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ORIGINAL ARTICLE

Condition-dependent immune function in a freshwater snail revealed by stable isotopes

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Abstract

1. The immune system is costly to maintain and use because it requires a lot of energy. This can make parasite resistance dependent on host nutritional state. The dependence of immune function on host condition can have broad ecological (e.g., disease dynamics) and evolutionary (e.g., expression of trade-offs related to parasite resistance) consequences.
2. Research on the dependence of immune function on host condition is typically conducted in laboratory experiments that manipulate either the quantity or composition of available resources. Such studies are essential in establishing conceptual frameworks, but their results are difficult to generalise to natural populations because the experimental treatments may deviate from natural variation in host resource level and use.
3. We examined the condition dependence of immune function in a generalist freshwater snail *Lymnaea stagnalis* by relating the activity of two immune parameters of snail haemolymph, phenoloxidase (PO)-like and antibacterial activity, to snail resource level and use using field-collected individuals. We measured several variables that reflect the snails' quality based on their recent (i.e., within the past few days; amount and stable isotope composition [$\delta^{15}N$ and $\delta^{13}C$] of produced faeces) and longer-term (i.e., weeks to months; stable isotope composition of tissues) resource use.
4. The PO-like activity of the snails' haemolymph was condition-dependent. Snails that had recently consumed food from higher trophic levels, presumably including more animal protein (high $\delta^{15}N$ values of faeces), had a stronger defence. Additionally, snails with low energy (i.e., lipid) reserves in their tissues (high $\delta^{13}C$ values of tissues) showed higher PO-like activity. The antibacterial activity of the snails' haemolymph was not condition dependent.
5. The finding of the importance of the composition of recently consumed food on immune function (namely PO-like activity) suggests that the parasite resistance of snails may change rapidly depending on the type of resources available in their environment. Thus, environmental variation may influence the outcome of host–parasite interactions on fine spatial and temporal scales. Furthermore, the

negative relationship between the snails' energy reserves and PO-like activity suggests substantial energetic costs of immune activity in *L. stagnalis*.

KEYWORD
ecological immunology, Gastropoda, immunocompetence, Mollusca, trade-off,

1 | INTRODUCTION

Several factors, from host genetic background and sex to environmental conditions, contribute to host susceptibility to parasite infections (Carius et al., 2001; Debes et al., 2017; Nunn et al., 2009; Wilson et al., 2002). Many of these effects are at least partly mediated by differences in host immune function, which is the primary physiological defence against parasites (reviewed in Janeway et al., 2005) and an essential determinant of organismal fitness (reviewed in Seppälä, 2015). The immune system is costly to maintain and use because it requires a lot of energy (Moret & Schmid-Hempel, 2000; Sheldon & Verhulst, 1996). Therefore, parasite resistance typically depends on host nutritional state, which can have broad ecological and evolutionary consequences. For example, individuals in poor physiological condition can be more susceptible to infections (Knutie et al., 2017; Kolluru et al., 2006; Murray et al., 1998; Wiehn & Korpimäki, 1998). Thus, deteriorating living conditions may predispose populations to disease outbreaks (reviewed in Lloyd, 1995; Wakelin, 1989). Additionally, variation in host resource level can affect the expression of trade-offs related to parasite resistance (Brzęk & Konarzewski, 2007; McKean et al., 2008; Moret & Schmid-Hempel, 2000) and maintain genetic polymorphism in host defences through genotype-by-environment (G × E) interactions (Blanford et al., 2003; Mitchell et al., 2005; Seppälä et al., 2011).

Research on the condition dependence of host immune function is typically conducted in laboratory experiments that manipulate either the quantity or composition of available resources (Brunner et al., 2014; Brzęk & Konarzewski, 2007; Ponton et al., 2020; Siva-Jothy & Thompson, 2002; Slater & Keymer, 1986; Stahlschmidt et al., 2013). Such experiments are essential because they demonstrate the condition dependence of immune defence while controlling for possible confounding factors (e.g., individuals may differ in their foraging efficiency) and they prove causality. However, such experiments often compare extreme resource levels (e.g., ad libitum food supply vs. no food). In nature, the variation in host resource level is likely to be more subtle at any given time point, making it difficult to generalise the results of such simplified laboratory experiments to natural populations. Therefore, expanding the work on condition dependence of immune defence to consider natural variation in host physiological condition in field populations is a high priority.

