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Ecological determinants of variation in phenotypic selection on quantitative immune defence traits

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Immune defence is an important determinant of organismal fitness. While theoretical models based on tradeoffs in resource allocation predict quantitative immune traits to be subject to stabilizing selection due to associated energetic costs and self-harm, empirical studies report mainly positive directional selection. This discrepancy may arise from multiple ecological factors that vary in nature and could influence selection. We examined if selection on immune activity varies depending on immune challenge/infection risk, between immune traits and among populations in the freshwater snail *Lymnaea stagnalis*. We assessed selection on the phenoloxidase-like and antibacterial activity of snail haemolymph while manipulating the level of immune challenge imposed by environmental microbes. We did this using snails from multiple populations and also quantified within-population family-level variation (i.e. evolutionary potential) in the snails' immune activity. We found that the strength of immune challenge and the examined immune trait determined selection on the snails' immune function. Thus, variation in infection risk can be an important factor in maintaining genetic variation in defence traits. Additionally, immune traits showed low among-population differentiation but high within-population genetic variation. This pattern could arise if natural snail populations are exposed to higher temporal than spatial variation in infection risk.

Keywords: ecological immunology, Gastropoda, immunocompetence, Mollusca, parasite resistance, selection gradient

Introduction

Organismal fitness (i.e. offspring contributed to the next generation) is a composite of many phenotypic traits that affect individual survival and reproductive success. One set of potentially important traits are those that constitute the immune system. This is because parasites present a severe threat to the survival and fecundity of free-living organisms, and immune function is the primary physiological barrier against infections (reviewed by Murphy and Weaver 2016). Therefore, variation in the expression of immune defence traits can contribute to among-individual fitness variation in nature,

immune activity being subject to natural selection (reviewed by Seppälä 2015, Seppälä et al. 2021a). Understanding the type and strength of such selection is critical for predicting the evolution of parasite resistance.

Predicting phenotypic selection on immune activity is not trivial. While immune defence eliminates harmful (i.e. virulent) parasites, it may come at a considerable energetic cost in terms of the maintenance and mounting of defence (Kraaijeveld and Godfray 1997, Moret and Schmid-Hempel 2000). Additionally, high immune activity can inflict self-harm through autoreactivity (Graham et al. 2010, Khan et al. 2017). Because of such costs, theoretical models based on tradeoffs in resource allocation between fitness-related traits predict stabilizing selection (i.e. intermediate trait values lead to the highest fitness) on immune activity (van Baalen 1998, Shudo and Iwasa 2001, Houston et al. 2007). This is typical for quantitative immune traits that reflect hosts' capacity for non-specific defences that are likely to be polygenic (i.e. evolve through quantitative genetic variance (Christe et al. 2000, Cotter et al. 2004); defences with high specificity (e.g. against different parasite genotypes) may evolve through other models of selection such as negative frequency-dependent selection (Hamilton 1980, May and Anderson 1983)). However, contrary to the theoretical predictions, empirical data mainly support positive directional selection on quantitative immune defence traits (i.e. the highest trait values lead to the highest fitness; reviewed by Seppälä 2015). Only a few empirical studies show stabilizing selection on such immune parameters (Råberg and Stjernman 2003, Kim et al. 2013, Langeloh et al. 2017, Soulsbury et al. 2018). This discrepancy between theoretical predictions and empirical observations, and the variation in the form of observed selection in empirical studies, highlight the need for examining underlying factors that influence the form and strength of natural selection on immune function.

Various ecological factors may contribute to selection on immune activity (reviewed by Seppälä 2015). In general, natural selection on phenotypic traits often shows spatial and temporal variation (Siepielski et al. 2009, 2011). Selection on immune defence traits could be strongly influenced by infection risk that varies across locations/habitats (Lindström et al. 2004, Scharsack et al. 2007) and over time (e.g. epidemics; Altizer et al. 2006). Such variation can make the optimal defence strategy context-dependent, displaying positive directional selection on immune activity when the risk of infection is high. Another related factor is the type of parasites hosts are exposed to because different immunological mechanisms are used against different parasites. Thus, differences in the form and strength of selection among immune traits may occur even when quantified simultaneously (Råberg and Stjernman 2003, Langeloh et al. 2017). Moreover, factors that contribute to the magnitude of phenotypic variation among individuals (i.e. potential for selection; Fairbairn and Reeve 2001) are important in determining the observed selection. For example, if variation in immune activity is low in the examined population, it may cover only a fraction of its 'overall' fitness function (i.e. fitness function over the broadest possible range of phenotypes), thus making the detection of stabilizing selection unlikely. Additionally, in organisms that show condition-dependent immune activity (Feder et al. 1997, Siva-Jothy and Thompson 2002, Seppälä and Jokela 2010), variation in resource availability/acquisition could obscure trade-offs between immune defence and life-history traits (reviewed by Reznick et al. 2000), potentially confounding the estimates of selection. Consequently, the observed fitness functions could appear as positive directional if resource availability for organisms was not limited (Kraaijeveld and Godfray 1997).

Here, we examined if selection on immune activity varies depending on infection risk, between immune traits and among populations in the freshwater snail *Lymnaea stagnalis*. We did not include the effect of variation in resource availability because stabilizing selection has been detected without strong resource limitation in an earlier study (Langeloh et al. 2017). Using snail survival and fecundity as fitness proxies, we assessed selection on two immune defence traits, the phenoloxidase (PO)-like and antibacterial activity of haemolymph, when experimentally manipulating the level of immune challenge/infection risk imposed by environmental microbes at different densities (low versus high). We conducted the experiment using offspring of individuals from multiple natural populations to test if the variation in their immune activity covers different parts of the overall fitness functions of the examined immune traits. Furthermore, we assessed family-level variation (i.e. evolutionary potential) in immune activity in the experimental snails. We found that the infection risk and examined immune trait were important determinants of the observed selection on immune activity. Additionally, the examined immune traits showed within-population genetic variation detected at the family level.

