *S.s.t*	0.303	SPwild: Ctr - *S.s.t*	0.887
SC2wild: Ctr - *D.p.*	0.029	NST: Ctr - *D.p.*	<0.001	SPwild: Ctr - *D.p.*	0.181
SC1wild: Ctr - BSA	1	IBB: Ctr - BSA	0.195		
SC1wild: Ctr - LPS	0.004	IBB: Ctr - LPS	<0.001		
SC1wild: Ctr - PWM	0.007	IBB: Ctr - PWM	0.168		
SC1wild: Ctr - *S.s.b*	0.087	IBB: Ctr - *S.s.b*	0.005		
SC1wild: Ctr - *S.s.t*	1	IBB: Ctr - *S.s.t*	0.197		
SC1wild: Ctr - *D.p.*	0.001	IBB: Ctr - *D.p.*	<0.001		

Fig. 2B: host origins - number of viable cells
Two-way RM ANOVA, *n* fish = 185, $p < 0.001$

NO: Ctr - BSA	0.599	GPS: Ctr - BSA	0.285	IBBwild: Ctr - BSA	0.414
NO: Ctr - LPS	<0.001	GPS: Ctr - LPS	<0.001	IBBwild: Ctr - LPS	<0.001
NO: Ctr - PWM	0.695	GPS: Ctr - PWM	0.425	IBBwild: Ctr - PWM	0.313
NO: Ctr - *S.s.b*	0.006	GPS: Ctr - *S.s.b*	<0.001	IBBwild: Ctr - *S.s.b*	0.001
NO: Ctr - *S.s.t*	0.532	GPS: Ctr - *S.s.t*	0.017	IBBwild: Ctr - *S.s.t*	0.341
NO: Ctr - *D.p.*	0.007	GPS: Ctr - *D.p.*	<0.001	IBBwild: Ctr - *D.p.*	<0.001
SC2wild: Ctr - BSA	0.735	NST: Ctr - BSA	0.549	SPwild: Ctr - BSA	0.248
SC2wild: Ctr - LPS	<0.001	NST: Ctr - LPS	0.002	SPwild: Ctr - LPS	0.087
SC2wild: Ctr - PWM	0.745	NST: Ctr - PWM	<0.001	SPwild: Ctr - PWM	0.366
SC2wild: Ctr - *S.s.b*	<0.001	NST: Ctr - *S.s.b*	0.002	SPwild: Ctr - *S.s.b*	0.032
SC2wild: Ctr - *S.s.t*	0.376	NST: Ctr - *S.s.t*	0.111	SPwild: Ctr - *S.s.t*	0.489
SC2wild: Ctr - *D.p.*	0.387	NST: Ctr - *D.p.*	0.1	SPwild: Ctr - *D.p.*	0.239
SC1wild: Ctr - BSA	0.155	IBB: Ctr - BSA	1		
SC1wild: Ctr - LPS	<0.001	IBB: Ctr - LPS	<0.001		
SC1wild: Ctr - PWM	0.285	IBB: Ctr - PWM	0.551		
SC1wild: Ctr - *S.s.b*	<0.001	IBB: Ctr - *S.s.b*	<0.001		
SC1wild: Ctr - *S.s.t*	0.062	IBB: Ctr - *S.s.t*	0.334		
SC1wild: Ctr - *D.p.*	0.002	IBB: Ctr - *D.p.*	<0.001		

