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Substrate specificity of phenoloxidase-like activity in an ecoimmunological model species *Lymnaea stagnalis*

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ABSTRACT

Ecoimmunological research on molluscs and other invertebrates frequently quantifies phenoloxidase (PO) activity to estimate the strength of the immune function. PO enzymes form different families whose relative roles in oxidative reactions are typically unknown. Understanding this could allow enzyme-specific assays with higher accuracy than in commonly used nonspecific assays. We tested the contribution of different PO enzyme families to haemolymph PO-like activity in *Lymnaea stagnalis* snails using substrates specific to enzymes detected in *L. stagnalis* transcriptome data (*p*-phenylenediamine, specific to laccases; L-tyrosine, specific to tyrosinases) and compared the reactions to those with a nonspecific substrate (L-dopa). We found laccase-like but no tyrosinase-like activity. However, reactions with L-dopa were the strongest, possibly due to other oxidative enzymes in snail haemolymph. Laccase-like activity is common in molluscs, and we propose the use of enzyme-specific assays in future ecoimmunological studies of this taxon. The lack of tyrosinase-like activity in *L. stagnalis* contradicts earlier transcriptome data, which calls for investigating the expression of PO enzymes in *L. stagnalis* at the proteome level.

INTRODUCTION

The freshwater snail *Lymnaea stagnalis* (Heterobranchia: Lymnaeida) is a model organism in multiple biological disciplines, including ecological immunology (reviewed by Fodor *et al.*, 2020). Earlier research in this species has focused on, for example, understanding the evolutionary ecology of immune function (e.g. natural selection: Langeloh, Behrmann-Godel & Seppälä, 2017; Langeloh *et al.*, 2023; evolutionary potential in immune activity: Seppälä & Jokela, 2010; Seppälä & Langeloh, 2016; Leicht, Seppälä & Seppälä, 2017) and the effects of various environmental factors on immunity (e.g. resource availability: Seppälä & Jokela, 2010; Seppälä, Yohannes & Salo, 2022; temperature: Leicht, Jokela & Seppälä, 2013; Leicht *et al.*, 2017; Salo *et al.*, 2017, 2019; chemical pollution: Gust *et al.*, 2013; Boisseaux *et al.*, 2014; Salo *et al.*, 2017).

In *L. stagnalis* (see Seppälä & Leicht, 2013), like in other molluscs (Scheil *et al.*, 2013; Unlu & Ekici, 2021; Friesen *et al.*, 2022) and other invertebrates in general (e.g. Adamo, 2004; Moret & Schmid-Hempel, 2009; Joop *et al.*, 2014), immune activity is frequently estimated by quantifying phenoloxidase (PO) activity of haemolymph. The proPO activating system is a component of humoral innate immunity with oxidative and cytotoxic effects (e.g. melanization; reviewed by Cerenius & Soderhäll, 2021). PO enzymes form three families based on their molecular recognition mechanisms, leading to partial substrate specificity. These families are as follows: (1) catecholases that oxidize *o*-diphenols; (2) laccases that oxidize *o*-diphenols, *p*-diphenols and *p*-diamines; and (3) tyrosinases that catalyse hydroxylation of monophenols and oxidation of *o*-diphenols (Thurston, 1994; Rescigno *et al.*, 1998; Dittmer & Kanost, 2010; Gorman *et al.*, 2012). Invertebrates express all three PO activities, but their relative roles in oxidative reactions are typically unknown. This is because PO activity is most often quantified using L-dopa (*o*-diphenol, oxidized by all PO enzymes) as a substrate. However, understanding the relative importance of different PO enzymes could be highly beneficial. If a certain PO enzyme dominates the reaction, then an enzyme-specific assay could improve measurements by providing stronger and more accurate reactions. This is because L-dopa is also oxidized by other enzymes, such as haemocyanins (reviewed by Cerenius & Soderhäll, 2021), and it is susceptible to auto-oxidization (e.g. Luna-Acosta *et al.*, 2010). However, if multiple PO enzymes contribute to the reaction, then the L-dopa assay may provide a useful composite measure for overall PO-like activity.

Recent transcriptome sequencing of *L. stagnalis* suggests that laccases and tyrosinases are likely to contribute to the PO activity in this species (Seppälä *et al.*, 2021; note that the presence of catecholases in the transcriptome data has not been examined). This observation calls for examining the relative roles of different PO enzymes in determining the observed activity. For instance, in the related *Biomphalaria* species, laccases contribute most strongly to the haemolymph PO activity (Le Clec'h, Anderson & Chevalier, 2016).