Material and methods

Study system and experimental animals

Lymnaea stagnalis is a hermaphroditic freshwater snail that inhabits the littoral zones of stagnant and slowly-flowing water bodies in the Northern Hemisphere (Fodor et al. 2020). It is predominantly an annual species that rarely survives past its first breeding season (Boycott 1936, Brown 1979). In the wild, *L. stagnalis* populations are often infected with digenetic trematodes (i.e. flukes) that use snails as intermediate hosts in their life cycles (Väyrynen et al. 2000, Faltýnková et al. 2007). Trematode prevalence, the proportion of infected hosts, can be high (Loy and Haas 2001, Louhi et al. 2013), and infected snails have reduced fitness owing to parasite-induced castration and increased mortality rate (Karvonen et al. 2004, Seppälä et al. 2013). Therefore, infections can impose selection on snail immune function (Langeloh et al. 2017). Furthermore, previous studies using full-sib families and maternal sibships have demonstrated genetic variation in both immune activity (Seppälä and Jokela 2010, Seppälä and Langeloh 2016, Leicht et al. 2017)

and parasite resistance (Seppälä et al. 2011) of *L. stagnalis*, suggesting potential for evolutionary responses to parasite-mediated selection.

In this study, we used snails from six laboratory stock populations each established in summer 2012 by collecting adult snails from different ponds in northern Switzerland (Adlisberg: 47°22′N, 8°34′E, n = 54; Eschenberg: 47°28′N, 8°43′E, n = 101; Irchel: 47°23′N, 8°32′E, n = 82; Warth 1: 47°34′N, 8°51′E, n = 148; Warth 2: 47°34′N, 8°51′E, n = 37; Zürichberg: 47°23′N, 8°33′E, n = 87). During the time of collecting, copulations between snails were frequent. Because *L. stagnalis* prefers outcrossing over self-fertilization (Puurtinen et al. 2007, Nakadera et al. 2017) and can store allosperm from previous matings (Nakadera et al. 2014), offspring of the field-collected individuals were likely to adequately represent the genetic variation in the source populations. We maintained each stock population in a water tank (volume: 800 l) filled with aged tap water (temperature: 18 ± 2°C), and fed the snails with fresh lettuce and *Spirulina* powder ad libitum. In spring 2013, we isolated individual juvenile snails (shell length: 6.2–14.5 mm) from the F_2 generation of each lab stock (15–31 individuals per population). We maintained these snails in cups (volume: 0.2 l) and fed them with fresh lettuce. After all individuals had reached maturity, we gave each of them one mating partner from their respective stock population. We kept the mating pairs together for two weeks to maximize the probability of mating. We then removed the mating partners and allowed the snails to lay egg clutches for 26 days. After that, we removed the parental snails from the cups and collected a sample of head–foot tissue from each individual for microsatellite genotyping (Supporting information). From the produced offspring, we raised 146 families for the experiment (2–18 siblings per family, variation in family size arose from differences in mortality rate).

Experimental design

After the experimental snails had reached maturity (shell length ≥ 20 mm), we conducted a laboratory experiment in which we exposed them to different immune challenge treatments and measured their immune activity, survival, growth and reproductive output over a six-week time period. Considering that the reproductive season of *L. stagnalis* lasts three to four months (Nakadera et al. 2015), the experiment covered a significant proportion of the snails' reproductive period.

To manipulate the immune challenge for the snails, we exposed them to different concentrations of environmental microbes that invade snails as a part of the normal exchange between snail haemolymph and the surrounding water during snail movement. For example, snails expel haemolymph when retreating into their shells and take in water when resuming activity (Rigby and Jokela 2000). This water can contain microorganisms that need to be eliminated by the snail's immune system. We manipulated the strength of the immune challenge/infection risk by keeping the snails in either 'standard' or 'microorganism-enriched' water (Moret et al. 2010). In the standard water treatment, we used the same aged tap water in which the stock populations were maintained. Thus, the water contained microbes in a comparable density to the earlier culturing conditions of the experimental snails. For the microorganism-enriched water treatment, we supplemented the aged tap water with 2 ml l^{-1} of LB medium (20 mg l^{-1} of Tryptone, 20 mg l^{-1} of NaCl, 10 mg l^{-1} of yeast extract in de-ionized water), 1 ml l^{-1} of algae medium (10.1 mg l^{-1} of KNO_3, 0.1 mg l^{-1} of KH_2PO_4 , 1.1 mg l^{-1} of $MgSO_4$ in de-ionized water) and 1.5 g l^{-1} of sugar to provide additional resources for the contained microbes to enhance their growth. We allowed the microbes to grow in this solution for five days and diluted it with aged tap water in a 1:3 ratio before use in the experiment. In earlier studies, microorganism-enriched water has been shown to activate snail immune defence (Seppälä and Leicht 2013) and reduce fitness (Rigby and Jokela 2000). It is important to note that both microbial communities consisted of naturally-occurring environmental microbes, some of which may be opportunistic pathogens. The treatments were unlikely to contain any specialist snail pathogens because, for example, microorganism-enriched water was prepared without contact with snails. In fact, the immune challenge/pathogenicity of microorganism-enriched water was modest compared to standard water. This is because although the snails' mortality rate was almost doubled compared to the standard water treatment, it was generally low (standard water: 3.7%; microorganism-enriched water: 6.6%). Furthermore, although the species diversity in both microbial communities is likely to be high, their composition is not known.

