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Examining the role of parasites in limiting unidirectional gene flow between lake and river sticklebacks

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Abstract

1. Parasites are important selective agents with the potential to limit gene flow between host populations by shaping local host immunocompetence.
2. We report on a contact zone between lake and river three-spined sticklebacks (*Gasterosteus aculeatus*) that offers the ideal biogeographic setting to explore the role of parasite-mediated selection on reproductive isolation. A waterfall acts as a natural barrier and enforces unidirectional migration from the upstream river stickleback population to the downstream river and lake populations.
3. We assessed population genetic structure and parasite communities over four years. In a set of controlled experimental infections, we compared parasite susceptibility of upstream and downstream fish by exposing laboratory-bred upstream river and lake fish, as well as hybrids, to two common lake parasite species: a generalist trematode parasite, *Diplostomum pseudospathaceum*, and a host-specific cestode, *Schistocephalus solidus*.
4. We found consistent genetic differentiation between upstream and downstream populations across four sampling years, even though the downstream river consisted of ~10% first-generation migrants from the upstream population as detected by parentage analysis. Fish in the upstream population had lower genetic diversity and were strikingly devoid of macroparasites. Through experimental infections, we demonstrated that upstream fish and their hybrids had higher susceptibility to parasite infections than downstream fish. Despite this, naturally sampled upstream migrants were less infected than downstream residents. Thus, migrants coming from a parasite-free environment may enjoy an initial fitness advantage, but their descendants seem likely to suffer from higher parasite loads.
5. Our results suggest that adaptation to distinct parasite communities can influence stickleback invasion success and may represent a barrier to gene flow, even between close and connected populations.

KEYWORDS

Diplostomum pseudospathaceum, ecological speciation, local adaptation, migration, parasite-mediated selection, reproductive isolation, *Schistocephalus solidus*, stickleback

1 | INTRODUCTION

Natural selection can foster rapid adaptive ecological divergence when different populations adapt to divergent resources or habitats (Rundle, 2000; Rundle & Nosil, 2005). While dispersal and gene flow can counteract local adaptation by disrupting adaptive genetic combinations, strong divergent ecological selection can also limit gene flow between diverging populations. Indeed, maladapted dispersing individuals may face decreased survival and reproductive isolation, through the establishment of reproductive barriers such as assortative mating and hybrid inviability or inferiority (Lenormand, 2002; Nosil, Vines, & Funk, 2005; Rundle & Nosil, 2005). Evaluating which ecological factors limit gene flow and contribute to reproductive isolation between diverging habitats can shed light onto the ecological speciation process (Nosil et al., 2005; Rundle & Nosil, 2005), and growing evidence indicates that, aside from specialization on resources and abiotic habitat features (Hendry, Taylor, & McPhail, 2002; Nosil & Crespi, 2004; Via, Bouck, & Skillman, 2000), parasites may be one of these factors (El Nagar & MacColl, 2016; Kaufmann, Lenz, Kalbe, Milinski, & Eizaguirre, 2017; MacColl & Chapman, 2010; Raeymaekers et al., 2013).

Parasite infections exert a strong but heterogeneously distributed selection force on hosts and are a potential source of diversifying selection between habitats (Karvonen & Seehausen, 2012; Wilson et al., 2002). Host immune defences are shaped by host–parasite interactions but are costly and impose trade-offs in energy allocation on other life-history traits, such as growth or reproduction (Lochmiller & Deerenberg, 2000; Schmid-Hempel, 2003). Further, indirect costs are linked to immunopathology (e.g. oxidative stress) and autoimmune diseases (Graham, Allen, & Read, 2005). Hence, under a sustained reduction of parasite pressure, the selection on host resistance is expected to relax and resistance might decrease or be lost (Duncan, Fellous, & Kaltz, 2011; Keogh, Miura, Nishimura, & Byers, 2017). Thus, host immunocompetence, that is the ability to overcome or cope with potential parasite infections, may vary through local adaptation and influence the success of migrants in new habitats, shaping host dispersal patterns and gene flow (Schmid-Hempel & Ebert, 2003).

The three-spined stickleback (*Gasterosteus aculeatus*) is an excellent model to study the role of ecological factors in shaping population divergence (Reusch, Wegner, & Kalbe, 2001b; Rundle & Nosil, 2005). Following the last glaciation, marine sticklebacks have rapidly colonized and adapted to freshwater habitats across the Northern Hemisphere, as demonstrated by radiations into various stickleback ecotypes (see review by Hendry, Bolnick, Berner, & Peichel, 2009). In particular, lake and river stickleback populations experience different parasite pressures, which appears to reinforce reproductive barriers by decreasing migration success and gene flow between neighbouring populations (Eizaguirre et al., 2011; Feulner et al., 2015; Kaufmann et al., 2017; MacColl & Chapman, 2010; Reusch, Wegner, et al., 2001b; Scharsack, Kalbe, Harrod, & Rauch, 2007a).

Here, we focused on a lake–river system in which a waterfall creates the conditions for unidirectional migration of sticklebacks from a macroparasite-free, upstream river site towards a highly parasitized, downstream lake population. Given the natural movement of individuals from one parasitization extreme to another, we could test the role of parasites in selection against migrants. We hypothesized that the absence of macroparasites in the upstream population would lead to low host immunocompetence, which would subsequently result in higher infection levels in migrants moving into the parasite-rich lake habitat. Over four sampling years, we assessed gene flow and parasite pressure between these populations. We also experimentally infected laboratory-bred fish with two parasites, the generalist trematode *Diplostomum pseudospathaceum* and the host-specific cestode *Schistocephalus solidus*. We tested whether (a) gene flow was occurring from upstream to downstream populations; (b) whether parasite communities differed across sites and were stable over time; and (c) whether the upstream population differed in its resistance to parasite infections.

