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Negative associations between parasite avoidance, resistance and tolerance predict host health in salmonid fish populations

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Abstract

Genetic variation in defence against parasite infections is fundamental for host-parasite evolution. The overall level of defence of a host individual or population includes mechanisms that reduce parasite exposure (avoidance), establishment (resistance) or pathogenicity (tolerance). However, how these traits operate and evolve in concert is not well understood. Here, we investigated genetic variation in and associations between avoidance, resistance and tolerance in a natural host-parasite system. Replicated populations of Atlantic salmon (*Salmo salar*) and sea trout (an anadromous form of brown trout, *Salmo trutta*) were raised under common garden conditions and infected with the eye fluke *Diplostomum pseudospathaceum*. We demonstrate significant genetic variation in the defence traits across host populations and negative associations between the traits, with the most resistant populations showing the weakest avoidance and the lowest infection tolerance. These results are suggestive of trade-offs between different components of defence and possibly underlie the genetic variation in defence traits observed in the wild. Because the three defence mechanisms affect host-parasite evolution in profoundly different ways, we emphasize the importance of studying these traits in concert.

Key words: avoidance, host defence, resistance, trade-off, trematode parasite, tolerance

Introduction

Genetic variation in the ability of hosts to defend against parasite infections is essential for the evolution of host-parasite interactions. Hosts can (i) prevent or reduce invasion by parasites (behavioural avoidance), (ii) reduce parasite load during or after establishment (resistance) and (iii) minimize damages caused by established infections (tolerance) [1-4]. These three branches of defence differ fundamentally so that avoidance and resistance have negative effects on parasites and thus select for counter-adaptations, while tolerance does not reduce parasite fitness [5, 6]. Consequently, the investment into different defence mechanisms can have profound consequences on host-parasite evolution [3, 4, 7]. However, most studies on parasite defence focus on one mechanism at a time, making conclusions on epidemiological and evolutionary dynamics difficult.

Much theory has focused on elucidating the phenotypic trade-offs between investment in defence and other fitness correlated host traits [8-11]. Most free-living animals are infected at some point of their life [12], showing that perfect defence is rare. In fact, theory predicts that the optimal level of defence includes accepting some risk of infection, the level of which is scaled by the costs of defence [8]. Given that these costs are often substantial [9, 10], trade-offs may also arise between defence traits. On the other hand, selection may favour positive associations between these traits for a highly effective defence. Both negative and positive associations between host defence traits have specific implications for host-parasite evolution. However, relationships between the defence traits in animals have been considered only in a handful of studies, mostly using laboratory host-parasite systems. For example, strains of *Drosophila* and laboratory colonies of house mice vary in resistance and tolerance, with more resistant strains being less tolerant [13, 14]. Positive and negative relationships have been demonstrated between behavioural parasite avoidance and resistance. Sheep that have been selected for resistance to gastrointestinal nematodes are also more effective in behaviourally avoiding these parasites than more susceptible selection lines [15]. On the other hand, house finches that avoid conspecifics exhibiting sickness behaviours, invest less into innate immune responses than conspecifics with weaker behavioural defences [16]. However, if and how avoidance, resistance and tolerance act in concert in a single system has not been unequivocally established [17].

Here, we study these three branches of host defence in replicated populations of closely related salmonid species, Atlantic salmon (*Salmo salar*) and sea trout (an anadromous form of brown trout, *Salmo trutta*), infected with their natural parasite, the trematode fluke *Diplostomum pseudospathaceum*. The eye fluke is a common freshwater parasite, which emerges in high numbers to the water column following asexual reproduction in the first intermediate aquatic snail host [18]. After contact with the second intermediate fish host, the parasite penetrates its epithelium and migrates through tissues to settle in the eye lens [18]. As the lens lacks blood circulation, the host immune system can attack the parasite only during migration, which typically takes no longer than 24h [18]. In the lens, the parasite induces cataracts, which can impact host vision and consequently fitness [19-21]. We first determined the behavioural avoidance of fish against the parasite, which can include two components. First, exposure risk increases with personality traits such as boldness or exploration [22-25], suggesting that different personality types could be favoured in environments with different infection risk [26, 27]. We refer to this as “indirect avoidance”. Second, fish are able to detect the presence of parasitic larvae in the water and respond to exposure by moving away [17, 28, 29] (referred here to as “direct avoidance”). In addition to the two behavioural variables, we measured resistance as parasite load in the eye following an experimental exposure, and tolerance as size of eye cataracts (degree of pathology in the lens) as a function of parasite load. We studied whether the defence traits show genetic variation among the host populations and explored associations between these traits for an overall view on host defence.