In families consisting of ten or more siblings (n = 72), we assigned half of the individuals to each water quality treatment, while in families consisting of fewer than ten siblings (n = 74), we randomly assigned all individuals to one water quality treatment. Of these smaller families, the standard water treatment received 38 families, while the microorganism-enriched water treatment received 36 families. We did not split the families with small sizes into both treatments to minimize family-by-treatment combinations with too low level of replication for statistical analyses such as the analysis of variance (ANOVA). In the following selection-gradient analyses, we did not consider among-family variation explicitly. Still, a design in which as many families as possible are split into both treatments maximizes the similarity of the genetic background in each treatment.

We maintained the experimental snails individually in perforated 0.2-l plastic cups partially submerged in 40 l water baths (40 cups per water bath; temperature: 18 ± 2°C), each containing water according to the immune challenge treatment. We equipped the water baths with immersion pumps coupled with filter mats (growing surface for microbes) to circulate the water and reduce nutrient levels. We partly replaced the water in each water bath every five days to maintain constant water quality. For logistic reasons, we conducted the experiment in two blocks that started on consecutive days

(block 1: n = 604; block 2: n = 599). We fed the snails ad libitum with fresh lettuce during the experiment.

Measurements

Sixty hours after the beginning of the exposure to water quality treatments, we measured the shell length of each experimental snail to the nearest 0.1 mm and collected haemolymph samples for immunological analyses (PO-like and antibacterial activity of haemolymph). Afterwards, we quantified these parameters every two weeks until the end of the experiment (this sampling frequency does not jeopardize snail survival or immune activity (Boisseaux et al. 2016)). Both quantified immune traits are inducible (Seppälä and Leicht 2013). Thus, in the microorganism-enriched water treatment, repeated sampling allowed examining the initial response to immune challenge (the first measurements) as well as the snails' ability to maintain immune function over prolonged exposure to microbes (subsequent measurements). Starting from the second measuring event, we additionally assessed the snails' fecundity during the previous one week period. We also tracked the snails' survival once a week. At the end of the experiment, we collected a sample of head–foot tissue from each individual for microsatellite genotyping to determine whether the snails had been produced through outcrossing (Supporting information).

For immunological measurements, we obtained two haemolymph samples (one per examined immune trait) from each snail at each sampling event. To stimulate the expulsion of haemolymph, we gently tapped the undersides of the snails' feet until they retreated into their shells, simultaneously releasing haemolymph through the hemal pore (Sminia 1981). This response is normal predator-avoidance behaviour in *L. stagnalis* (Rigby and Jokela 2000). We took one sample to quantify the PO-like activity of haemolymph. PO is a component of oxidative defences (reviewed by Cerenius and Soderhäll 2021) in various taxa, including molluscs (Mitta et al. 2000, Hellio et al. 2007, Le Clec'h et al. 2016). For this assay, we mixed 10 µl of haemolymph with 100 µl of phosphate-buffered saline (PBS) in a 1.5 ml reaction tube. We took another sample to quantify the antibacterial activity of haemolymph, which reflects snails' ability to destroy microbial cells. For this assay, we collected 100 µl of haemolymph into a 1.5 ml reaction tube. We immediately snap-froze all samples in liquid nitrogen and stored them at −80°C until processing.

Later, we measured the PO-like and antibacterial activity of the samples spectrophotometrically using a microtiter plate reader (Spectra-Max 190, Molecular Devices) following the protocol outlined in Seppälä and Leicht (2013). In short, to measure the PO-like activity, we mixed haemolymph with L-Dopa and measured the increase in the optical density (OD) of the solution. The reaction is due to the enzymatic oxidization of L-Dopa by PO. Based on the transcriptome profiling of *L. stagnalis*, this measure may reflect the activity of two PO-enzyme families, namely tyrosinases and laccases (Seppälä et al. 2021b). To measure the antibacterial activity, we mixed haemolymph with lyophilized *Escherichia coli* cells and measured the decrease in OD of the solution. This reaction is caused by the lysis of bacteria cells by antibacterial factors, and likely reflects the activity of multiple antibacterial peptides and proteins (e.g. macins, lipopolysaccharide-binding/bactericidal permeability-increasing proteins; Seppälä et al. 2021b). It is important to note that the above measures reflect snails' capacity for certain types of immune responses on a broad scale. Thus, selection gradients for them may deviate from selection on individual immune factors/proteins that contribute to the measured reactions. This is because selection can vary even among closely related immune factors (Sparks et al. 2018). To estimate the repeatability (R) of the applied immunological assays, we analyzed duplicate haemolymph samples for both measurements from 44 randomly selected snails per trait. Repeatability describes the proportion of variance in a variable owing to differences among individuals rather than from stochastic variation between samples taken from the same individual. It is calculated from variance components derived from an ANOVA using individual as a factor (Krebs 1989). The repeatability of both immunological assays was high (PO-like activity: $R = 0.871$, $F_{43,43} = 6.758$, $p < 0.001$; antibacterial activity: $R = 0.793$, $F_{43,43} = 3.909$, $p < 0.001$).

To estimate the snails' fecundity, we collected the egg clutches deposited by each snail during a one-week period before each sampling event. We photographed the egg clutches from above on a millimeter paper using a digital camera. We measured the two-dimensional area covered by eggs in each clutch utilizing ImageJ software ver. 1.48s and used the sum of the areas of clutches collected from each snail during the experiment as a measure of its total reproductive output. Owing to a strong positive correlation between the number of eggs in an egg clutch and the area these eggs cover (Langeloh et al. 2017), this measure is a suitable proxy for snail fecundity.