2 | MATERIALS AND METHODS

2.1 | Field sampling and parasite screening

Over four years (2009, 2010, 2012 and 2013) in September, we sampled three-spined sticklebacks from the lake Skogseidvatnet and its headwater the river Orraelva (Hordaland, Fusa, Norway, 60°14′38″N, 05°54′51″E; Figure S1). A six-metre waterfall at approximately 1.5 km upstream of the lake creates a natural barrier to migration from the lake up the river. We characterized three distinct sampling sites: the river above the waterfall (RA), the river below the waterfall (RB) and the lake (L). Each year, sticklebacks (1+ years old) were caught with unbaited minnow traps or dipnets, killed with an overdose of MS222 (tricaine methanesulfonate, 1 mg/ml) and sampled for DNA. We dissected a subset of the captured fish; most fish were dissected shortly after capture in Norway, but some were transferred alive, transferred frozen or frozen after being transferred alive (hereafter refer to as dissection protocol) before being processed at the Max Planck Institute for Evolutionary Biology (Plön, Germany). For each fish, we recorded standard body length (tip of rostrum to tip of caudal peduncle, ±1 mm), body weight and weight of all internal organs (±0.1 mg). We screened eyes and all inner organs for macroparasites, according to a standard protocol (Kalbe, Wegner, & Reusch, 2002). In total, 663 fish were collected and genetically analysed, 420 fish had their inner organs weighed, and 389 out of 420 fish were screened for parasites (Tables S1 and S2).

2.2 | Fish genotyping

We extracted genomic DNA from caudal fins using the DNeasy® 96 Blood & Tissue Kit (Qiagen, Germany). To assess population genetic structure and gene flow among the three sampling sites and across years, we estimated genetic variation at nine neutral microsatellite loci (*Gac1097*, *Gac1125*, *Gac4170*, *Gac5196*, *Gac7033*, *STN18*, *STN32*, *STN75* and *STN84*; Largiadèr, Fries, Kobler, & Bakker, 1999; Peichel et al., 2001). We amplified microsatellite loci in two multiplex

polymerase chain reactions (Kalbe et al., 2009) and performed fragment analysis with GeneMarker 1.95 (SoftGenetics). We used MICRO-CHECKER 2.2.3 (Van Oosterhout, Hutchinson, Wills, & Shipley, 2004) to check for the presence of genotyping errors and null alleles.

2.3 | Population genetic diversity, differentiation and Bayesian structure

We assessed genetic diversity and differentiation between sampling sites for each sampling year by calculating the number of alleles per locus (*A*), allelic richness (A_R; allel.rich function, PopGenReport library in R; Adamack & Gruber, 2014), observed heterozygosity (H_o), expected heterozygosity (H_s) and pairwise F_{st} values using ARLEQUIN 3.5 (Excoffier & Lischer, 2010), and identified private alleles using CONVERT 1.31 (Glaubitz, 2004). We tested all microsatellite loci for neutrality within each sampling site using default parameters on LOSITAN (Antao, Lopes, Lopes, Beja-Pereira, & Luikart, 2008), and for deviations from Hardy–Weinberg equilibrium and linkage disequilibrium at each sampling site for each year using GENEPOP 4.2 on the Web (Rousset, 2008). We performed genetic clustering for each survey year independently and for all years combined using STRUCTURE 2.3.4 (Pritchard, Stephens, & Donnelly, 2000). We ran the admixture model, with 100,000 burn-in followed by 1,000,000 Markov chain Monte Carlo simulations and 20 iterations for each *K* (*K* = 1–13 for the complete dataset or *K* = 1–4 for a given sampling year). Results were retrieved using STRUCTURE HARVESTER (Earl & VonHoldt, 2012), CLUMPP 1.1.2 (Jakobsson & Rosenberg, 2007) and DISTRUCT (Rosenberg, 2003). We used GENECLASS2 (Piry et al., 2004) to detect putative first-generation migrants. We used NEWHYBRIDS 1.0 (Anderson & Thompson, 2002) to assess admixture by assigning individuals to six hybrid or parental categories: pure upstream, pure downstream, F1, F2 and both backcrosses. The individual-specific option (*z*) was used to specify that individuals sampled in the RA sampling site were of pure upstream genotype. We estimated recent migration rates with BAYESASS (Wilson & Rannala, 2003), using adjusted mixing parameters (migration rate prior *m* = 0.05, inbreeding coefficient prior *f* = 0.35) to reach optimal acceptance rates, 1,000,000 burn-in and 10,000,000 iterations.

2.4 | Fish laboratory-breeding

To test for differences in parasite resistance between Skogseidvatnet fish populations, wild fish from RA and L sampling sites were caught in September 2012, brought to the laboratory, and kept under standardized conditions. Pure river (RAxRA) and pure lake (LxL) fish families were generated in spring and summer 2013. The same parents were also used to generate reciprocal hybrid families RAxL (river mothers) and LxRA (lake mothers). Hereafter, we refer to the classes of laboratory-bred fish (RAxRA, LxL, RAxL, LxRA) as "genetic types" and to the hybrid families as "RA-" or "L-maternal hybrid" (RAxL and LxRA, respectively). In separate experiments, these fish were exposed to two flatworm parasites, a generalist trematode and a specialist cestode.

2.5 | Experimental infection with *Diplostomum pseudospathaceum*

The generalist trematode *Diplostomum pseudospathaceum* (Kalbe & Kurtz, 2006) infects fish via free-swimming larvae (cercariae), which then migrate to the eye lens within 24 hr, where they can escape the immune system and develop into metacercariae (Whyte, Secombes, & Chappell, 1991). The number of metacercariae in the eye lens serves as a proxy for the innate immunity efficacy (Scharsack & Kalbe, 2014). For each RAxRA, LxL, RAxL and LxRA fish family, 9–18 fish were individually exposed to 100 cercariae pooled from five naturally infected *Lymnaea stagnalis* snails from northern Germany in November 2013 (see Appendix S1, Table S3). At 4 weeks post-exposure, fish were killed with an overdose of MS222 and dissected. Metacercariae were counted in each eye lens.