Here, we examined the condition dependence of immune function in a generalist freshwater consumer, *Lymnaea stagnalis* (Gastropoda), using field-collected individuals. We quantified the activity of two immune parameters of snail haemolymph that represent different branches of the immune system, phenoloxidase (PO)-like (a component of oxidative defences) and antibacterial activity (Langeloh et al., 2017; Seppälä & Jokela, 2010; Seppälä & Leicht, 2013). These immune traits respond to immune challenges (Seppälä & Leicht, 2013), contribute to snail fitness (Langeloh et al., 2017) and show considerable within-population genetic variation (i.e., evolutionary potential; Leicht et al., 2017; Seppälä & Jokela, 2010; Seppälä & Langeloh, 2016). Furthermore, snail immune activity depends on environmental factors such as food availability (Seppälä & Jokela, 2010; Seppälä et al., 2021) and ambient temperature (Leicht et al., 2013, 2017; Salo et al., 2017, 2019; Seppälä et al., 2021). When the access of snails to food is removed experimentally under laboratory conditions, the PO-like activity is strongly decreased within a day, whereas the level of the antibacterial activity reduces a few days later (Seppälä & Jokela, 2010). These findings suggest that variation in resource availability in nature could contribute to disease outbreaks in snail populations. Furthermore, the dependence of the snail immune system on food availability shows within-population family-level variation (i.e., a G × E interaction determining immune activity; Seppälä & Jokela, 2010). This interaction suggests that variation in environmental conditions may promote the maintenance of genetic variation in snail defences.

To relate the variation in immune activity to the resource level of the snails, we measured several factors that reflect their condition based on the quantity and composition of resources consumed both recently (i.e., within the past few days; amount and stable isotope composition [^{15}N:^{14}N ratio denoted as $\delta^{15}N$ and ^{13}C:^{12}C ratio denoted as $\delta^{13}C$] of produced faeces) and over a longer time period (i.e., weeks to months; stable isotope composition of tissues; Li et al., 2018). We found that under natural conditions (i.e., natural variation in snail resource level and use), the PO-like activity of the snails' haemolymph was condition dependent. Snails that had recently consumed food with high $\delta^{15}N$ values had a stronger defence. Considering the covariation between $\delta^{15}N$ and C:N ratio of faeces, the result suggests that resource consumption from higher trophic levels, potentially including more animal protein, enhances the haemolymph PO-like activity. Additionally, snails with high $\delta^{13}C$ values in tissues had high PO-like activity. Based on the covariation between $\delta^{13}C$ and C:N ratio of tissues, this result suggests a negative relationship between the snails' lipid reserves and the PO-like activity, which could arise from the energetic costs of immune activity.

2 | METHODS

2.1 | Study system

Lymnaea stagnalis is a hermaphroditic pulmonate snail with a large geographic distribution in the Holarctic region (Fodor et al., 2020). It inhabits the littoral zone of stagnant and slowly flowing water bodies such as lakes and ponds. In these habitats, *L. stagnalis* can reach high population densities (Yurlova et al., 2006) and thus be an important resource for natural enemies such as parasites and predators. In fact, *L. stagnalis* is commonly infected by many species of digenetic trematodes (i.e., flukes) that use snails as intermediate hosts in their life cycles (Faltýnková et al., 2007; Väyrynen et al., 2000). These parasites castrate the snails and increase their mortality rate (Karvonen et al., 2004; Seppälä et al., 2013). Because the infection prevalences (i.e., the proportion of individuals infected; see Bush et al., 1997) of trematodes can be high in natural snail populations (Louhi et al., 2013; Loy & Haas, 2001), trematodes form a severe threat to snails. This makes parasite resistance an important determinant of snail fitness (Langeloh et al., 2017). *Lymnaea stagnalis* is a generalist consumer that utilises diverse food sources ranging from microalgae and macrophytes to plant detritus and animal corpses (Doi et al., 2010; Elger et al., 2004; Zhang et al., 2018). Because of such diversity in resource use, the immune function of *L. stagnalis* could show high variation depending on the composition of consumed resources.

2.2 | Sampling and measurements

On 20 August 2015, we collected 101 *L. stagnalis* snails from a small forest pond (area: 590 m^2) in Zürich (47°23′N, 8°33′E), Switzerland. The pond has one inflow (an intermittent headwater stream) and an outflow, and its catchment area is entirely forested. We collected the snails from a 15 × 3 m area next to the shoreline of the eastern bank of the pond (no snails were collected from the immediate vicinity of the inflow or the outflow). Immediately after collecting, we measured the shell length of the snails to the nearest 0.1 mm (range: 21.3–48.1 mm) and sampled snail haemolymph for immunological measurements. We stimulated the expulsion of haemolymph by gently tapping the undersides of the snails' feet until they retreated into their shells, simultaneously releasing haemolymph through the haemal pore (see Sminia, 1981). This behaviour is a normal antipredatory response of *L. stagnalis* (Rigby & Jokela, 2000). From the released haemolymph, we obtained two samples per snail. We took one sample (10 µl of haemolymph mixed with 100 µl of phosphate-buffered saline in a 1.5-ml reaction tube) to quantify PO-like activity that is a component of oxidative defences (Cerenius & Soderhäll, 2021) in various taxa, including molluscs (Hellio et al., 2007; Le Clec'h et al., 2016; Mitta et al., 2000). We took the other sample (100 µl of haemolymph in a 1.5-ml reaction tube) to quantify antibacterial activity that reflects the ability of haemolymph to destroy microbial cells. We immediately snap-froze all samples in liquid nitrogen and stored them at −80°C for later processing (see the immunological assays in the fourth paragraph of this section).