Therefore, in this study, we tested the contribution of different PO enzymes to haemolymph PO-like activity in *L. stagnalis* using two substrates that are specific to the enzymes detected in the earlier transcriptome data: *p*-phenylenediamine (PPD), which is specific to laccases, and L-tyrosine, which is specific to tyrosinases. Additionally, we used a nonspecific substrate, L-dopa, to compare the efficiency of enzyme-specific assays to the nonspecific assay used in earlier ecoimmunological research on *L. stagnalis*. In these tests, we confirmed the role of PO activity in the observed reactions using a PO inhibitor. We found that laccases, but not tyrosinases, contribute to the PO-like activity in *L. stagnalis* haemolymph. Additionally, using the nonspecific substrate, L-dopa led to the strongest reaction, suggesting the potential contribution of additional enzymes to PO-like activity. These findings suggest the possibility of using enzyme-specific assays in future ecoimmunological studies on *L. stagnalis* and potentially other molluscs.

MATERIAL AND METHODS

Study organisms

We used snails from a genetically diverse laboratory stock generated by combining parental snails from different natural populations. This 'mixed' population was established earlier to create experimental snails that represent our study species more generally than individual natural populations that are typically genetically highly differentiated (Kopp, Wolff & Jokela, 2012). We started the stock population during the breeding season of 2016 (late May) by collecting adult snails from six ponds in Northern Switzerland: Irchel (47°23′N, 8°32′E, $n = 30$), Zürichberg (47°23′N, 8°33′E, $n = 33$), Dättnau (47°29′N, 8°41′E, $n = 17$), Eschenberg (47°28′N, 8°43′E, $n = 36$), Kyburg (47°27′N, 8°44′E, $n = 33$) and Ittingen (47°35′N, 8°51′E, $n = 29$) (Supplementary Material Fig. S1). We brought the field-collected snails to the lab, placed them in a water tank (volume: 800 l; water temperature: 18 ± 2 °C), and allowed them to lay eggs for 11 days. Although a simultaneous hermaphrodite, *Lymnaea stagnalis* prefers reproduction through cross-fertilization (Puurtinen *et al*., 2004; Nakadera *et al*., 2017) and can store allosperm from matings for over 2 months (Nakadera, Blom & Koene, 2014). Therefore, the offspring of field-collected snails that started the stock population can be expected to adequately represent the genetic variation across populations in the study region. The stock population was maintained through mass breeding (*ad libitum* feeding with fresh lettuce and Spirulina powder) for 3 years (7–10 generations) before the experiment.

Experimental design

To test the substrate specificity of *L. stagnalis* PO-like activity, we used 42 haphazardly chosen adult snails (shell length: 31.5–41.7 mm) from the above laboratory stock. We conducted the experiment once, testing the haemolymph from each snail (i.e. 42 biological replicates) against three substrates (L-dopa, PPD and L-tyrosine) with and without a PO inhibitor sodium diethyldithiocarbamate (DETC). We could not include technical replicates because the amount of haemolymph per snail was limited. Note that in these tests, we used haemolymph rather than a pure source of PO enzymes. Haemolymph contains a broad range of enzymes, some of which are likely to have oxidative potential and could thus confound our results. However, haemolymph is currently the only practical target for investigation in ecoimmunological research across the study systems used.

We blot dried the snails on a paper towel and collected haemolymph samples from each individual by gently tapping the undersides of their feet with a pipet tip until they retreated into their shells, simultaneously releasing haemolymph through the haemal pore (Sminia, 1981). This behaviour is a normal antipredatory response in *L. stagnalis* (Rigby & Jokela, 2000). We immediately transferred six 50-μl samples of haemolymph from each snail to microcentrifuge tubes (a total of 300 μl of haemolymph per snail), snap-froze the samples in liquid nitrogen and stored them at −80 °C. Snap-freezing disrupts haemocytes, thus releasing enzymes that they contain. On average, the number of detectable cells in *L. stagnalis* haemolymph decreases by 26% owing to snap-freezing (K. Seppälä, personal communication). Mechanical homogenization of haemolymph does not increase enzyme activity (K. Seppälä, personal communication), suggesting that the remaining cells after snap-freezing are damaged. Storing the haemolymph at −80 °C could reduce its PO activity compared to fresh samples. However, the magnitude of such an effect in *L. stagnalis* is unknown and should be investigated in the future. This is because immediate sample processing is impossible in ecoimmunological research on *L. stagnalis* because of large sample sizes (see, e.g. Seppälä & Jokela, 2010; Seppälä & Langeloh, 2016; Leicht *et al*., 2017; Langeloh *et al*., 2023) and, in some studies, because of sampling in the field (Langeloh *et al*., 2017; Seppälä *et al*., 2022).

We next examined the relative importance of different PO enzyme families on haemolymph PO-like activity by testing the oxidative potential of the collected haemolymph samples on three different substrates that vary in their specificity to different PO enzymes: L-dopa (metabolized by all PO enzymes), PPD (metabolized by laccases) and L-tyrosine (metabolized by tyrosinases). Note that the detailed biochemical characterization of PO activities (see, e.g. Quinn *et al*., 2020) was beyond the scope of this study because of its limited use in ecoimmunology. We also did not activate the 'total PO' of haemolymph using, for example, chymotrypsin (e.g. Waite & Wilbur, 1976; Laughton & Siva-Jothy, 2011) because we were interested in the active forms of PO that reflect a snail's ability to respond to infections.