2.6 | Experimental infection with *Schistocephalus solidus*

The cestode *Schistocephalus solidus* is specific to three-spined sticklebacks. It grows to an enormous size in the fish's body cavity before being eaten by the final bird host. Within about two weeks of exposure, the innate immune system can clear infections (Scharsack, Koch, & Hammerschmidt, 2007b). Once parasites do establish, their growth is affected by the host's allelic diversity at the major histocompatibility complex (MHC) genes, which are central to adaptive immunity (Kurtz et al., 2004).

We used six *S. solidus* individuals from naturally infected three-spined sticklebacks in Lake Skogseidvatnet to produce three independent *S. solidus* laboratory-bred families in an in vitro system (Smyth, 1946; Wedekind, 1997). Laboratory-cultured copepods (*Macrocyclops albidus*) were exposed to one tapeworm larvae each (for details, see Scharsack, Koch, et al., 2007b) and microscopically checked for infection. After allowing worms to fully develop in copepods for 18 days, each fish was exposed to a single worm larva. To minimize family effects, we balanced the exposure of six fish families (three RAxRA families and three LxL families) to three worm families (*n* = 8–12 fish for each combination). As controls, 8–10 fish from each family were handled similarly to exposed fish but not fed any infected copepod (Table S4). In this experiment, we only used pure RA and L fish, as there were insufficient numbers of RAxL and LxRA hybrids for infection. At 8 weeks post-exposure, fish were killed with an overdose of MS222 and dissected. The recovered worms were weighed. Plerocercoid weight relative to fish somatic weight was used as a measure of *S. solidus* virulence and host adaptive immunity efficacy (Kurtz et al., 2004).

2.7 | Proxies for fish condition and immuno-status

Fish weight relative to length was used as a proxy for general fish condition (García-Berthou, 2001). We also examined organ weight, relative to fish weight, for three organs (liver, spleen and head kidney). Liver size is a predictor of energy reserves (Wootton, Evans, &

Mills, 1978). An enlargement of the two major fish immune organs, spleen and head kidney, can indicate immune activation in response to parasite infections (Macnab, Katsiadaki, & Barber, 2009).

2.8 | Statistical analyses

All analyses were carried out using R 3.5.1 (R Core Team, 2018). We summarized the parasites communities observed in the field survey by calculating the Shannon diversity index for each individual fish (diversity function, vegan library; Oksanen et al., 2019). Fish harbouring more parasite species and more parasite individuals have higher Shannon values. We built a series of linear models to test hypotheses. We started with a model that included variables that were not of interest but that we wished to control for, like fish sex, sampling year and dissection protocol. Then, we added the following terms in succession and compared models with likelihood-ratio tests: site (to compare river and lake fish, as well as those above and below the waterfall), the interaction between site and year (to test whether site differences are stable) and fish genotype (to examine whether migrants have more parasites than residents). Genotypes were determined in the population genetic analyses (see Sections 3.1–3.3.), and three clusters were defined: upstream, downstream and admixed. However, each genotype was not found in every sampling site; only five combinations of site and genotype were represented: (a) upstream genotype residing in RA, (b) upstream genotype migrating into RB, (c) downstream genotype residing in RB, (d) downstream genotype residing in L, and (e) admixed genotype residing in RB and L. Therefore, we could not test a full genotype by site interaction. Instead, when we add genotype to the model, we are testing whether admixed genotypes and migrants from above to below the waterfall have different parasite communities than the genetic residents.

The Shannon index, in addition to being prone to zero inflation (Figure S2), does not tell us which parasite species differ between sites or genotypes. Therefore, we also took a multivariate approach, in which the abundance of each parasite species was simultaneously modelled with negative binomial regressions (manyglm function, mvabund library; Wang, Naumann, Wright, & Warton, 2012). The model terms and comparisons were the same as above.

Condition and immune status proxies from the field study were also assessed with linear models. The same model-building approach was used except that the initial models included a size covariate. In the case of fish somatic weight (excludes gonads and *S. solidus* weights), the covariate was fish standard length, while for organs, it was fish somatic weight. Fish length, weight and organ weights were log-transformed to meet normality and homoscedasticity assumptions. The condition and immune proxies for fish from the two experimental infections were analysed using the same size covariates and log transformations. However, the condition analyses are not completely comparable between field and experimental fish, as model structures differed (e.g. family effects were included in experiments but not the field).

In the *D. pseudospathaceum* experiment, we compared parasite abundance between river (RAxRA), lake (LxL) and hybrid (RAxL, LxRA) fish. We used a generalized mixed effect model with Poisson errors, sex and genetic type as fixed effects, and maternal and paternal identity as random effects (glmer function, lme4 library; Bates, Mächler, Bolker, & Walker, 2015). In the *S. solidus* exposure, we used linear mixed effect models to test for the effect of the fish genetic type (RAxRA or LxL) on worm weight. We included sex as a fixed factor, and fish family and *S. solidus* family as random factors. We used a general mixed effect model with binomial family, and sex and fish family as random effects to assess the effect of the genetic type on the proportion of infected fish. Tukey *post hoc* tests were used for *post hoc* comparisons (lsmeans function in R, lsmeans library; Lenth, 2016).

All data and codes are available from the Dryad Digital Repository.

3 | RESULTS

3.1 | Fish above the waterfall exhibit lower genetic diversity

Microsatellite loci showed an average of 3, 19 and 18 alleles per loci in RA, RB and L, respectively. Genetic diversity was extremely reduced in RA, with half the heterozygosity (0.47 on average) observed in RB or L. The upstream population (RA) shared all its alleles with the downstream populations (RB and L) and those alleles were among the most commonly found alleles in the downstream population (Figure S3). By contrast, rare alleles were found in several loci for both RB and L (RB: 17 private alleles in 7 loci; L: 10 private alleles in 6 loci; Table S5).