Methods

Fish and parasite origins

Five populations of Atlantic salmon and five populations of sea trout were obtained through a breeding programme of the Natural Resource Institute Finland (Luke). The populations originated from different river systems in Finland and Russia: the Rivers Ii, Neva, Oulu, Simo and Tornio for salmon and the rivers Ii, Ingarskila, Iso, Lesti and Musta for trout (see Table S1 for coordinates). Offspring were produced in 2016 using 40-189 parent fish per population and crossing each female with 1-5 males as well as each male with 1-5 females. The eggs were

reared at Kainuu Fisheries Research Station (KFRS, www.kfrs.fi) of the Natural Resource Institute Finland. For the purpose of other experiments, the populations were raised in replicated tanks with either standard or enriched (supplemented with gravel and shelters) rearing conditions as described in Hyvärinen and Rodewald [30]. For this study, individuals from the rearing treatments were combined in equal numbers for each population. Five months before the experiments, in February 2017, all individuals used in the experiment were marked with PIT tags (Biomark, HDX 12 mm) under light anaesthesia (MS-222, 100 mg l⁻¹). Subsequently, the fish species were kept separately, but the populations were maintained together in four 3.2 m² tanks for each species (total 8 tanks).

Infective larval stages (cercariae) of *D. pseudospathaceum* were obtained from 26 naturally infected *Lymnaea stagnalis* snails. The snails were collected 3-5 weeks before the experiments from Lake Vuojärvi (Central Finland, 62° N, 25° E). The parasite does not show a detectable genetic population structure in the snail populations across a large geographic scale [31], which is why the origin of the parasites was unlikely to affect the results. The snails were kept in 1 l of lake water at 4 °C and fed with lettuce *ad libitum*. To induce cercarial shedding, the snails were transferred to room temperature. Single genotype infections in the snails were verified by genotyping 20 haphazardly collected cercariae using four highly polymorphic microsatellite loci [Diplo06, Diplo09, Diplo23 and Diplo29; 32] as described in Louhi *et al.* [31].

Indirect avoidance (personality assays)

For the behavioural trials (indirect and direct avoidance), 480 individuals, 48 of each population, were transferred from KFRS to Konnevesi Research Station in June 2017. Fish were evenly distributed among four identical 180 l holding tanks supplied with lake water (17 °C). Each tank held either salmon or trout and equal numbers of individuals originating from different populations. Indirect avoidance was assessed through two personality traits, boldness and exploration. The assays were conducted twice with a time interval of 14-16 days in 120 x 20 x 20 cm flow-through tanks (24 l, see supplementary Figure S1) as described in Klemme *et al.* [33]. Briefly, fish were placed individually into a closed refuge at one end of the tank and after five minutes of acclimation, they were released and their behaviour recorded for 30 minutes with a video camera. An observer, who was blind to fish origin, scored: (1) the latency until the fish first emerged completely from the refuge, and for the subsequent 5 minutes (2) the number of

lines the fish crossed in the open area of the tank longitudinally divided into 5 equal zones [33]. Latency to emerge is related to boldness, measured as response to a novel environment with bolder individuals emerging earlier. Lines crossed represents exploration, measured as space use in a novel environment with more explorative individuals crossing more lines [see 33]. Nine individuals in the first round of the assays and two in the second round did not leave the refuge. These individuals were included in the analysis using the maximum score of 1800 seconds for (1) and the minimum score of 0 crosses for (2). An unexpected infection with the ectoparasitic flagellate *Ichthyobodo necator* resulted in the loss of 135 of 480 fish (28.1%) between the first and the second personality assay. All remaining individuals were treated for the infection and allowed to recover for eight days before the experiment continued. Thus, the total number of fish tested in both assays was 345, but due to technical problems with video recordings, data were available for 330 fish (see Table S2).