After haemolymph sampling, we placed the snails individually in plastic containers prefilled with 0.1 L of artificial pond water (deionised water with 0.25 g/L of Dennerle Osmose ReMineral+ [Dennerle GmbH]; GH = 8.5). We transported the snails to a laboratory in these containers and maintained them in a climate chamber (18 ± 1°C) for two days without food. We did this to allow the snails to empty their intestines (the snails stopped producing faeces after 1–2 days of fasting) for later quantification of the amount and stable isotope composition of faeces that they produced (proxies for quantity and composition of recently [i.e., within the past few days] consumed resources in the field; see the measurements in the third and fifth paragraphs of this section). The use of artificial pond water prevented organic material in natural pond water from influencing the measurements.

After the snails had emptied their intestines, we removed them from the containers and dissected their soft body tissues under a stereomicroscope to examine whether the snails carried trematode infections previously obtained in the field. We cut the snails' feet off and placed them individually in 1.5 ml reaction tubes. We immediately froze the tissue samples in liquid nitrogen and stored them at −80°C for later analysis of their stable isotope compositions (see details in the last three paragraphs of this section). To quantify the amount of faeces produced by the snails, we filtered the water in each container using pre-weighed (after 24 hr drying at 50°C) GF/F glass microfiber filters (GE Healthcare Life Sciences). After filtering, we dried the filters again for four days and measured their weight to the nearest 0.01 mg. We used the difference between the final and initial weights of each filter as a measure for the amount of produced faeces. In a simultaneously run laboratory test, we found that the amount of faeces provides a good estimate for the amount of previously consumed food (see Supporting Information). After weighing, we placed the filters individually in 1.5-ml reaction tubes and stored them at −80°C for later analysis of the stable isotope composition of faeces (see details in the last three paragraphs of this section).

We measured the PO-like and antibacterial activity of snail haemolymph spectrophotometrically using a microtiter plate reader (Spectra-Max 190; Molecular Devices) as described in Seppälä and Leicht (2013). In short, to quantify PO-like activity, we mixed haemolymph with L-dopa and measured the increase in the optical density of the solution. This reaction is due to the oxidisation of L-dopa by PO enzymes. Based on the recent transcriptome profiling of *L. stagnalis*, this measure probably reflects the activities of two PO-enzyme families, namely laccases and tyrosinases (Seppälä et al., 2021). To quantify antibacterial activity, we mixed haemolymph with lyophilised *Escherichia coli* cells and measured the decrease in the optical density of the solution. This reaction is due to the lysis of bacteria cells by antibacterial proteins and a likely composite measure for the activities of multiple antibacterial peptides and proteins (e.g., macins, lipopolysaccharide-binding/bactericidal permeability-increasing proteins; Seppälä et al., 2021). To estimate the repeatability (*R*) of the applied immunological assays, we analysed duplicate haemolymph

samples for both parameters from 18 randomly selected snails per trait. Repeatability describes the proportion of variance in a variable that arises from differences among individuals rather than from stochastic variation among samples taken from the same individual. It is calculated from variance components derived from an analysis of variance (ANOVA) using individual as a factor (see Krebs, 1989). The repeatability of both immunological assays was high (PO-like activity: $R = 0.908$, $F_{17,18} = 10.465$, $p < 0.001$; antibacterial activity: $R = 0.876$, $F_{17,18} = 7.450$, $p < 0.001$).