Statistical analyses

The fecundity or immune activity of 30 snails could not be measured (2.5% of all individuals) because of technical issues. We excluded these snails from the data. To estimate the variation in the snails' performance arising from the experimental factors (immune challenge/infection risk, block) and the genetic background of snails (population, family), we analyzed the variation in the snails' immune activity and reproductive output using mixed-model ANOVAs. In these models, we included the water quality treatment as a fixed factor, and population, family (nested within population) and block as random factors. Additionally, we included interaction terms between factors other than the block. In the ANOVAs, we excluded water quality-by-family combinations with fewer than three observations (these individuals were not removed from analyses for selection). Thus, the number of snails used in the ANOVAs varied among the examined traits.

Because the PO-like activity of snail haemolymph decreased in microorganism-enriched water after the first

measuring event (Supporting information), we divided the data from different phases of the experiment into different analyses. First, we conducted the ANOVAs for the PO-like and antibacterial activity at the first measurement event (i.e. 60 h after the beginning of the water quality treatments). These measurements reflect 'early-stage' immune activity during the experiment, and in microorganism-enriched water, they show the freshly activated levels of defence. Note that the exact time of peak immune activity in snails after exposure to microorganism-enriched water is not known and may deviate from the measurements conducted in this study. In these analyses, we square root transformed early-stage PO-like activity to fulfil the assumptions of ANOVA. Second, we repeated the analyses using the average of the next three measurements of immune activity (measuring events 2–4; hereafter called 'later-stage immune activity'; two outlier individuals were removed from these and subsequent analyses). These measurements more strongly reflect the ability of the snails to maintain immune function during a prolonged immune challenge. Note that we used only individuals that survived until the end of the experiment for the latter measurements to avoid temporal patterns in the data (Supporting information) from biasing the results after the death of some snails during the study. Therefore, the downstream analyses of selection on the later-stage immune activity reflect selection only through variation in snail fecundity. In these analyses, we square root transformed later-stage PO-like activity to fulfil the assumptions of ANOVA. Additionally, we conducted the above analyses using only families verified to be produced through outcrossing to understand genetic variation in the snails' immune defence at the level of full-sib families (i.e. without families that could be produced through self-fertilization). Lastly, we analyzed the variation in the snails' total reproductive output during the experiment using a similar ANOVA.

Because the snails' immune activity depended on the immune challenge (i.e. water quality treatment), we analyzed selection on the levels of the early-stage and later-stage activity of the immune traits separately in different water quality treatments (i.e. standard water, microorganism-enriched water). The examined populations showed similar immune activity, which is why we pooled them for analyses of selection. We report the results for selection from analyses in which variation between blocks was not removed from the variables. We did this to maintain the original scales of the variables, and because results for selection from analyses using the residuals of variables from a model including only block were qualitatively similar.

We applied regression-based selection gradient analyses described by Lande and Arnold (1983) to estimate selection on the examined immune traits. As a fitness measure, we used the snails' total reproductive output over the course of the experiment, which was transformed into relative fitness (individual fitness/mean fitness; the data included individuals with zero fitness, but their proportion was low (12.2%)). Additionally, we standardized the trait values for each immune parameter ((individual trait value − mean trait value)/standard deviation of trait values). In the analyses, we estimated both linear (β) and quadratic (γ) univariate selection coefficients and their standard errors (SE) (Lande and Arnold 1983). We doubled the coefficients and SEs for the second-order terms (Stinchcombe et al. 2008). While selection gradients are sufficient for identifying directional selection, stabilizing and disruptive selection cannot be detected solely based on the statistical significance of the quadratic term in the analysis (Schluter 1988). Additionally, an intermediate fitness maximum (stabilizing selection) or minimum (disruptive selection) is required in the fitness function. Therefore, we visualized selection using cubic spline estimates (Schluter 1988) for each trait (early-stage and later-stage levels separately). These univariate selection gradients measure the combined effect of direct and indirect selection on the examined immune traits in the case of covariation between them. A multivariate selection gradient analysis estimates direct selection on each trait. In this study, correlations between immune parameters were weak ($|r| \leq 0.097$ for all comparisons of the early-stage and later-stage levels of immune activity in different water quality treatments), which is why we did not calculate multivariate selection gradients.

The obtained estimates of selection for the early-stage levels of defence include fitness variation arising through differences in both the survival and fecundity of the snails. Thus, we calculated selection gradients for each fitness component separately. To measure selection through variation in the snails' survival, we used the data on whether or not an individual survived until the end of the study. Because this variable is binomial (survived, died), we used logistic regression instead of linear regression to estimate selection (Janzen and Stern 1998). We refer to the first-order and second-order terms from these models as $\beta_{logistic}$ and $\gamma_{logistic}$, respectively. To measure selection through variation in the snails' fecundity, we used the total reproductive output of those individuals that survived to the end of the experiment. Because the estimates of selection for the later-stage levels of immune activity were based on the individuals that survived until the end of the study, they reflect selection arising through variation in the snails' fecundity. We performed all statistical analyses using IBM SPSS ver. 26.0 software and produced cubic spline estimates to visualize fitness functions using the R package 'mgcv' (Wood et al. 2016).

Results

The early-stage (i.e. the first measuring event after the beginning of the experiment) level of the PO-like activity was higher in the microorganism-enriched water treatment (i.e. high immune challenge/infection risk, estimated marginal mean (± SE): 0.253 ± 0.003) compared to the standard water treatment (i.e. low immune challenge/infection risk, estimated marginal mean (± SE): 0.215 ± 0.003, Table 1). This indicates immune activation owing to the exposure to microbes. However, immune-challenged snails were not able to maintain elevated PO-like activity over the duration of

Table 1. Mixed-model analyses of variance for the haemolymph PO-like activity (early-stage and later-stage levels), haemolymph antibacterial activity (early-stage and later-stage levels), shell length and total reproductive output of *L. stagnalis* snails by immune challenge/infection risk treatment (standard water, microorganism-enriched water), population (6 populations), family (nested within population), block (two blocks) and their relevant interactions.