Direct avoidance (response to cercarial presence)

Direct parasite avoidance was assessed 5-10 days after the second personality test in choice preference assays between a compartment containing *D. pseudospathaceum* and a compartment without parasites [see 17, 29]. The tanks used for the personality assays were modified so that they had three compartments connected through round holes (see supplementary Figures S2). The fish were placed individually into the middle compartment with the connecting holes blocked. A solution containing lake water and approximately 1800 cercariae (combined from five snails) was introduced evenly into a randomly chosen outer compartment and a control solution of lake water without parasites to the other. After 10 minutes of acclimation, the connections were opened remotely and the behaviour of the fish was video recorded for 15 minutes. Earlier experiments have verified that cercariae remain well within their designated compartment for the duration of the experiment [17]. The time spent in each of the two choice compartments was scored from the videos, blind to compartment treatments and fish origin. Time spent in the compartment with parasites is strongly positively related to parasite load acquired during the test and therefore a good predictor of avoidance behaviour [17, 29]. Of the 284 individuals tested, six never left the middle compartment and 13 visited only one of the choice compartments. As these individuals did not exercise a true choice, they were excluded from the data, resulting in avoidance behaviour data for 265 individuals (see Table S2).

Resistance

Host resistance was quantified at KFRS as parasite load resulting from exposure to 300 cercariae of *D. pseudospathaceum*. Ninety-four fish from each population (total N = 940) were individually placed into containers with 1 l of lake water (14.5-15.0 °C) and exposed to one of seven parasite genotypes for 30 min. The total number of fish exposed to each parasite genotype varied between 80-180, depending on the cercarial output of each snail. Exposures were conducted in 2-3 blocks for each genotype (see Table S3 for details) and each block had the same number of fish from each population. Resistance was determined after 10 weeks (see estimation of tolerance) by euthanizing the fish (overdose of MS-222) and dissecting the number of parasites in the eye lenses of each fish under a microscope. Fish length was also recorded. During the 10 weeks, 39 fish were lost, resulting in resistance data for 901 individuals (Table S2). Further, 45 unexposed individuals (2-10 per population) were dissected to account for possible uncontrolled exposures from the tank water. This examination showed infection in one control individual by a single parasite, indicating that the level of uncontrolled infection was negligible.

Tolerance

Tolerance was assessed as the slope of the degree of pathology (eye cataracts) against parasite load following the experimental exposures described above. The proportional volume (10, 20, 30, ... 100 %) of the parasite-induced cataracts was determined using slit-lamp microscopy (Kowa SL-15) [34]. To account for size differences among fish individuals, the diameter of the eye lenses (in mm) was measured under the microscope and transformed to volume. Tolerance was assessed as reaction norm between the lens volume covered by cataracts (average of both lenses, mm³) and parasite load. Fifty-four of the 901 exposed fish (6.0%) that did not become infected and thus had no cataracts were excluded from the analysis of tolerance (Table S2).