The analyses for stable isotope compositions ($\delta^{15}N$ and $\delta^{13}C$) of tissue and faeces samples were conducted at the Stable Isotope Lab at the University of Konstanz using a Micro Cube elemental analyser (Elementar, Analysensysteme, Germany) gas chromatography and an Isoprime (Micromass, Manchester, UK) isotope ratio mass spectrometer (see Yohannes et al., 2017). The analyses aimed to track the nitrogen and carbon sources of the snails' diet on a broad scale to estimate among-individual variation in the composition of consumed resources. The analyses did not aim to link the isotope composition of individual snails to specific food sources in the field. Because ^{15}N is enriched from one trophic level to the next (DeNiro & Epstein, 1981; Post, 2002), we used $\delta^{15}N$ values to estimate variation in the effective trophic level among the snails (tissue samples; based on the isotopic turnover rates in *L. stagnalis* this measure reflects the average food consumption over several weeks; Li et al., 2018) and the food they had consumed recently before being collected (faeces samples; the snails emptied their guts after 1–2 days of fasting; note that metabolic wastes cannot bias the measurements from faeces because snails excrete urine through the pneumostome [see de With & van der Schors, 1984]). Variation in $\delta^{15}N$ values could arise from differences in the proportional resource use of snails from different trophic levels, with high values potentially indicating increased animal protein consumption. Alternatively, high $\delta^{15}N$ values in tissue could indicate fasting during which individuals consume their own tissues as an energy source, thus leading to the enrichment of ^{15}N (Hobson et al., 1993; Olive et al., 2003). To estimate whether variation in $\delta^{15}N$ values of faeces is likely to arise from variation in protein content, we also examined the C:N ratio of the samples (high $\delta^{15}N$ values together with a low C:N ratio would support the notion that $\delta^{15}N$ reflects protein content. In our data, increasing carbon content and decreasing N content led to a higher C:N ratio in both sample types (multiple linear regression: $|t| \geq 13.838$, $p < 0.001$ for all).

Additionally, because lipids contain more ^{12}C than other biochemical fractions (DeNiro & Epstein, 1977; Pinnegar & Polunin, 1999), we used $\delta^{13}C$ values to estimate variation in the energy (i.e., lipid) content of the snails' tissue samples and the food they had recently consumed (faeces samples). Alternatively, $\delta^{13}C$ values can reflect variation in the consumption of resources with different origins such as terrestrial, limnetic, and benthic (Batt et al., 2012; Solomon et al., 2011). In fact, ponds and lake littorals inhabited by *L. stagnalis* contain numerous alternative food sources, and snails are known to consume them broadly (Doi et al., 2010; Salo et al., 2018). However, low $\delta^{13}C$ values together with a high C:N ratio would support the notion that $\delta^{13}C$ values reflect lipid content. Nonetheless, because individual organic compounds (e.g., amino acids, fatty acids) can vary in their isotope composition (Bec et al., 2011; Whiteman et al., 2019), future studies would ideally examine the macromolecular composition and isotopic values of food-web components using novel approaches such as compound-specific stable isotope analyses. Such studies would shed more light on factors determining variation in isotope composition detected among snails in this study.

The availability of different isotopes in the habitat could also vary spatially, thus contributing to the variation in isotope composition among examined snails. Such variation could depend on, for example, the inflow of water into the water body. Considering the small size, low inflow and homogeneous catchment area of the examined pond (see the first paragraph of this section), the potential effects of such factors are likely to be small. Furthermore, the mobility of snails (over 2 m/h; Dalesman & Lukowiak, 2010; Pavlova, 2010) is high enough for individuals to effectively move between different parts of the pond and thus be similarly exposed to spatially variable factors that may influence their isotope composition. In fact, the $\delta^{15}N$ values of the faeces and tissue samples support this notion. This is because the means of different sample types were similar (paired-samples *t*-test: $t_{96} = -1.617$, $p = 0.109$). However, variance in $\delta^{15}N$ values was higher in the faeces samples (*F*-test: $F_{96,96} = 10.305$, $p < 0.001$), suggesting that snails vary in their resource use over time and tissue samples reflect average resource consumption over longer time periods. The $\delta^{13}C$ values showed similar variance between sample types (*F*-test: $F_{96,96} = 1.201$, $p = 0.186$) but were higher in tissue samples (paired-samples *t*-test: $t_{96} = 6.202$, $p < 0.001$). This suggests higher lipid content in snail tissues than in their food sources.

2.3 | Data analyses

The stable isotope composition of the faeces could not be measured from four snails, which is why we excluded those individuals from the data. To examine the suitability of various univariate and multivariate statistical approaches for the data set, we analysed covariation between the examined immune traits (i.e., PO-like and antibacterial activity of haemolymph) and variables representing the snails' short-term (i.e., amount and stable isotope composition of produced faeces) and longer-term resource use (i.e., stable isotope composition of tissues) using Pearson's correlations. We also examined the correlations between variables representing snail resource level and use, snail shell length, and the C:N ratio of both faeces and tissues. This was because variation in snail size could be a confounding factor in further analyses if it covaried with other variables, and the relationship between isotope values and C:N ratio is important for interpreting the results (see the last three paragraphs of the previous section). We used the following transformations to meet the assumptions of downstream analyses: immune traits: sqrt(x), amount of faeces: ln($x + 2$), $\delta^{15}N$ faeces: sqrt(x)$^{-1}$, $\delta^{15}N$ tissue: x^2, $\delta^{13}C$ tissue: sqrt($x + 34$), C:N ratio tissue: x^{-7}, shell length: x^3. The $\delta^{13}C$ values and C:N ratio of faeces did not require a transformation. The PO-like and antibacterial activity of haemolymph did not correlate with

each other (Pearson correlation: $r = -0.003$, $n = 97$, $p = 0.975$), but correlations between other variable pairs were common (Table S1).