Within each sampling site and for each year, no locus deviated significantly from neutrality or from Hardy–Weinberg equilibrium after Bonferroni correction except for *STN32* in RB2012 and RB2013, and for *STN75* in RA2009 and RB2013 (Table S6). A few locus pairs in RB2010, RB2012, RB2013 and L2012 showed genotypic linkage disequilibrium. Loci *Gac7033*, *STN32* and *STN75* in RA and RB presented signs of homozygote excess and potential null alleles. We investigated whether the problem loci *Gac7033*, *STN32* and *STN75* might bias the analysis by running population genetic differentiation and STRUCTURE analyses with and without these three loci. Both analyses showed similar results. We will hereafter present the results of the analysis with all nine loci.

3.2 | Upstream and downstream populations are genetically different

While the RA population was consistently and significantly differentiated from both RB and L, the latter two did not diverge in pairwise F_{ST} estimates (Figure S4). Bayesian population structure analysis indicated two source populations (K = 2). Each year showed a consistent pattern where the upstream (RA) and downstream fish (RB and L) formed two distinct clusters with a few migrants from the RA cluster found in RB but not in L (Figure S5). The four sampling years combined revealed the same result (K = 2; Figure 1). We refer to these genetic clusters as upstream and downstream clusters.

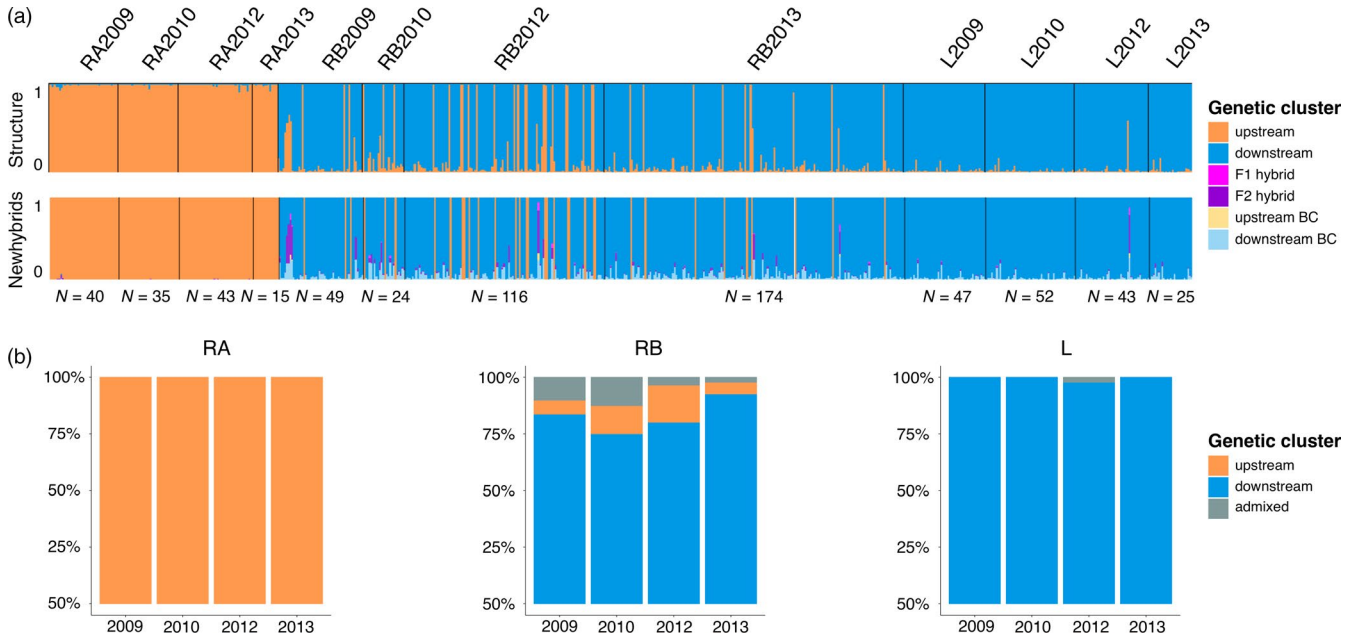

FIGURE 1 (a) STRUCTURE and NewHybrids assignments using microsatellite data from three sampling sites (RA, RB and L) of three-spined sticklebacks over four survey years (2009, 2010, 2012, 2013). STRUCTURE: Two genetic clusters ($K = 2$) correspond to the upstream population (in orange) and the downstream population (in blue). The *y*-axis shows individual probabilities of assignment grouped by sampling populations. NEWHYBRIDS: the *y*-axis shows individual posterior probability of assignment to four classes (pure upstream, pure downstream, F1 hybrid, F2 hybrid, upstream backcross BC, downstream backcross BC). (b) Proportion of individuals from each genetic cluster in sampling sites over four survey years

3.3 | Migration is unidirectional

Using a threshold for cluster membership coefficient $Q = 0.2$ to interpret the STRUCTURE analysis (Vähä & Primmer, 2006), individuals could be split into three groups: upstream population ($Q = 0.9159–0.9960$), downstream population ($Q = 0.0030–0.1994$) and admixed individuals ($Q = 0.2156–0.6551$). All fish sampled above the waterfall belonged to the upstream cluster: no fish from the downstream cluster and no admixed fish were found above the waterfall. Each year, between 5.2% and 16.4% (10.0% ±5.3 fish on average over four sampling years) of fish sampled in RB belonged to the upstream population and were identified as first-generation migrants using GENECLASS2. Similar results were obtained with BAYESASS with an estimated 4.6% migration rate overall years from RA into RB (Table S7), and the same individuals being identified as first-generation migrants. In total, 34 of 363 fish sampled in RB were putative first-generation migrants from the upstream population (Table S1). No fish from the upstream population were identified further downstream in the lake. Seventeen fish had admixed genotypes (RB: 16; L: 1; Table S1) with high likelihood to belong to both upstream and downstream populations. This suggests limited admixture between the upstream migrants and downstream fish. NEWHYBRIDS categorization of the pure upstream and downstream population clusters was congruent with the STRUCTURE analysis (pure upstream cluster: $p = 0.934–1.000$; pure downstream cluster: $p = 0.679–1.000$, with 462 out of 479 fish exceeding 0.800). All but one of the 17 individuals identified as admixed in STRUCTURE were not assigned to one specific genotype class in NEWHYBRIDS ($p < 0.75$). Instead, the probability of those individuals to belong to a given genotype frequency was spread across pure downstream ($p = 0.059–0.709$), F2 hybrids ($p = 0.096–0.530$) and downstream backcross ($p = 0.160–0.284$). One individual sampled in RB in 2010 was classified as admixed in STRUCTURE and pure downstream in NEWHYBRIDS ($p = 0.789$).