Statistical analysis

Behavioural variables from the personality tests were log+1 transformed for data normality. Bolder individuals (short latency to emergence) were found to be also more explorative (more line crosses; round 1: $r = -0.404$, $p < 0.001$, round 2: $r = -0.393$, $p < 0.001$). Consequently, a

single personality variable ('personality score') capturing both boldness and exploration was produced for each round using principal component analysis (PCA), with negative values indicating relatively bolder and faster exploring individuals. Both PCAs retained one component with eigenvalue > 1 on which both behaviours loaded significantly (Table S4). The personality score was highly repeatable across the two tests for both species combined (Intraclass correlation coefficient ICC = 0.47, 95% CI = 0.34 – 0.57, $p < 0.001$), and separately for salmon (ICC = 0.41, 95% CI = 0.19 – 0.56, $p < 0.001$) and trout (ICC = 0.54, 95% CI = 0.37 – 0.56, $p < 0.001$). Individual mean personality scores were then calculated across both rounds of tests and used in subsequent analyses.

Variation in indirect and direct avoidance was analysed using general linear models. For indirect avoidance, personality score was entered as response variable (normal error, identity link), and fish species and population nested within species as factors. For direct avoidance, time spent in the compartment with parasites (in seconds) was fitted as response variable (negative binomial error, log link), species and population nested within species as factors, and the log-transformed total time spent in both outer compartments (parasite + control) as offset variable (denominator). This yielded proportional time spent in the parasite compartment, with the 95% confidence intervals outside 0.5 indicating significant avoidance or preference for the parasite compartment [35].

Variation in resistance and tolerance were analysed using mixed linear models, including parasite genotype as random factor. For resistance, parasite load was fitted as response variable (negative binomial error, log link), fish species and population nested within species as factors and fish length as covariate. For tolerance, cataract coverage (mm^3) was used as response variable (normal error, identity link) and fish species and population nested within species as factors. As tolerance is quantified as slope of a regression of host health against parasite load within a group of given host type [4], interactions between parasite load and fish species or population were included in the model. To test for a nonlinear relationship between cataract coverage and parasite load, the original model included also the quadratic term of parasite load [4]. However, this term was not significant and thus excluded from the final model. As cataracts were never observed in uninfected individuals, and all infected individuals had at least some cataracts, the intercept of the slopes was set at zero [17].

Associations among indirect avoidance, direct avoidance, resistance and tolerance were investigated using Spearman correlation on population means. Because the means and variances of the traits differed between salmon and trout, the species were analysed separately. However, for pairwise relationships that had the same direction (positive or negative) in both species, Fisher's meta-analysis [36] was used to combine the species-specific correlations. This analysis sums the ln-transformed one-tailed p-values of each correlation (2, 1 per species), multiplies it by -2 and compares the resulting value to a χ^2 distribution with $df = 4$ ($2 \times$ the number of tests). Data were analysed using SPSS (ICC and PCA, IBM statistics 24) and SAS 9.4 (linear models).

Results

Variation in defence traits

Personality scores were lower in salmon than in trout, indicating higher boldness and exploration, and lower indirect avoidance ($F_1 = 9.97$, $p = 0.002$, Figure 1A). Additionally, the populations within each fish species differed in indirect avoidance ($F_8 = 47.13$, $p < 0.001$, Figure 1A), suggesting significant variation in behavioural strategies of the populations.

There were also differences in the direct avoidance of the infection. Overall, the fish spent a lower proportion of time in the compartment with parasites compared to a random distribution (0.36, CI = 0.33-0.39, GLM, $\chi^2 = 616.51$, $p < 0.001$). However, salmon spent more time in the parasite compartment than trout, with two populations (Ii and Neva) showing no preference for either side, which indicates lower direct avoidance ($\chi^2 = 5.80$, $df = 1$, $p = 0.016$, Figure 1B). Populations of each species, on the other hand, did not differ in their proportional time spent in the parasite compartment ($\chi^2 = 6.82$, $df = 8$, $p = 0.556$, Figure 1B).

Experimental exposures indicated variation also in resistance and tolerance. Trout were more resistant than salmon ($F_{1, 883} = 100.89$, $p > 0.001$) and populations within each species differed significantly ($F_{8, 883} = 4.63$, $p > 0.001$, Figure 1C), suggesting genetic variation in resistance. Cataract volume was strongly positively associated with parasite load in all populations (Table 1, Figure 2A and B). However, a significant interaction between fish population and parasite load indicated genetic variation in infection tolerance as well (Table 1, Figure 2A and B).