To examine the variation in the snails' immune activity, we first tested whether trematode-infected snails differed from uninfected individuals in their immune activity using a multivariate analysis of variance (MANOVA). A MANOVA was not possible for variables representing snail resource level and use and shell length because of covariation among them (Table S1). Therefore, we tested if the infection status affected these variables using univariate analyses of variance (ANOVA). Because of a difference in shell length between infected and uninfected snails (see Section 3) that could confound the use of snail size as an explanatory variable when examining the condition dependence of immune activity, and too limited a number of infected snails for a separate multivariate test (see the next paragraph), we excluded individuals infected with trematodes from further analyses.

Excluding trematode-infected snails did not change the covariation among the examined variables (immune traits: Pearson correlation: $r = 0.044$, $n = 75$, $p = 0.708$; resource level and use, shell length: Table 1). Because of moderate correlations between variables representing snail resource use, we analysed their effects on the snails' immune activity using multiple linear regression analyses (method: enter) conducted separately for each immune trait. In these models, we included the amount of faeces and the stable isotope composition ($\delta^{15}N$ and $\delta^{13}C$) of both faeces and tissue as explanatory variables. Additionally, we included snail shell length in the models because snail immune function and food consumption can depend on it (Salo et al., 2017). We also examined the interactive effects between explanatory variables with biologically relevant interpretations (e.g., interaction between $\delta^{15}N$ and $\delta^{13}C$ of faeces) on immune activity, but we did not include them in the final models because such effects were non-significant (results not shown). We performed all statistical analyses using IBM SPSS Statistics version 26 software (IBM Corp.).

3 | RESULTS

Twenty-two of the examined snails (22.7%) were infected by trematode parasites (Plagiorchiidae family; morphological identification). Trematode-infected and uninfected snails did not differ in their immune activity (MANOVA: Pillai's trace = 0.007, $F_{2,94} = 0.343$, $p = 0.710$). Variables representing snail resource level and use did not depend on infection status (ANOVAs: $F_{1,95} \leq 2.651$, $p \geq 0.107$ for all). However, infected individuals were 6% larger than uninfected ones (estimated marginal mean with upper and lower *SE*: infected snails: 40.4 mm [upper *SE* = 41.3 mm, lower *SE* = 39.5 mm]; uninfected snails: 38.2 [upper *SE* = 38.7 mm, lower *SE* = 37.6 mm]; ANOVA: $F_{1,95} = 4.149$, $p = 0.044$). Owing to the difference in shell length, we excluded infected snails from further analyses.

In the group of uninfected snails, regressing the haemolymph PO-like and antibacterial activity against variables representing snail resource level and use revealed condition dependence of immune function (Table 2, Figure 1). The PO-like activity increased with higher $\delta^{15}N$ values of both snails' faeces and tissues (Table 2, Figure 1a,b). In the faeces samples, the $\delta^{15}N$ and C:N ratio had a

TABLE 1 Pearson correlation coefficients (*r*) between variables representing the short-term (amount, stable isotope composition and C:N ratio of produced faeces) and longer-term resource use (stable isotope composition and C:N ratio of tissues) of snails uninfected with trematodes ($n = 75$) and their statistical significances (*p*)

	Amount of faeces	$\delta^{15}N$ faeces	$\delta^{13}C$ faeces	C:N ratio faeces	$\delta^{15}N$ tissue	$\delta^{13}C$ tissue	C:N ratio tissue	Shell length
Amount of faeces	1	**0.365**	**0.230**	**0.462**	−0.177	**0.259**	−0.154	0.225
		$p = 0.001$	$p = 0.048$	$p < 0.001$	$p = 0.129$	$p = 0.025$	$p = 0.188$	$p = 0.052$
$\delta^{15}N$ faeces		1	0.068	**−0.292**	−0.212	0.210	0.056	0.062
			$p = 0.564$	$p = 0.011$	$p = 0.068$	$p = 0.070$	$p = 0.632$	$p = 0.598$
$\delta^{13}C$ faeces			1	0.165	−0.155	**0.279**	0.039	0.172
				$p = 0.158$	$p = 0.185$	$p = 0.016$	$p = 0.740$	$p = 0.141$
C:N ratio faeces				1	0.150	0.081	**−0.348**	0.010
					$p = 0.200$	$p = 0.488$	$p = 0.002$	$p = 0.931$
$\delta^{15}N$ tissue					1	**−0.655**	0.155	0.028
						$p < 0.001$	$p = 0.183$	$p = 0.809$
$\delta^{13}C$ tissue						1	**−0.513**	−0.200
							$p < 0.001$	$p = 0.086$
C:N ratio tissue							1	0.217
								$p = 0.062$
Shell length								1

Note: Correlations between these variables and snail shell length are also shown. Transformations used for each variable are described in the main text. Statistically significant correlations are in bold.