3.4 | Parasite pressure differs between sites, but migrants are not more severely infected

We found upstream fish in RA to be consistently devoid of macroparasites, whereas fish in downstream sites RB and L harboured a diverse parasite community with four species of cestodes, four trematodes and two nematodes (Figures 2 and S6, Tables S8 and S9). The RA site therefore clearly differed from the other sites (Figure 2a). To ensure site differences were not exclusively caused by the RA site, we excluded it from the analysis. The Shannon index was significantly affected by site (LR test, $\chi^2_1 = 6.020$, $p < 0.001$; Figure 2a); lake fish had higher parasite diversity than fish in the river below the waterfall (coefficient = −0.412, $p < 0.001$). This pattern held for 3 of the 4 sampling years but was reversed in 2009, resulting in a significant site by year interaction (LR test, $\chi^2_3 = 2.486$, $p = 0.001$). Adding fish genotype to the model was also an improvement (LR test, $\chi^2_2 = 2.491$, $p < 0.001$), as upstream migrants had lower parasite diversity than downstream river residents (coefficient = −0.355, $p = 0.008$; Figure 2b). Admixed fish exhibited a similar level of parasite diversity to downstream residents (coefficient = −0.049, $p = 0.675$).

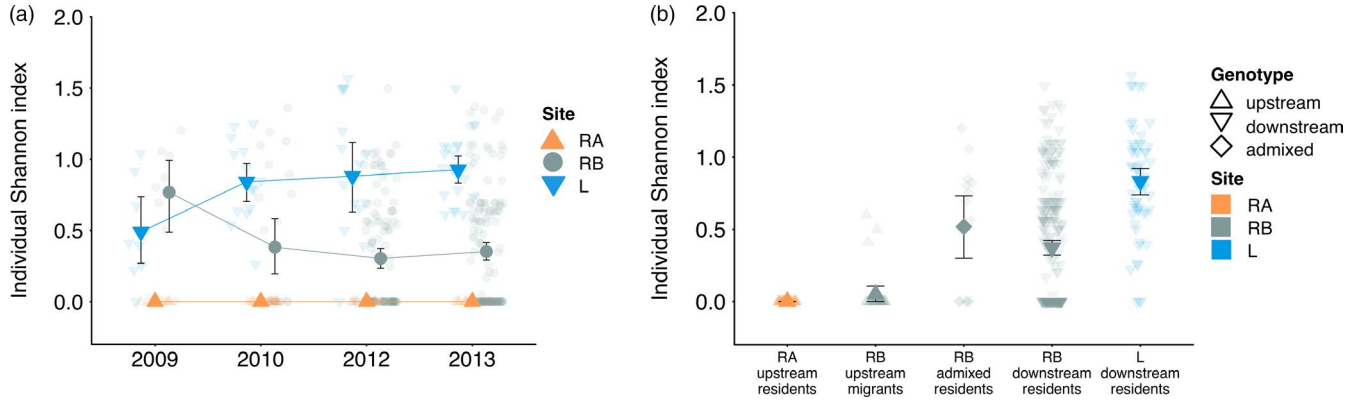

FIGURE 2 Individual Shannon index for the different genotypes found in the field (a) across four survey years (2009, 2010, 2012 and 2013) and (b) over all years. The *x*-axis designates the site (RA, RB or L), the genetic cluster (upstream, downstream or admixed) and the migration status (migrant or resident) of the genotype (see Table S2 for sample sizes). Means with error bars (±95% CI)

A multivariate approach yielded similar results; we found significant effects of site (manyGLM, Wald = 9.559, *df* = 1, $p = 0.001$) and genotype (Wald = 8.074, *df* = 2, $p = 0.001$), although the site by year interaction was no longer significant (Wald = 3.652, *df* = 1, $p = 0.146$). The common parasite species (*Apatemon* sp., *Diplostomum* sp. and *Diphyllobothrium* sp.) showed the same pattern of differences across genotypes (Figure S7), indicating these genotype differences are not caused by a particular parasite species.

3.5 | Upstream fish are more susceptible to experimental infections with two parasites

Laboratory-bred upstream fish were more susceptible than downstream fish to *D. pseudospathaceum* and *S. solidus*. All fish exposed to *D. pseudospathaceum* were infected with at least one metacercaria, but the mean abundance significantly differed among genetic types (LR test, $\chi^2_2 = 11.736$, $p = 0.003$; Figure 3a): RA fish had more parasites than L fish (Tukey's HSD, $p = 0.006$) or hybrids (Tukey's HSD, $p = 0.002$). The two maternal hybrid lines did not significantly differ from each other (ANOVA, $F_3 = 16.122$, $p = 0.001$; Tukey's HSD, $p = 0.768$) and were combined. Hybrids had intermediate numbers of parasites compared to pure families (Hybrids vs. RA: Tukey's HSD, $p = 0.002$; Hybrids vs. L: Tukey's HSD, $p = 0.006$; Figure 3a).

RA fish were about twice as likely to become infected with *S. solidus* than L fish (43.7% vs. 20.7% of infected fish; LR test, $\chi^2_1 = 7.230$ $p = 0.007$; Figure 3b). Worms infecting RA fish grew on average one and a half time bigger than those infecting L fish (112.6 mg, range = 62.6–176.7; 95.0 mg, range = 40.9–147.6; ANOVA, $F_{1,46} = 16.003$, $p < 0.001$; Figure 3c).