Associations among defence traits

Among the correlations between different defence traits, the relationships between direct avoidance and resistance, and between resistance and tolerance, were negative in both species (Table 2). The combined correlations confirmed these negative associations (direct avoidance and resistance: $\chi^2 = 11.83$, $df = 4$, $p = 0.019$, Figure 3A; resistance and tolerance: $\chi^2 = 11.19$, $df = 4$, $p = 0.025$, Figure 3B). The relationship between direct avoidance and tolerance was positive in trout, but only weakly so in salmon (Table 2, Figure S3). Consequently, the combined relationship was not significantly positive ($\chi^2 = 7.06$, $df = 4$, $p = 0.133$). Indirect avoidance (personality) was positively associated with tolerance in both fish species, but the combined relationship was not significant (Table X, $\chi^2 = 6.02$, $df = 4$, $p = 0.198$, Figure S3). The relationships between indirect and direct avoidance, and between indirect avoidance and resistance, were weak and not significant (Table 2, Figure S3).

Discussion

Host defence against parasites includes several mechanisms that contribute cumulatively to host and parasite fitness. Very few studies, however, have taken more than one of the three key components of defence, avoidance, resistance and tolerance, into account in one host-parasite system. By using replicated populations of two salmonid species, we show genetic variation in host avoidance, resistance and tolerance against a natural parasite. Importantly, we demonstrate rare evidence for negative associations between these traits, with the most resistant populations showing the lowest avoidance and tolerance to infection. However, indirect avoidance was not related to the other defence traits, suggesting that selection on host personality is shaped by other factors.

Understanding genetic variation in parasite avoidance, resistance and tolerance is essential when considering the evolution of host defence strategies. In the simplest case, the degree of parasite-inflicted damage and subsequent reduction in host fitness is a direct function of parasite load, and the variation in damage corresponds to genetic variation in host ability to avoid or resist the infection. In addition, the extent of damages caused by a given parasite load

may vary among host populations if there is variation in host ability to withstand the infection (tolerance). Our data support all three mechanisms. Moreover, it appears that in populations with low resistance, hosts avoid the parasites better, but also the health consequences per parasite capita are less. This is consistent with the idea of trade-offs between the main arms of host defence, and implies variation in defence strategies among the populations. Given the overall high costs of defence due to trade-offs with other life-history activities [10, 37], such negative associations between defence traits may be common and are predicted by theory [7], although demonstrated only in a handful of studies in animal systems [13, 14, 16, 38]. These associations may have profound consequences on evolutionary interactions between hosts and parasites, because the relative investment in tolerance compared to avoidance and resistance will influence parasite fitness [6, 39]. This has also practical implications as it suggests that selection for high resistance, for example in livestock, may lead to a reduction in other defence traits [4]. Thus, our work emphasizes the importance of multidimensional approaches on host defence, not only for understanding host-parasite interactions, but also for managing diseases.

It is also possible that negative associations between defence traits result simply from functional redundancy with, for example, complete immune-mediated resistance making avoidance and tolerance unnecessary [40, 41]. Demonstrating trade-offs requires determining the actual fitness costs associated with each of the defence traits. While these costs have not been evaluated in the present system, evidence from other systems strongly supports costs associated with avoidance [42] resistance [9, 10] and tolerance [4, 41]. Moreover, none of the three defences in the present system is complete. Direct avoidance is likely triggered by physical stimuli caused by penetrating parasites [43] and thus, at least some larvae can enter the host before avoidance mechanisms are activated. Fish possess immune functions that reduce the infectivity of *D. pseudospathaceum* [18], but probably due to the short time window the immune system can act on the parasite, some parasites are able to establish in the eye. Finally, although we found evidence for tolerance decreasing the severity of cataracts, some cataracts still occurred (Figure 2). Thus, it could be expected that selection would favour all defence traits operating in concert to reduce the negative fitness effects of the infection.