Trait	Source	df	MS	F	η^2 (%)	p
PO-like activity (early-stage)	Treatment (T)	1	0.238	54.717[a]	3.0	< 0.001
	Population (P)	5	0.021	3.763[b]	1.3	0.112
	Family(population) (F(P))	128	0.014	1.432[c]	23.6	0.055
	Block	1	0.007	1.349	0.1	0.246
	T × P	5	0.004	0.377[c]	0.2	0.863
	T × F(P)	64	0.010	1.873	8.2	< 0.001
	Error	924	0.005			
PO-like activity (later-stage)	Treatment (T)	1	0.038	18.986[a]	1.5	0.004
	Population (P)	5	0.011	2.538[b]	2.1	0.085
	Family(population) (F(P))	124	0.006	2.155[c]	27.3	< 0.001
	Block	1	0.001	0.522	< 0.1	0.470
	T × P	5	0.002	0.765[c]	0.4	0.578
	T × F(P)	63	0.003	1.317	6.4	0.055
	Error	805	0.002			
Antibacterial activity (early-stage)	Treatment (T)	1	0.000	4.449[a]	0.5	0.082
	Population (P)	5	0.000	3.068[b]	2.8	0.096
	Family(population) (F(P))	128	0.000	1.665[c]	14.5	0.012
	Block	1	0.000	3.601	0.3	0.058
	T × P	5	0.000	1.886[c]	0.6	0.107
	T × F(P)	65	0.000	0.824	4.4	0.837
	Error	932	0.000			
Antibacterial activity (later-stage)	Treatment (T)	1	0.000	0.056[a]	< 0.1	0.821
	Population (P)	5	0.000	4.661[b]	4.1	0.009
	Family(population) (F(P))	125	0.000	2.633[c]	22.5	< 0.001
	Block	1	0.000	1.571	0.1	0.210
	T × P	5	0.000	1.113[c]	0.4	0.361
	T × F(P)	63	0.000	0.865	4.3	0.763
	Error	869	0.000			
Reproductive output	Treatment (T)	1	33 668.236	0.272[a]	0.0	0.618
	Population (P)	5	1 357 314.175	3.625[b]	3.3	0.015
	Family(population) (F(P))	128	494 428.591	2.848[c]	30.5	< 0.001
	Block	1	117 621.211	0.885	0.1	0.347
	T × P	5	118 056.541	0.687[c]	0.3	0.635
	T × F(P)	65	173 568.997	1.305	5.4	0.057
	Error	943	132 967.860			

[a] T × P as the error term.
[b] F(P)) + T × P − T × F(P) as the error term.
[c] T × F(P) as the error term.

the experiment (i.e. later-stage PO-like activity (measuring events 2–4), estimated marginal means (± SEs): microorganism-enriched water: 0.207 ± 0.002, standard water: 0.221 ± 0.002, Table 1). Snail populations did not differ in their performance except for the later-stage level of the antibacterial activity (range of estimated marginal means (± SEs): 0.037 ± 0.000 to 0.041 ± 0.001, Table 1) and reproductive output (range of estimated marginal means (± SEs): 574.224 ± 32.763 to 837.990 ± 33.014, Table 1). However, these effects were weak, explaining 4.1% and 3.3% of the total variance in the examined traits, respectively (Table 1). There was no statistically significant difference between blocks in the examined traits (Table 1).

Because of these results, we analyzed selection on the snails' immune activity separately in standard and microorganism-enriched water, but pooled the snails originating from different populations in the analyses. We found that selection on the early-stage levels of immune activity varied between the examined immune traits (Fig. 1). The selection on the PO-like activity was negative directional in both water quality treatments, indicating that the expression of this immune trait was costly under the tested experimental conditions (Fig. 1a, c). This effect arose from negative directional selection on both the survival and fecundity of snails, although the effects through each fitness component were mostly statistically non-significant (standard water: survival: $\beta_{logistic} \pm SE = -0.401 \pm 0.212$, $p = 0.059$; fecundity (of survivors): $\beta \pm SE = -0.047 \pm 0.026$, $p = 0.071$; microorganism-enriched water: survival: $\beta_{logistic} \pm SE = -0.210 \pm 0.156$, $p = 0.178$; fecundity (of survivors): $\beta \pm SE = -0.062 \pm 0.023$, $p = 0.007$).

The selection on the early-stage level of the antibacterial activity, on the other hand, depended on the strength of