Fig. 2C: host origins - G/L ratio
Two-way RM ANOVA, *n* fish = 185, $p < 0.001$

NO: Ctr - BSA	0.529	GPS: Ctr - BSA	1	IBBwild: Ctr - BSA	0.098
NO: Ctr - LPS	0.428	GPS: Ctr - LPS	0.996	IBBwild: Ctr - LPS	0.005
NO: Ctr - PWM	1	GPS: Ctr - PWM	0.029	IBBwild: Ctr - PWM	0.02
NO: Ctr - *S.s.b*	0.807	GPS: Ctr - *S.s.b*	0.565	IBBwild: Ctr - *S.s.b*	0.017
NO: Ctr - *S.s.t*	0.002	GPS: Ctr - *S.s.t*	<0.001	IBBwild: Ctr - *S.s.t*	<0.001
NO: Ctr - *D.p.*	0.713	GPS: Ctr - *D.p.*	1	IBBwild: Ctr - *D.p.*	0.096
SC2wild: Ctr - BSA	0.275	NST: Ctr - BSA	0.94	SPwild: Ctr - BSA	0.764
SC2wild: Ctr - LPS	0.086	NST: Ctr - LPS	0.345	SPwild: Ctr - LPS	0.829
SC2wild: Ctr - PWM	0.001	NST: Ctr - PWM	<0.001	SPwild: Ctr - PWM	0.105
SC2wild: Ctr - *S.s.b*	0.054	NST: Ctr - *S.s.b*	0.066	SPwild: Ctr - *S.s.b*	0.999
SC2wild: Ctr - *S.s.t*	<0.001	NST: Ctr - *S.s.t*	<0.001	SPwild: Ctr - *S.s.t*	1
SC2wild: Ctr - *D.p.*	0.397	NST: Ctr - *D.p.*	0.972	SPwild: Ctr - *D.p.*	0.929
SC1wild: Ctr - BSA	0.2	IBB: Ctr - BSA	0.527		
SC1wild: Ctr - LPS	0.046	IBB: Ctr - LPS	0.213		
SC1wild: Ctr - PWM	0.042	IBB: Ctr - PWM	0.017		
SC1wild: Ctr - *S.s.b*	0.108	IBB: Ctr - *S.s.b*	0.166		
SC1wild: Ctr - *S.s.t*	<0.001	IBB: Ctr - *S.s.t*	<0.001		
SC1wild: Ctr - *D.p.*	0.302	IBB: Ctr - *D.p.*	0.796		

Fig. 3A, 5A: sympatric/allopatric combinations - respiratory burst activity
Two-way RM ANOVA, *n* fish = 154, $p < 0.001$

NO: Ctr - LPS	<0.001	GPS: Ctr - LPS	<0.001	IBB: Ctr - LPS	<0.001
NO: Ctr - PWM	<0.001	GPS: Ctr - PWM	<0.001	IBB: Ctr - PWM	<0.001
NO: Ctr - NO*b*	0.001	GPS: Ctr - NO*b*	<0.001	IBB: Ctr - NO*b*	<0.001
NO: Ctr - SC3*b*	<0.001	GPS: Ctr - SC3*b*	<0.001	IBB: Ctr - SC3*b*	0.002
NO: Ctr - GPS*b*	<0.001	GPS: Ctr - GPS*b*	<0.001	IBB: Ctr - GPS*b*	<0.001
NO: Ctr - NST*b*	0.004	GPS: Ctr - NST*b*	<0.001	IBB: Ctr - NST*b*	0.002
NO: Ctr - IBB*b*	<0.001	GPS: Ctr - IBB*b*	<0.001	IBB: Ctr - IBB*b*	<0.001
NO: Ctr - SP*b*	<0.001	GPS: Ctr - SP*b*	<0.001	IBB: Ctr - SP*b*	<0.001
NO: Ctr - NO*t*	0.004	GPS: Ctr - NO*t*	<0.001	IBB: Ctr - NO*t*	0.276
NO: Ctr - SC3*t*	0.003	GPS: Ctr - SC3*t*	<0.001	IBB: Ctr - SC3*t*	0.148
NO: Ctr - GPS*t*	0.005	GPS: Ctr - GPS*t*	<0.001	IBB: Ctr - GPS*t*	0.022
NO: Ctr - NST*t*	0.003	GPS: Ctr - NST*t*	0.148	IBB: Ctr - NST*t*	0.003
NO: Ctr - IBB*t*	0.004	GPS: Ctr - IBB*t*	<0.001	IBB: Ctr - IBB*t*	<0.001
NO: Ctr - SP*t*	<0.001	GPS: Ctr - SP*t*	<0.001	IBB: Ctr - SP*t*	<0.001
SC1: Ctr - LPS	<0.001	NST: Ctr - LPS	<0.001	SP: Ctr - LPS	<0.001
SC1: Ctr - PWM	0.006	NST: Ctr - PWM	0.006	SP: Ctr - PWM	<0.001
SC1: Ctr - NO*b*	<0.001	NST: Ctr - NO*b*	<0.001	SP: Ctr - NO*b*	0.002
SC1: Ctr - SC3*b*	<0.001	NST: Ctr - SC3*b*	<0.001	SP: Ctr - SC3*b*	0.005
SC1: Ctr - GPS*b*	<0.001	NST: Ctr - GPS*b*	<0.001	SP: Ctr - GPS*b*	0.002
SC1: Ctr - NST*b*	<0.001	NST: Ctr - NST*b*	<0.001	SP: Ctr - NST*b*	0.004
SC1: Ctr - IBB*b*	<0.001	NST: Ctr - IBB*b*	<0.001	SP: Ctr - IBB*b*	0.002
SC1: Ctr - SP*b*	<0.001	NST: Ctr - SP*b*	<0.001	SP: Ctr - SP*b*	<0.001
SC1: Ctr - NO*t*	0.007	NST: Ctr - NO*t*	0.043	SP: Ctr - NO*t*	0.015
SC1: Ctr - SC3*t*	0.003	NST: Ctr - SC3*t*	0.009	SP: Ctr - SC3*t*	0.013
SC1: Ctr - GPS*t*	0.001	NST: Ctr - GPS*t*	0.008	SP: Ctr - GPS*t*	0.011
SC1: Ctr - NST*t*	0.009	NST: Ctr - NST*t*	0.024	SP: Ctr - NST*t*	0.014
SC1: Ctr - IBB*t*	<0.001	NST: Ctr - IBB*t*	0.006	SP: Ctr - IBB*t*	0.011
SC1: Ctr - SP*t*	0.012	NST: Ctr - SP*t*	0.002	SP: Ctr - SP*t*	0.003