Optimal investment in different forms of defence likely varies in different environments, because parasitism is typically structured in both space and time. This is well illustrated in amphibians, where species with low exposure risk owing to short spatiotemporal overlap with an

aquatic parasite, are showing stronger avoidance behaviour (low-cost), but weaker tolerance (high-cost) than species with high exposure risk [38]. In the present system, costs of direct avoidance in terms of time devoid of other important behaviours are likely to be low, but it may not be beneficial to invest into avoidance if exposure risk and parasite encounter rates are high. Similarly tolerance, which could be related to repair mechanisms such as tissue regeneration in the eye [44], should be most beneficial if infection risk, and consequently parasite loads, remain moderate as cataracts cannot be completely prevented. Resistance, on the other hand, may be cost-efficient only under high infection risk due to high maintenance and deployment costs [45, 46]. Particularly in the present system, efficient resistance should be rapidly deployed, as the immune responses can operate against the infection only for a short time. Although the natural levels of exposure in our experimental fish populations are unknown, differences in infection risk experienced by these populations in the past represents one possible explanation for the variation in allocation to different defence traits [8].

We also found that indirect avoidance, i.e. personality traits that are expected to affect parasite infection risk [26, 27], was not related to any of the other host defence traits. Recent studies have explored how history of host populations with parasites could affect their personality profiles, but evidence for parasite-mediated selection on personality remains so far inconclusive [47, 48]. It is possible that individual or cumulative effects of other biological and environmental factors such as predation pressure [49-51] or resource availability [52] have played a stronger role in the evolution of animal personalities. In the present system, personality does not appear to act as behavioural defence mechanism and the factors underlying the observed genetic variation in personality remain to be identified.

To conclude, we found that all studied traits showed significant genetic variation. As natural selection is expected to erode genetic variation in fitness-related traits [53], one central question in evolutionary biology has been to understand why such variation persists in natural populations [10, 13, 42]. Our results suggest that genetic correlations between different defence traits, arising for example from linkage or pleiotropy between involved genes, could contribute to the variation in host defence. Due to the differential impact of the defence traits (avoidance and resistance vs. tolerance) on parasite fitness, our results emphasize the importance of multidimensional approaches on host defence.

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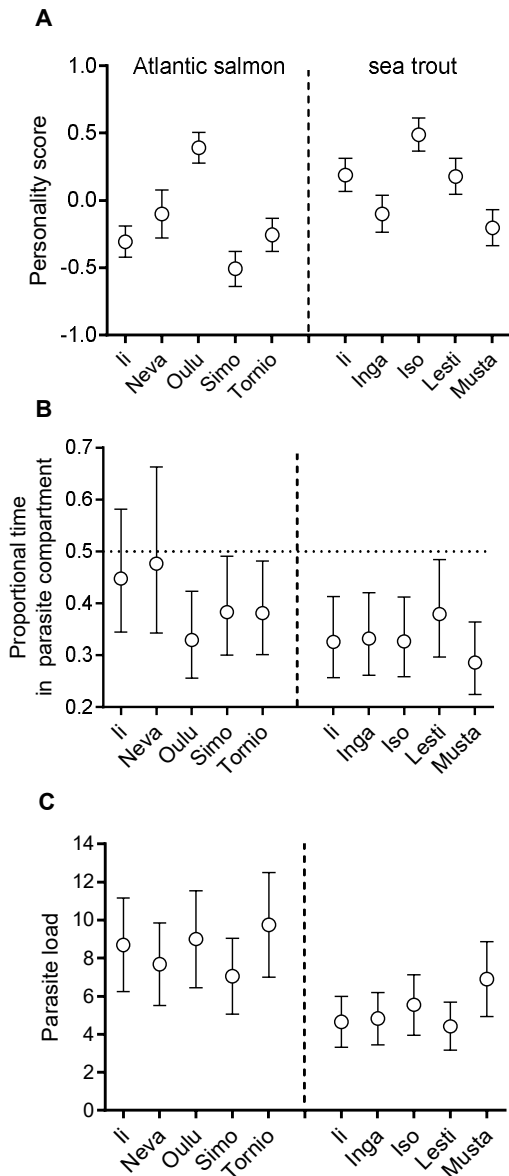
Table 1 Results of a mixed linear model on eye cataract coverage (mm³) caused by the trematode *D. pseudopathaceum* in five host populations of Atlantic salmon and five host populations of sea trout. Fish species, fish population nested within species and parasite load were used as factors, and parasite genotype as random factor.