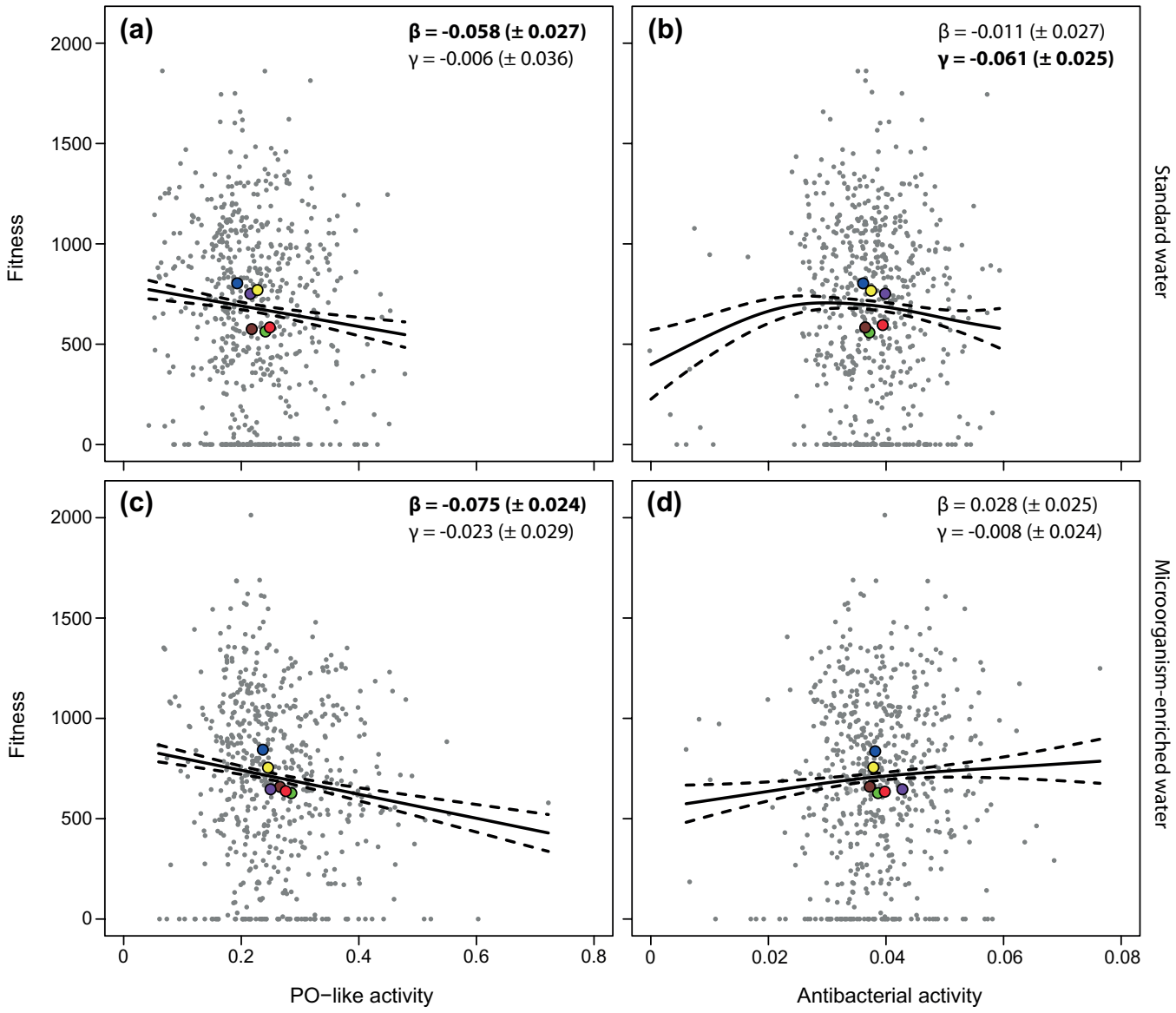

Figure 1. The fitness of the snails (sum of areas covered by eggs in all egg clutches collected from snails at the four measuring events of the experiment in mm²) as functions of the 'early-stage' levels (i.e. the first measurement event after the beginning of the experiment) of the PO-like and antibacterial activity of haemolymph in OD (vertical panels) in different water quality treatments (standard and microorganism-enriched water; horizontal panels). The solid black lines show fitness functions estimated using the cubic spline method, and the dashed lines their standard errors. Grey dots represent the raw data points, and coloured circles show the mean fitness and immune activity in the examined populations (calculated from raw data; a total of six populations; circles with the same colour represent one population). Univariate linear (β) and quadratic (γ) selection coefficients (± SE) are given in each plot.

immune challenge in the environment (Fig. 1). Under low immune challenge/infection risk (standard water treatment), the selection was stabilizing (Fig. 1b). Again, this effect arose from selection through both examined components of fitness (survival: $\gamma_{logistic} \pm SE = -0.183 \pm 0.061$, $p = 0.003$; fecundity (of survivors): $\gamma \pm SE = -0.055 \pm 0.026$, $p = 0.033$). However, most of the experimental individuals, and all population means, were in the part of the fitness function where higher immune activity led to reduced fitness (Fig. 1b), indicating that the population would evolve towards lower immune activity. Under high immune challenge/infection risk (microorganism-enriched water treatment), the fitness benefits of high antibacterial activity increased, which made the fitness function flat (Fig. 1d).

Selection gradients on the later-stage levels of the PO-like activity remained negative directional (Fig. 2a, c), but significant selection gradients on the antibacterial activity were not detected (Fig. 2b, d). The latter may be because the phenotypic variation among individuals, and thus the potential for selection, was reduced at the later-stage level compared to the early-stage level of defence (i.e. very few individuals showed low antibacterial activity at the later-stage level).