Fig. 3B, 5B: sympatric/allopatric combinations - number of viable cells
Two-way RM ANOVA, *n* fish = 155, $p < 0.001$

NO: Ctr - LPS	<0.001	GPS: Ctr - LPS	<0.001	IBB: Ctr - LPS	<0.001
NO: Ctr - PWM	0.44	GPS: Ctr - PWM	1	IBB: Ctr - PWM	0.003
NO: Ctr - NO*b*	<0.001	GPS: Ctr - NO*b*	0.006	IBB: Ctr - NO*b*	<0.001
NO: Ctr - SC3*b*	<0.001	GPS: Ctr - SC3*b*	0.468	IBB: Ctr - SC3*b*	0.025
NO: Ctr - GPS*b*	<0.001	GPS: Ctr - GPS*b*	0.907	IBB: Ctr - GPS*b*	0.582
NO: Ctr - NST*b*	0.003	GPS: Ctr - NST*b*	0.575	IBB: Ctr - NST*b*	0.654
NO: Ctr - IBB*b*	<0.001	GPS: Ctr - IBB*b*	<0.001	IBB: Ctr - IBB*b*	<0.001
NO: Ctr - SP*b*	<0.001	GPS: Ctr - SP*b*	<0.001	IBB: Ctr - SP*b*	<0.001
NO: Ctr - NO*t*	<0.001	GPS: Ctr - NO*t*	0.019	IBB: Ctr - NO*t*	0.046
NO: Ctr - SC3*t*	<0.001	GPS: Ctr - SC3*t*	<0.001	IBB: Ctr - SC3*t*	<0.001
NO: Ctr - GPS*t*	0.743	GPS: Ctr - GPS*t*	1	IBB: Ctr - GPS*t*	0.625
NO: Ctr - NST*t*	0.315	GPS: Ctr - NST*t*	0.218	IBB: Ctr - NST*t*	0.779
NO: Ctr - IBB*t*	0.7	GPS: Ctr - IBB*t*	0.012	IBB: Ctr - IBB*t*	0.026
NO: Ctr - SP*t*	1	GPS: Ctr - SP*t*	<0.001	IBB: Ctr - SP*t*	0.772
SC1: Ctr - LPS	<0.001	NST: Ctr - LPS	<0.001	SP: Ctr - LPS	0.026
SC1: Ctr - PWM	0.188	NST: Ctr - PWM	0.195	SP: Ctr - PWM	0.742
SC1: Ctr - NO*b*	<0.001	NST: Ctr - NO*b*	0.002	SP: Ctr - NO*b*	<0.001
SC1: Ctr - SC3*b*	0.002	NST: Ctr - SC3*b*	0.002	SP: Ctr - SC3*b*	0.024
SC1: Ctr - GPS*b*	0.007	NST: Ctr - GPS*b*	0.063	SP: Ctr - GPS*b*	0.008
SC1: Ctr - NST*b*	0.225	NST: Ctr - NST*b*	0.781	SP: Ctr - NST*b*	0.038
SC1: Ctr - IBB*b*	<0.001	NST: Ctr - IBB*b*	<0.001	SP: Ctr - IBB*b*	<0.001
SC1: Ctr - SP*b*	<0.001	NST: Ctr - SP*b*	<0.001	SP: Ctr - SP*b*	<0.001
SC1: Ctr - NO*t*	0.002	NST: Ctr - NO*t*	0.903	SP: Ctr - NO*t*	<0.001
SC1: Ctr - SC3*t*	<0.001	NST: Ctr - SC3*t*	1	SP: Ctr - SC3*t*	<0.001
SC1: Ctr - GPS*t*	0.549	NST: Ctr - GPS*t*	0.085	SP: Ctr - GPS*t*	0.021
SC1: Ctr - NST*t*	0.52	NST: Ctr - NST*t*	0.096	SP: Ctr - NST*t*	0.055
SC1: Ctr - IBB*t*	0.515	NST: Ctr - IBB*t*	0.038	SP: Ctr - IBB*t*	0.55
SC1: Ctr - SP*t*	0.061	NST: Ctr - SP*t*	0.021	SP: Ctr - SP*t*	0.154