Factor	<i>df</i> <i>numerator</i>	<i>df</i> <i>denominator</i>	<i>F</i>	<i>p</i>
Species	2	817	10.21	<0.001
Population(species)	8	817	1.34	0.218
Parasite load	1	817	1215.59	<0.001
Species × parasite load	1	817	2.64	0.104
Population(species) × parasite load	8	817	6.45	<0.001

Table 2 Spearman correlation coefficients for population level relationships between indirect avoidance (ind. avoidance = personality score), direct avoidance (proportional time in parasite compartment), resistance (parasite load) and tolerance (reaction norm between parasite inflicted damage and parasite load), separately for two host species (Atlantic salmon N = 5 populations, sea trout N = 5 populations).

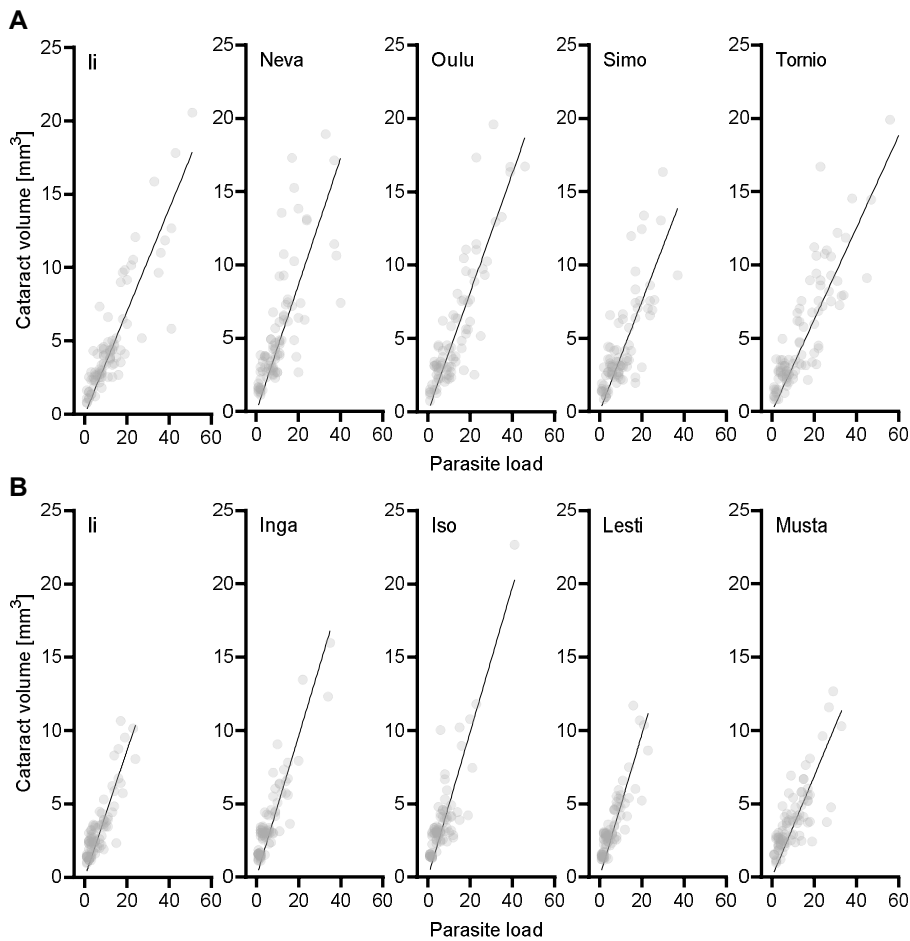
Salmon	Ind. avoidance	Direct avoidance	Resistance
Ind. avoidance			
Direct avoidance	-0.300		
Resistance	0.500	-0.600	
Tolerance	0.500	0.300	-0.500
Trout			
Ind. avoidance			
Direct avoidance	0.100		
Resistance	-0.300	-0.900	
Tolerance	0.400	0.700	-0.900