3.6 | Proxies for condition and immuno-status differ between sites

A detailed summary of the fish condition and organ weight analyses is in the supplementary material (Appendix S2, Figure S8, Tables S10–S12). Here, we report only the main results.

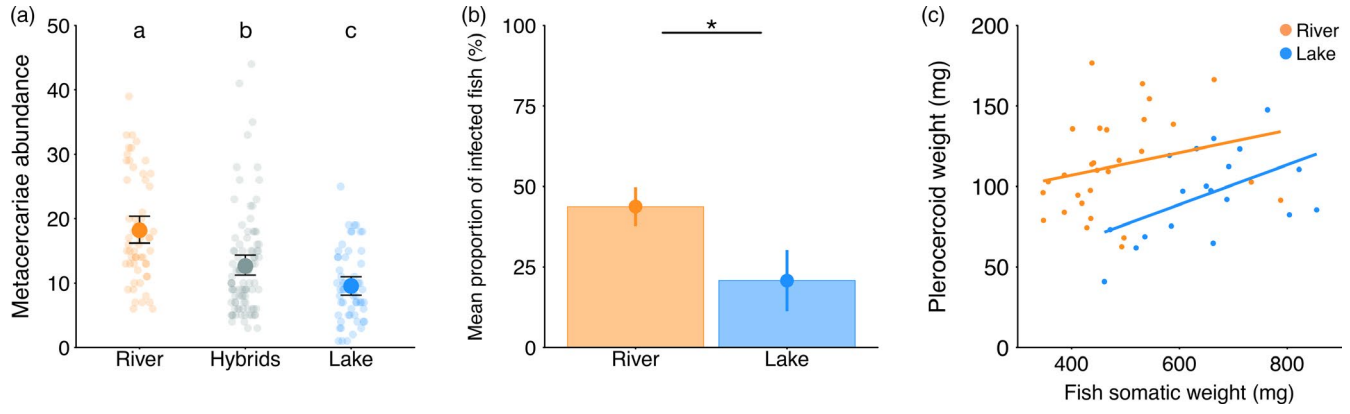

FIGURE 3 Results of the experimental infections for the different Skogseidvatnet laboratory-bred fish genetic types (River: "River Above" RAxRA, Hybrids: "River Above" maternal hybrids RAxL and lake maternal hybrids LxRA, combined, and Lake: lake LxL). (a) Abundance of *Diplostomum pseudospathaceum* metacercariae in the eye lenses. (b) Mean proportion of fish infected with *Schistocephalus solidus* per fish family and (c) *S. solidus* plerocercoid weight as a function of fish somatic weight. Means with error bars (±95% CI); letters and asterisks are significantly different (Tukey's HSD, $p < 0.050$; see Tables S3 and S4 for sample sizes)

Fish condition and relative organ weights differed between sites (LR tests for body condition: $\chi^2_2 = 0.075$, $p < 0.001$; liver: $\chi^2_2 = 0.201$, $p < 0.001$; spleen: $\chi^2_2 = 8.869$, $p < 0.001$; head kidney: $\chi^2_2 = 0.495$, $p = 0.001$). In particular, fish above the waterfall tended to be heavier (both relative to their length and in absolute values; coefficient = 4.628e−02, $p < 0.001$), have slightly smaller livers (coefficient = −0.079, $p < 0.001$), substantially smaller spleens (coefficient = −0.473, $p < 0.001$), and slightly smaller head kidneys (coefficient = −0.109, $p = 0.002$) than fish below the waterfall and in the lake.

Two observations suggest these site differences are environmental, not genetic. First, differences varied from year-to-year for body (LR test, $\chi^2_6 = 0.040$, $p = 0.001$) and liver weights (LR test, $\chi^2_6 = 0.227$, $p = 0.003$), although not for spleen (LR test, $\chi^2_6 = 0.188$, $p = 0.681$) and head kidney weights (LR test, $\chi^2_6 = 0.178$, $p = 0.562$). Second, the patterns were not reproduced in the laboratory-raised fish. For example, in both experiments, RA fish had larger spleens than L fish (*D. pseudospathaceum*: ANOVA, $\chi^2_2 = 22.943$, $p < 0.001$; *S. solidus*: LR test, $\chi^2_1 = 3.625$, $p = 0.057$), which is the opposite of what was observed in the field-collected fish.

3.7 | Migrants are not in worse condition

Genotype effects on condition were neither strong nor consistent in the field survey (LR tests for body condition: $\chi^2_3 = 0.012$, $p = 0.085$; liver: $\chi^2_3 = 0.186$, $p = 0.001$; spleen: $\chi^2_3 = 0.065$, $p = 0.716$; head kidney: $\chi^2_3 = 0.060$, $p = 0.648$; Figure 4). Migration from RA to RB did not seem to worsen fish condition. On the energetic measures (body and liver weight), upstream migrants resembled upstream residents, while on the immune measures (spleen and head kidney weight), they looked more like the downstream residents. This was also true for the admixed genotypes, except that they had relatively large livers.

4 | DISCUSSION

In this study, we identified two distinct genetic clusters, one upstream and one downstream of a waterfall, and these clusters were consistent across 4 years. While it is not surprising that the upstream population, separated by a natural barrier, was genetically isolated