Immune trait	Effect	β	*SE*	*t*	*p*
PO-like activity	Amount of faeces	−0.266	0.552	−0.483	0.631
	δ^{15}N faeces	**2.894**	1.361	2.127	0.037
	δ^{13}C faeces	−0.089	0.138	−0.640	0.524
	δ^{15}N tissue	**0.170**	0.053	3.181	0.002
	δ^{13}C tissue	**2.086**	0.860	2.425	0.018
	Shell length	−0.000	0.000	−0.384	0.702
Antibacterial activity	Amount of faeces	−0.318	0.173	−1.845	0.069
	δ^{15}N faeces	0.330	0.426	0.776	0.441
	δ^{13}C faeces	**−0.118**	0.043	−2.713	0.008
	δ^{15}N tissue	0.011	0.017	0.670	0.505
	δ^{13}C tissue	0.241	0.269	0.895	0.374
	Shell length	−0.000	0.000	−1.566	0.122

TABLE 2 Partial regression coefficients (β), their *SE*, and statistical significances (*p*) between the immune traits (haemolymph phenoloxidase [PO]-like and antibacterial activity) and variables representing the short-term (amount and stable isotope composition of produced faeces) and longer-term resource use (stable isotope composition of tissues) of uninfected snails ($n = 75$) based on multiple linear regressions (each immune trait analysed separately)

Note: Snail shell length was included in the analyses because it covaried with some of the variables (Table 1). The analyses examine the independent effects of each variable representing the snails' resource level and use after considering the covariation with other variables. Statistically significant effects are in bold.

negative correlation (Table 1), suggesting that the variation in δ^{15}N values probably reflects resource consumption from different trophic levels with different amounts of animal protein. Additionally, the PO-like activity increased with higher δ^{13}C values of the snails' tissues (Table 2, Figure 1c). The δ^{13}C values of the tissue samples had a negative correlation with their C:N ratio (Table 1), which suggests that δ^{13}C reflects the lipid content of tissues. The antibacterial activity of snail haemolymph increased with decreasing δ^{13}C values of the faeces (Table 2, Figure 1d). However, δ^{13}C did not correlate with the C:N ratio of the faeces samples (Table 1). Thus, δ^{13}C values are more likely to reflect variation in the origin of consumed resources (e.g., limnetic, benthic) than their lipid content.

4 | DISCUSSION

Host immune function is often condition dependent (Brzęk & Konarzewski, 2007; Siva-Jothy & Thompson, 2002; Slater & Keymer, 1986; Stahlschmidt et al., 2013), which arises from the energetic costs of maintaining and using the immune system (Moret & Schmid-Hempel, 2000; Sheldon & Verhulst, 1996). Research on the condition-dependence of immune function is typically conducted in laboratory experiments that manipulate either the quantity or composition of available resources (Cotter et al., 2011; Seppälä & Jokela, 2010). The results of such studies, however, are difficult to generalise to natural conditions, which is why the potential role of variation in host resource use in mediating host defence traits in field populations is still poorly understood. Here, we report condition-dependent immune (namely PO-like) activity in *L. stagnalis* snails under field conditions. The relationship between the snails' immune function and condition was driven by the composition of recently consumed food and the energy reserves of the snails. Specifically, snails that had recently consumed resources from higher trophic levels, with a potentially higher proportion of animal protein (i.e., high δ^{15}N values of faeces, δ^{15}N had a negative correlation with C:N ratio of faeces), had a stronger defence. Additionally, snails with low lipid reserves (i.e., low δ^{13}C values of tissues, δ^{13}C had a positive correlation with C:N ratio of tissues) showed high PO-like activity, potentially indicating substantial energetic costs of immune activity in *L. stagnalis*.

The dependence of the PO-like activity of snail haemolymph on recently consumed resources is in line with earlier research conducted under laboratory conditions. In an experimental study, Seppälä and Jokela (2010) showed that the resource level of snails affected haemolymph PO-like activity within 1 day when individuals fed ad libitum were compared to those that were food-deprived. Together with that observation, our results suggest that the PO-like activity of snail haemolymph could vary on fine spatial and temporal scales in nature, thus creating variation in the outcome of host–parasite interactions depending on the prevailing environmental conditions. Such variation could have broad ecological effects on disease dynamics and contribute to the maintenance of genetic variation in immune activity in natural populations. The latter effect is possible because, under laboratory conditions, the condition-dependence of PO-like activity has been shown to express high within-population family-level variation (i.e., a G × E interaction determining immune activity; Seppälä & Jokela, 2010).