Figure 1



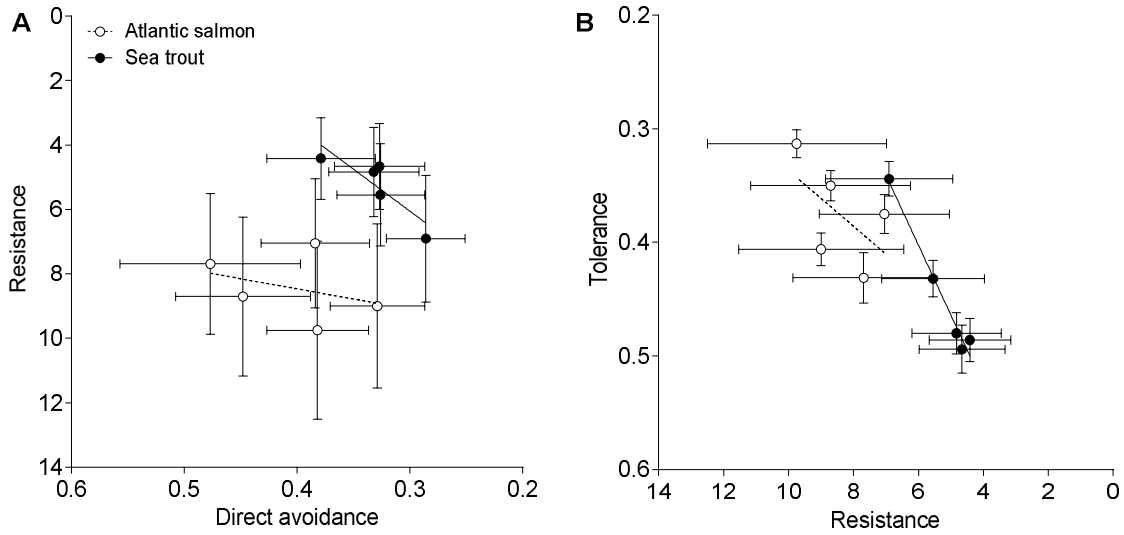
Variation in (A) personality score (mean \pm SE, indirect avoidance, negative values for relatively bold and fast exploring individuals), (B) proportional time in parasite compartment (mean \pm 95% CI, direct avoidance, CIs below 0.5 indicate significant avoidance) and (C) parasite load (mean \pm SE; resistance) for five populations of Atlantic salmon and five populations of sea trout infected with *D. pseudospathaceum* eye fluke.

Figure 2



Variation in tolerance expressed as slope of cataract volume (mm^3) against the load of *D. pseudospathaceum* for five populations of Atlantic salmon (A) and five populations of sea trout (B). Overlapping data are visualized as darker shade of the points. Fitted lines show linear regressions through the origin.

Figure 3



Associations between (A) direct avoidance and resistance and (B) resistance and tolerance to *D. pseudospathaceum* eye fluke infections in five populations of Atlantic salmon (open circles) and five populations of sea trout (filled circles). Fitted lines (dashed = salmon, solid = trout) are linear regressions to illustrate the direction of the relationships. Note that the axes have been reversed to facilitate interpretation, i.e. for direct avoidance (proportion of time spent in the parasite compartment), resistance (parasite load) and tolerance (slope of cataract volume against parasite load) lower values indicate higher performance.