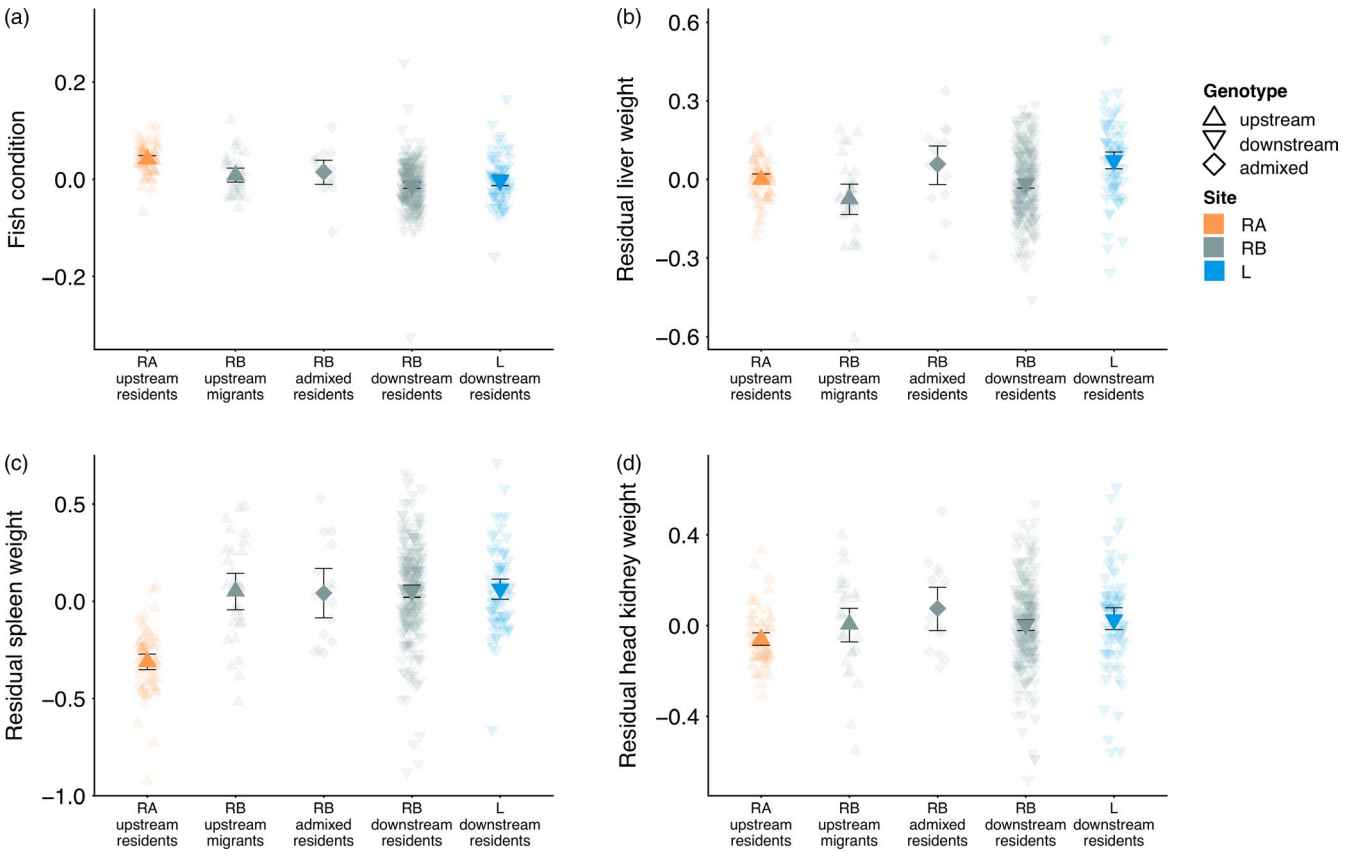

FIGURE 4 Fish condition and immune status proxies for the different genotypes found in the field over all years (a) fish condition, (b) liver weight, (c) spleen weight and (d) head kidney weight. Residuals of log-transformed organ and body weights from linear models including the effects of sex, dissection protocol, and as size covariate, log-transformed fish standard body length (fish condition) or fish somatic weight (liver, spleen, head kidney). The *x*-axis designates the site (RA, RB or L), the genetic cluster (upstream, downstream or admixed) and the migration status (migrant or resident) of the genotype (see Table S2 for sample sizes). Means with error bars (±95% CI)

from the rest of the system and presented a reduced genetic diversity (Crispo, Bentzen, Reznick, Kinnison, & Hendry, 2006; Paz-Vinas, Loot, Stevens, & Blanchet, 2015), it is striking that the downstream population stayed genetically distinct despite a continuous influx of upstream fish. We detected unidirectional migration from the upstream to the downstream population, as evidenced by 10.0% putative migrants on average. Yet, introgression appeared limited. Only 4.4% of fish had admixed genotypes, suggesting that hybridization occurs rarely. This low rate of admixture and the maintenance of genetic differentiation over time contrast with other river–lake contact zones (Berner, Grandchamp, & Hendry, 2009; Hanson, Moore, Taylor, Barrett, & Hendry, 2016). Instead, the level of admixture is comparable with the one found in reproductively isolated stickleback ecotypes, indicating that unidirectional gene flow was insufficient to alter downstream allele frequencies (Gow, Peichel, & Taylor, 2006, 2007; Jones, Brown, Pemberton, & Braithwaite, 2006; Taylor et al., 2006).

These results are consistent with reproductive isolation observed in other unilateral contact zones between ecologically diverging stickleback populations (Caldera & Bolnick, 2008; Ravinet et al., 2015). Many ecological factors could act to restrict gene flow from upstream to downstream, such as resource quality or competition, higher conspecific density, predation pressure and other abiotic factors (Bolnick, 2004; Crispo et al., 2006; Glover et al., 2012; Rundle, Vamosi, & Schluter, 2003; Svanbäck & Bolnick, 2007). We specifically looked at one striking ecological variable, the divergent macroparasite communities. Over 4 years, the upstream population was consistently devoid of macroparasites, whereas both downstream populations were dependably infected with parasites. Natural populations of three-spined sticklebacks have been extensively surveyed for macroparasites (Kalbe et al., 2002; Karvonen, Lucek, Marques, & Seehausen, 2015; Poulin, Blanar, Thieltges, & Marcogliese, 2011), and although river sticklebacks generally have fewer macroparasites than lake populations (Feulner et al., 2015; Karvonen et al., 2015; Scharsack, Kalbe, et al., 2007a), to the best of our knowledge, this is the first documented population with no evidence of macroparasites. In rivers, macroparasite abundance has been observed to decrease as one moves upstream (Barger & Esch, 2001; Blasco-Costa, Koehler, Martin, & Poulin, 2013), and physical barriers like waterfalls may induce breaks in host and parasite species distributions, exacerbating this infection gradient (Barger & Esch, 2001). Yet, the complete absence of macroparasites in an upstream fish population is an extreme case.