In this study, the antibacterial activity of snail haemolymph, which is also reduced by experimental food deprivation (Seppälä & Jokela, 2010), did not depend on the δ^{15}N of recently consumed food (estimated from produced faeces). However, the antibacterial activity covaried with the δ^{13}C values of faeces. δ^{13}C can reflect the energy (i.e., lipid) content of samples because lipids contain more ^{12}C than other elemental nutrient sources (DeNiro & Epstein, 1977; Pinnegar & Polunin, 1999). In our data, however, this interpretation was not supported because the C:N ratio of the samples did not correlate with δ^{13}C values. Alternatively, δ^{13}C could reflect variation in the consumption of resources from

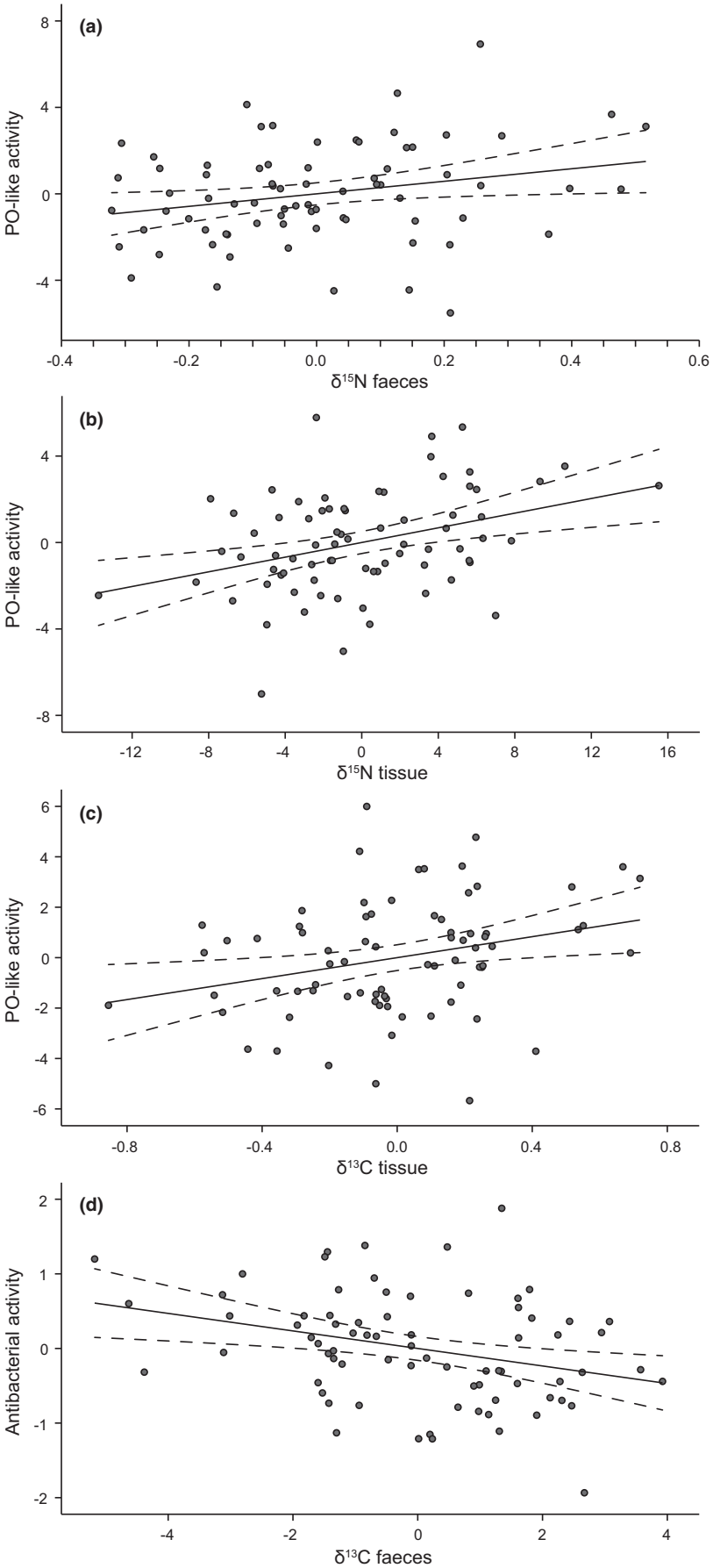

FIGURE 1 Partial regression plots for relationships between snail immune activity (phenoloxidase [PO]-like and antibacterial activity of haemolymph) and variables reflecting snail resource level and use that were statistically significant. (a) $\delta^{15}N$ of produced faeces, (b) $\delta^{15}N$ of tissue, (c) $\delta^{13}C$ of tissue, and (d) $\delta^{13}C$ of produced faeces. Solid lines show regression lines, and dashed lines their 95% confidence intervals. Transformations used for each variable are described in the main text

different origins such as terrestrial, limnetic, and benthic (Batt et al., 2012; Solomon et al., 2011). Typically, limnetic resources contain more light isotopes of C compared to benthic resources. However, the separation of terrestrial resources from other resource types is less clear and varies among studies (see Batt et al., 2012; Solomon et al., 2011). Therefore, variation in the use of resources from different origins could have contributed to the antibacterial activity of snail haemolymph.