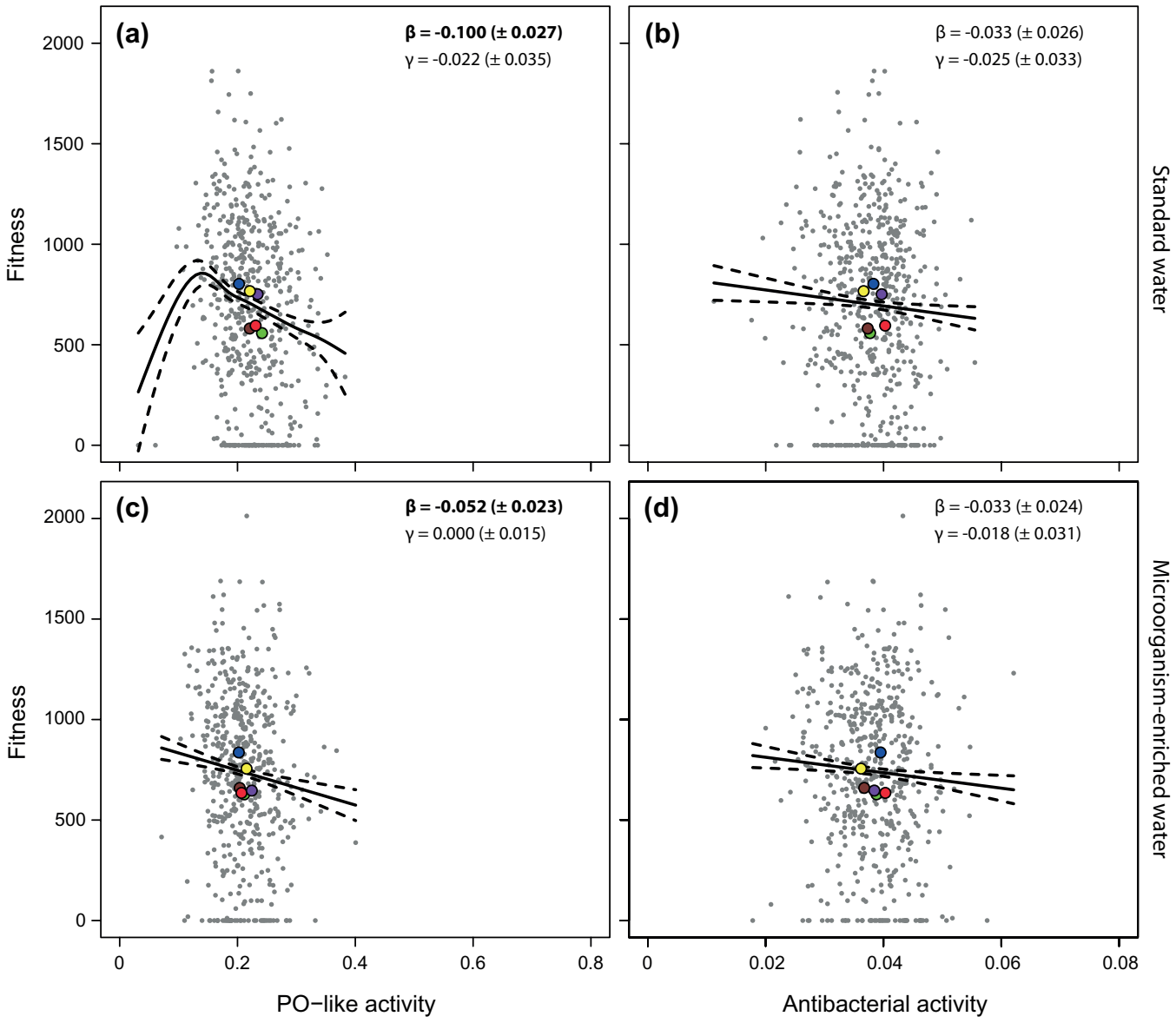

Figure 2. The fitness of the snails (sum of areas covered by eggs in all egg clutches collected from snails at the four measuring events of the experiment in mm^2) as functions of the 'later-stage' levels (i.e. the average of the last three measurement events (measuring events 2–4)) of the PO-like and antibacterial activity of haemolymph in OD (vertical panels) in different water quality treatments (standard and microorganism-enriched water; horizontal panels). The solid black lines show fitness functions estimated using the cubic spline method, and the dashed lines their standard errors. Grey dots represent the raw data points, and coloured circles show the mean fitness and immune activity in the examined populations (calculated from raw data; a total of six populations; circles with the same colour represent one population). Univariate linear (β) and quadratic (γ) selection coefficients (± SE) are given in each plot. The scales of the axes are the same as in Fig. 1 for easy comparison between the figures.

Of the investigated experimental (immune challenge/infection risk, block) and genetic (population, family) factors, family was the strongest determinant of variation in the snails' immune activity, explaining a total of 14.5–27.3% of the variation in the examined immune parameters (Table 1). When the same analyses were conducted using only families that were verified to be produced through outcrossing, the same pattern remained, although the proportion of variance arising from the family was slightly smaller (11.5–26.3% depending on the immune trait). These results indicate abundant genetic variation in the examined immune traits.

Discussion

Our findings indicate that the strength of immune challenge/infection risk in the environment and the examined immune defence trait were important determinants of the observed selection on immune activity. Under low immune challenge (i.e. in aged tap water used under standard culturing conditions), phenotypic selection on the early-stage level of the antibacterial activity of the snails' haemolymph (i.e. the first measurement event after the beginning of the water quality treatments) was stabilizing, individuals with intermediate levels of immune

activity having the highest fitness. Under high immune challenge (i.e. microorganism-enriched water), the relative fitness of individuals with high antibacterial activity increased, making the fitness function flat. This suggests that the cost–benefit ratio of antibacterial activity, and thus the form of selection on immune function, changes depending on infection risk in the environment. Therefore, a stronger immune challenge than used in this study could have led to positive directional selection on this trait. In fact, positive directional selection on the antibacterial activity of *L. stagnalis* has been shown under field conditions (Langeloh et al. 2017), where snails are likely to be exposed not only to opportunistic microbes but also to specialist pathogens. Selection gradients for the levels of the later-stage antibacterial activity (i.e. the average of the last three measurements) were flat in both immune challenge/water quality treatments, which may be at least partly because the phenotypic variation in antibacterial activity was lower at the later-stage levels compared to the early-stage levels (individuals with low later-stage levels were absent).