All macroparasite species detected were freshwater parasites with complex life cycles (Table S9). The majority infect fish-eating birds as final host, which could disperse parasite eggs through their faeces in all three sites (Fellis & Esch, 2005). Such life cycles, through their higher colonization potential, are expected to have a homogenizing effect on parasite distribution (Blasco-Costa et al., 2013; Esch, Kennedy, Bush, & Aho, 1988; Louhi, Karvonen, Rellstab, & Jokela, 2010). The absence of parasites in the upstream habitat, despite high host vagility, suggests local factors limit parasite colonization (Esch et al., 1988). Most of the identified parasite species infect invertebrate intermediate hosts, which are either eaten by sticklebacks or release free-swimming parasite larvae. Therefore, limiting local factors may include the invertebrate communities, low host population density and/or downstream drift preventing free-swimming infective stages or invertebrate hosts to remain in contact with fish (Blasco-Costa et al., 2013; Poulin et al., 2011; Thieltges, Jensen, & Poulin, 2008).

We expected the upstream population to adapt to the absence of parasites by having a reduced investment into immune functions (Lindström, Foufopoulos, Pärn, & Wikelski, 2004; Lochmiller & Deerenberg, 2000). Fish above the waterfall did have higher body condition, investing more in growth (bigger size) than in fatty reserves (small livers) or immunity (smaller spleen and head kidney). However, putative upstream migrants exhibited relatively low levels of infection and their condition and immuno-proxies were comparable to downstream river residents. Admixed individuals were also similar to the residents in their infection levels, condition and immune status. Thus, our field data do not support the idea that migration from above to below the waterfall has fitness costs in terms of higher infection or lower condition.

In light of this result, we used experimental infections to determine whether the absence of macroparasites in the upstream site was due to reduced exposure or higher resistance. First-generation, laboratory-bred upstream fish were more susceptible than lake fish to both *D. pseudospathaceum* and *S. solidus* infections. And they were less able to control parasite growth, harbouring significantly larger *S. solidus* parasites. These results show that upstream fish are devoid of macroparasites because they experience a low infection risk, not because they are more resistant. This is consistent with previous studies showing lower resistance of river fish compared to lake fish (Kalbe & Kurtz, 2006; Scharsack, Kalbe, et al., 2007a), but it is unclear whether this higher susceptibility is due to low genetic standing variation alone or parasite-mediated selection (Hale & Briskie, 2007). Hybrids had intermediate susceptibilities. Thus, in the presence of parasites, migrants and first-generation hybrids may be at a disadvantage compared to pure downstream fish (Nosil et al., 2005). Differential parasite pressure could potentially limit genetic flow, alone or in combination with other ecological and genetic factors. For instance, the diversity of immune genes, particularly the MHC, plays an important role in stickleback mate selection (Aeschlimann, Häberli, Reusch, Boehm, & Milinski, 2003; Reusch, Häberli, Aeschlimann, & Milinski, 2001a), so the lowered genetic variation in the upstream fish could promote mating barriers between migrants and residents in the downstream environment (Eizaguirre et al., 2011).

Upstream fish were more susceptible to laboratory infections, but putative migrants did not accumulate more parasites in the field. The simplest explanation for this discordant result is that migrants spent at least part of their life in a macroparasite-free environment. Although we do not know when fish migrated from above to below the waterfall, these migrants still likely had a lower cumulative exposure to parasites than residents, because even from a young age, sticklebacks prey on the small invertebrates that transmit macroparasites (Christen & Milinski, 2005).

Another explanation could be elevated mortality of the more parasitized migrants thereby biasing observations towards lower infection rates. Such "migratory culling" has been observed in experiments that transplanted sticklebacks between rivers and lakes (Kaufmann et al., 2017; Shaw, Sherman, Barker, & Zuk, 2018). The enlarged spleens of laboratory-infected, upstream fish hint that their immune response may be more aggressive, yet less effective, potentially contributing to mortality. Finally, upstream migrants may use the habitat differently than residents. They may be better adapted morphologically to swim in river current than downstream populations (Bolnick et al., 2009; Jiang et al., 2017) and spend less time exposed to higher infection risk in the lake. Or they could forage differently, affecting their exposure risk (Berner, Adams, Grandchamp, & Hendry, 2008; Webster & Hart, 2006).

5 | CONCLUSION

We highlighted the potential for parasite-mediated selection to promote local adaptation and population divergence in a system with natural, unidirectional migration from a low- to a high-parasitized habitat. We identified for the first time a stickleback river population where macroparasites are naturally absent, allowing inquiry into how parasites can prevent gene flow. Upstream fish and their hybrids had higher susceptibility to experimental infections, but migrants collected in the downstream environment did not have more parasites than residents. This discrepancy between field and laboratory infection patterns suggests dispersing individuals (first-generation migrants) may have an advantage, having spent some portion of their life unexposed to parasites. However, this may be negated by their descendants inheriting a higher susceptibility to parasites. Our results support the idea that adaptation to distinct parasite communities can reduce gene flow, even between close and connected populations.

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CONFLICT OF INTERESTS

We declare having no conflict of interest.

AUTHORS' CONTRIBUTIONS

N.I.E. and M.K. designed research; N.I.E., T.H., I.E.S., P.J.J. and M.K. collected field data; N.I.E. and M.K. performed experiments; N.I.E. and D.B. analysed data; and N.I.E. drafted the manuscript. All authors revised and approved the final manuscript.

DATA AVAILABILITY STATEMENT

Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.6837p85 (Erin et al., 2019).

ETHICS STATEMENT

All animal experiments were approved by the Ministry of Energy, Agriculture, the Environment and Rural Areas of the state of Schleswig-Holstein, Germany (reference number: V 313-72241.123-34).

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