Our results highlighting the importance of dietary proteins rather than lipids of available resources in determining snail haemolymph PO-like activity are in line with earlier research examining the relative importance of different macronutrients for immune function (Cotter et al., 2011; Lee et al., 2006; Ponton et al., 2020; Wilson et al., 2019). However, such dependence is not the case for all immune traits (Cotter et al., 2011; Ponton et al., 2020). For example, in a study on fruit flies, Ponton et al. (2020) showed that *Micrococcus luteus*-infected individuals shift to food rich in carbohydrates but poor in proteins, which increases the transcription of genes encoding for antimicrobial peptides. Such a shift in food preference could arise from increased energetic demands of immune-activated individuals (Demas et al., 2012; Sheldon & Verhulst, 1996). In our study, snails with low energy (i.e., lipid) reserves (low $\delta^{13}C$ values of tissues, $\delta^{13}C$ had a positive correlation with C:N ratio of tissues) showed high PO-like activity. The reason for this result is unknown, but it could arise if the snails varied in their exposure to parasites/pathogens before sampling, which could have activated their immune function differently. Thus, individuals with the highest immune challenge could have shown the highest levels of immune activity, which may have reduced their energy reserves. Additionally, the PO-like activity increased with $\delta^{15}N$ values of the snail tissue. This supports the above notion because $\delta^{15}N$ can increase when individuals consume their tissues as an energy source (Hobson et al., 1993; Olive et al., 2003). However, substantial energetic costs of immune activation would be required for these effects to arise owing to immune challenge. This is because trade-offs are often not visible in data sets that include variation in organismal physiological condition (reviewed in Reznick et al., 2000). Such variation is likely to be high in field data, including this study. Therefore, our results call for further laboratory experiments on energetic costs and trade-offs related to immune function/activation in *L. stagnalis*.

Parasites often impact their hosts' characteristics, ranging from physiological to life-history traits. In previous studies, parasite infections have been reported to influence host isotope composition in shrimps, water fleas, and marine snails (Miura et al., 2006; Pulkkinen et al., 2016; Sánchez et al., 2013). These effects may arise from altered host feeding behaviour and/or habitat use (Miura et al., 2006; Sánchez et al., 2013), and direct physiological effects of parasitism (Pulkkinen et al., 2016). However, similar to previous research on *L. stagnalis* (Doi et al., 2010) and marine molluscs (Dubois et al., 2009), our study did not indicate an effect of trematode infection on the snails' isotope composition. These findings suggest that the physiology and feeding behaviour (i.e., preference for different food types) of *L. stagnalis* are not altered by infection, although trematodes have other strong impacts on snails by, for example, castrating them (Karvonen et al., 2004; Seppälä et al., 2013).

To conclude, we found that the PO-like activity of snail haemolymph was related to snail resource level under natural conditions. Specifically, snails that had recently consumed food from higher trophic levels, potentially including more animal protein (i.e., high $\delta^{15}N$ of faeces), had the strongest defence. Additionally, snails with low energy (i.e., lipid) reserves in their tissues (high $\delta^{13}C$) that had possibly used their own tissues as an energy source (high $\delta^{15}N$) showed high PO-like activity. The importance of the composition of recently consumed food on immune function suggests that the parasite resistance of snails may change rapidly depending on the type of resources available in their environment. Thus, environmental variation may affect the outcome of host–parasite interactions on fine spatial and temporal scales. The present study, however, examined variation in the snails' resource level only in one location and at one point in time. Therefore, the consequences of variation in the composition of available resources both over space and time could not be estimated. Such variation may be larger and have a stronger influence on snail immune function than the variation detected in this study. Furthermore, the finding of the negative relationship between the snails' energy reserves and PO-like activity may indicate substantial energetic costs of immune activity in *L. stagnalis*. Considering the likely high variation in the physiological condition of the examined snails, which could hinder the expression of an energetic trade-off, the energetics of snail immune activity should be subjected to detailed experimental studies.

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AUTHORS' CONTRIBUTIONS

O.S. conceptualised the study; O.S., E.Y., and T.S. designed the study and collected the data; O.S. and T.S. analysed the data; O.S. led the writing of the manuscript; E.Y. and T.S. contributed to the draft. All authors gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data available from the Zenodo digital repository https://doi.org/10.5281/zenodo.6461677.

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