Fig. 3C, 5C: sympatric/allopatric combinations - G/L ratio
Two-way RM ANOVA, *n* fish = 155, $p = 0.001$

NO: Ctr - LPS	0.192	GPS: Ctr - LPS	1	IBB: Ctr - LPS	1
NO: Ctr - PWM	0.848	GPS: Ctr - PWM	1	IBB: Ctr - PWM	0.829
NO: Ctr - NO*b*	0.486	GPS: Ctr - NO*b*	1	IBB: Ctr - NO*b*	1
NO: Ctr - SC3*b*	0.645	GPS: Ctr - SC3*b*	1	IBB: Ctr - SC3*b*	1
NO: Ctr - GPS*b*	0.414	GPS: Ctr - GPS*b*	1	IBB: Ctr - GPS*b*	1
NO: Ctr - NST*b*	0.498	GPS: Ctr - NST*b*	1	IBB: Ctr - NST*b*	1
NO: Ctr - IBB*b*	0.203	GPS: Ctr - IBB*b*	1	IBB: Ctr - IBB*b*	0.527
NO: Ctr - SP*b*	0.149	GPS: Ctr - SP*b*	1	IBB: Ctr - SP*b*	0.59
NO: Ctr - NO*t*	0.491	GPS: Ctr - NO*t*	1	IBB: Ctr - NO*t*	0.154
NO: Ctr - SC3*t*	0.134	GPS: Ctr - SC3*t*	1	IBB: Ctr - SC3*t*	0.159
NO: Ctr - GPS*t*	0.109	GPS: Ctr - GPS*t*	1	IBB: Ctr - GPS*t*	0.236
NO: Ctr - NST*t*	0.043	GPS: Ctr - NST*t*	1	IBB: Ctr - NST*t*	0.002
NO: Ctr - IBB*t*	0.174	GPS: Ctr - IBB*t*	0.876	IBB: Ctr - IBB*t*	1
NO: Ctr - SP*t*	0.005	GPS: Ctr - SP*t*	<0.001	IBB: Ctr - SP*t*	<0.001
SC1: Ctr - LPS	0.035	NST: Ctr - LPS	0.062	SP: Ctr - LPS	0.346
SC1: Ctr - PWM	1	NST: Ctr - PWM	0.324	SP: Ctr - PWM	0.322
SC1: Ctr - NO*b*	0.004	NST: Ctr - NO*b*	0.582	SP: Ctr - NO*b*	0.144
SC1: Ctr - SC3*b*	0.144	NST: Ctr - SC3*b*	0.712	SP: Ctr - SC3*b*	0.01
SC1: Ctr - GPS*b*	0.02	NST: Ctr - GPS*b*	0.846	SP: Ctr - GPS*b*	0.028
SC1: Ctr - NST*b*	0.03	NST: Ctr - NST*b*	0.662	SP: Ctr - NST*b*	0.159
SC1: Ctr - IBB*b*	0.058	NST: Ctr - IBB*b*	0.028	SP: Ctr - IBB*b*	0.006
SC1: Ctr - SP*b*	1	NST: Ctr - SP*b*	0.023	SP: Ctr - SP*b*	0.038
SC1: Ctr - NO*t*	1	NST: Ctr - NO*t*	0.008	SP: Ctr - NO*t*	0.822
SC1: Ctr - SC3*t*	1	NST: Ctr - SC3*t*	<0.001	SP: Ctr - SC3*t*	0.062
SC1: Ctr - GPS*t*	1	NST: Ctr - GPS*t*	0.003	SP: Ctr - GPS*t*	0.321
SC1: Ctr - NST*t*	1	NST: Ctr - NST*t*	0.003	SP: Ctr - NST*t*	0.499
SC1: Ctr - IBB*t*	0.749	NST: Ctr - IBB*t*	0.669	SP: Ctr - IBB*t*	1
SC1: Ctr - SP*t*	0.032	NST: Ctr - SP*t*	0.006	SP: Ctr - SP*t*	<0.001