Selection gradients for the PO-like activity of the snails' haemolymph were negative directional in both immune challenge/water quality treatments. This suggests that the PO-like activity did not provide enough benefits to outweigh the costs associated with its production under the examined levels of immune challenge/infection risk. Low benefits of the PO-like activity could arise if, for example, PO is not important in defence against opportunistic microorganisms in *L. stagnalis*. Although PO is reported to act against various pathogens ranging from bacteria to multicellular parasites (reviewed by Cerenius and Soderhäll 2021), its effects are sometimes negligible. For example, fruit fly mutants that do not express PO maintain equal resistance to bacteria and fungi compared to wild-type flies (Leclerc et al. 2006). It is possible that the exposure of snails to other parasite types (e.g. trematodes) would have led to different selection gradients on the PO-like activity. In fact, stabilizing selection has been observed in a cage experiment, where snails were exposed to a natural parasite community, including trematodes, in the field (Langeloh et al. 2017). Together with the earlier findings by Langeloh et al. (2017) our results suggest that high antibacterial activity provides greater fitness benefits than high PO-like activity in *L. stagnalis*. This could be if PO-like activity is associated with higher costs arising not only through its production but potentially also through oxidative stress typical for oxidative defences (Dowling and Simmons 2009, Khan et al. 2017).

The result indicating stabilizing selection on the antibacterial activity is in line with theoretical predictions assuming tradeoffs between immune function and other fitness-related traits. However, our findings do not align with most empirical studies that report positive directional selection on immune activity (reviewed by Seppälä 2015). In addition to *L. stagnalis* (Langeloh et al. 2017, this study), stabilizing selection on quantitative immune defence traits has been reported in three bird species, blue tit (antibody responsiveness to diphtheria; Råberg and Stjernman 2003), common kestrel (PHA test; Kim et al. 2013) and black grouse (antibody responsiveness to tetanus; Soulsbury et al. 2018). Furthermore, negative directional selection on antibody responsiveness to tetanus has been detected in specific populations of the common side-blotched lizard that live under low infection risk (i.e. in low population densities; Svensson et al. 2001). If the variation in selection on immune function described in this and earlier studies (Svensson et al. 2001, Råberg and Stjernman 2003, Langeloh et al. 2017) is general across study systems, the discrepancy between theoretical and empirical work on phenotypic selection on immune function (i.e. stabilizing versus positive directional selection) could arise if empirical studies were typically conducted during periods of high infection risk (e.g. epidemics). Unfortunately, this information is often not available for studies conducted under field conditions (but see Wilcoxen et al. 2010).

Of the examined experimental and genetic factors, snail population contributed very little to the variation in the snails' immune activity. Instead, most of the variation was found at the individual level, suggesting that the opportunity for selection was similar among the six study populations. While *L. stagnalis* populations typically show significant population genetic structure in the wild (Puurtinen et al. 2004, Kopp et al. 2012), our results suggest that genetic differentiation based on neutral markers does not necessarily imply differences in immune phenotypes among populations. However, the examined immune traits showed within-population genetic variation detected at the family level. This finding indicates evolutionary potential in immune activity when subject to selection. Interestingly, family was the strongest determinant of variation in the expression of the snails' immune function (11.5–26.3% of variance was explained by family depending on the immune trait). The reason for the low differentiation among populations but high within-population family-level variation in immune activity is unknown, but it could have arisen from patterns in infection risk in the source populations. This is because variation in the type and strength of selection is generally one of the strongest factors contributing to genetic polymorphism in phenotypic traits (Haldane and Jayakar 1963). If selection varies spatially, optimal trait values may differ among populations leading to genetic and phenotypic differentiation among them. Temporally variable trait optima could instead maintain within-population genetic variation in fitness-related traits because the relative fitness of different genotypes becomes context-dependent. Hence, the observed genetic variation in immune activity across different levels (i.e. within and among populations) suggests that selection on *L. stagnalis* immune function may vary more over time than space in nature.

In conclusion, we found that the form of phenotypic selection on the immune activity of *L. stagnalis* snails depended on the immune challenge/infection risk in the environment and the examined immune defence trait. While the PO-like activity of the snails' haemolymph was subject to negative directional selection independently of the infection risk, selection gradients on the antibacterial activity varied in relation to the level of immune challenge imposed by environmental microbes. Under the low immune challenge, selection on the antibacterial activity was stabilizing. In contrast, under high immune challenge, the benefits of high antibacterial activity

increased, making the fitness function flat. Our results suggest that the form and strength of selection on immune function can vary in nature depending on infection risk. Additionally, selection can differ among immune defence traits depending on the type of parasites the hosts are exposed to. The contribution of such ecological factors may partly explain the discrepancy between theoretical predictions and earlier empirical observations on selection on immune activity (i.e. stabilizing versus positive directional selection). Additionally, our results indicated high within-population genetic variation in snail immune function. Such variation could have arisen/been maintained in nature if the source populations were exposed to temporal variation in infection risk in the past.

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Author contributions

Laura Langeloh: Conceptualization (equal); Data curation (lead); Formal analysis (equal); Investigation (lead); Project administration (equal); Writing – original draft (equal). **Jukka Jokela**: Formal analysis (supporting); Investigation (supporting); Resources (equal); Supervision (supporting); Writing – review and editing (equal). **Katri Seppälä**: Data curation (supporting); Investigation (supporting); Writing – review and editing (equal). **Otto Seppälä**: Conceptualization (equal); Formal analysis (equal); Funding acquisition (lead); Investigation (supporting); Project administration (equal); Resources (equal); Supervision (lead); Writing – original draft (equal).

Data availability statement

Data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.59zw3r2bn (Langeloh et al. 2022).

Supporting information

The Supporting information associated with this article is available with the online version.

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