Fig. 4: sympatric/allopatric combinations
A (respiratory burst) - body antigens: paired sample *t*-test, *n* fish = 154, *p* = 0.251
A (respiratory burst) - tegument antigens: paired sample *t*-test, *n* fish = 154, *p* = 0.896
B (number of viable cells) - body antigens: paired sample *t*-test, *n* fish = 155, *p* = 0.956
B (number of viable cells) - tegument antigens: paired sample *t*-test, *n* fish = 155, *p* = 0.014
C (G/L ratio) - body antigens: paired sample *t*-test, *n* fish = 155, *p* = 0.764
C (G/L ratio) - tegument antigens: paired sample *t*-test, *n* fish = 155, *p* = 0.752

Correlation between data and geographic distance (geo) respectively *S. solidus* prevalence differences (prev) between populations
Table shows sequential Bonferroni corrected *p*-values of Mantel-tests. For significant *p*-values the *r*-value is given.

geo/prev	assay	*b*/*t*	host/parasite	*p*-value	*r*-value
geo	respiratory burst activity	*b*	host	0.117	
geo	respiratory burst activity	*b*	parasite	0.166	
geo	respiratory burst activity	*t*	host	0.260	
geo	respiratory burst activity	*t*	parasite	0.422	
geo	number of viable cells	*b*	host	0.707	
geo	number of viable cells	*b*	parasite	0.152	
geo	number of viable cells	*t*	host	0.675	
geo	number of viable cells	*t*	parasite	0.864	
geo	G/L ratio	*b*	host	0.327	
geo	G/L ratio	*b*	parasite	0.968	
geo	G/L ratio	*t*	host	0.127	
geo	G/L ratio	*t*	parasite	0.065	
prev	respiratory burst activity	*b*	host	0.021	0.588
prev	respiratory burst activity	*b*	parasite	0.025	0.818
prev	respiratory burst activity	*t*	host	0.169	
prev	respiratory burst activity	*t*	parasite	0.144	
prev	number of viable cells	*b*	host	0.960	
prev	number of viable cells	*b*	parasite	0.092	
prev	number of viable cells	*t*	host	1	
prev	number of viable cells	*t*	parasite	0.896	
prev	G/L ratio	*b*	host	0.305	
prev	G/L ratio	*b*	parasite	0.950	
prev	G/L ratio	*t*	host	0.091	
prev	G/L ratio	*t*	parasite	0.170	