We thawed the frozen haemolymph samples on ice and centrifuged them at 4000 *g* for 15 min to remove cell debris. Centrifuging may lead to losing some of the active PO in haemolymph if PO enzymes adhere to each other and/or other proteins. In *L. stagnalis*, this loss is a maximum of 5% of enzyme activity (K. Seppälä, personal communication). We transferred 40 μl of the supernatant from each sample to a well of a 96-well flat-bottom microwell plate (Greiner Bio-One GmbH, Austria) and added 120 μl of either L-dopa (4 g/l in cacodylate buffer, that is, 10 mM sodium cacodylate and 10 mM calcium chloride; pH = 8.4), PPD (2.2 g/l in cacodylate buffer) or L-tyrosine (2 g/l in cacodylate buffer). We aimed to offer a high amount of substrate in all measurements to prevent their availability from limiting reactions. Variation in substrate solubility, however, led to differences in the used molar concentrations, L-tyrosine being lower compared to other substrates. In these tests, we used one haemolymph sample per snail per substrate. Additionally, we analysed one haemolymph sample per snail per substrate while adding sodium DETC (1.1 g/l in 10 mM cacodylate buffer), a specific PO inhibitor (Bai *et al*., 1997), into each well. To control for autoxidation of the substrates, we included five wells per microtiter plate in which haemolymph was substituted with nanopure water.

The oxidation of the substrates by PO enzymes produces dopachrome, which leads to an increase in the optical density (OD) of the solution. We quantified this change in each microtiter plate well spectrophotometrically, using a microtiter plate reader (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA). We used the wavelength of 490 nm to determine OD in wells with L-dopa and L-tyrosine, and 465 nm for wells with PPD as a substrate. These wavelengths provide the maximum absorption for each oxidized end product (Le Clec'h *et al*., 2016). In the reactions, we measured OD in 20-s intervals for 40 min at 37 °C (an optimal temperature in *Biomphalaria* spp.; Le Clec'h *et al*., 2016) and calculated PO-like activity as an increase in OD (i.e. OD after 40 min–OD at time 0; see examples of typical reactions in Supplementary Material Fig. S2). We could not estimate the

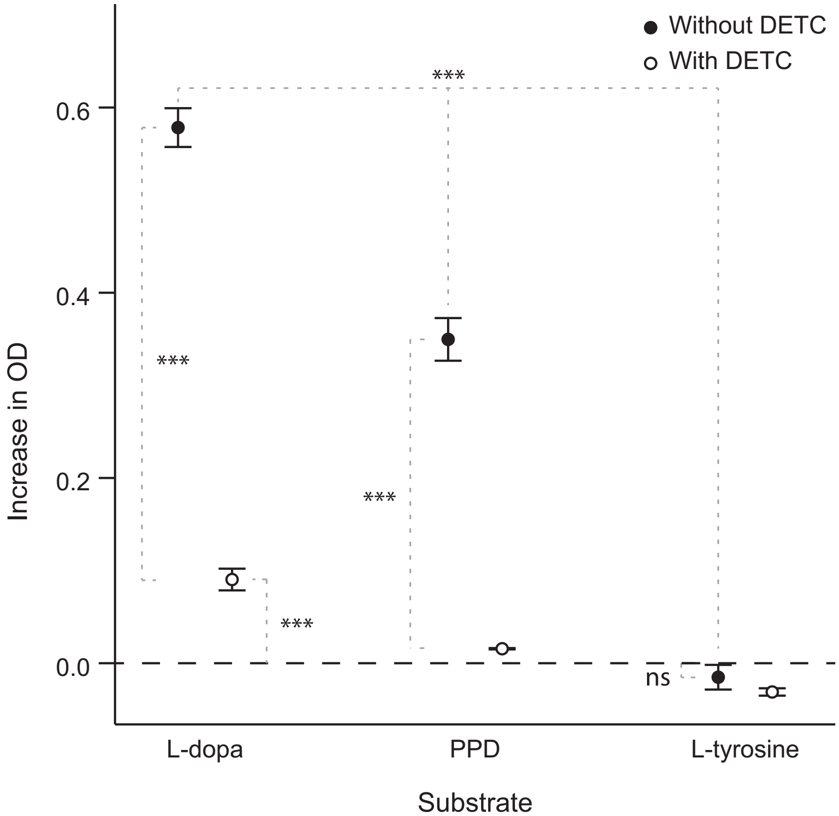

Figure 1. Phenoloxidase (PO)-like activity of *Lymnaea stagnalis* haemolymph (42 snails) measured as an increase in optical density (mean ± SE) using three different substrates with distinct enzyme specificities. L-dopa is oxidized by all PO enzymes (catecholases, laccases and tyrosinases) and also by other enzymes, such as haemocyanins. PPD and L-tyrosine are enzyme-specific substrates oxidized by laccases and tyrosinases, respectively. Black error bars show the total activity of haemolymph, and white error bars show the activity with a PO inhibitor sodium DETC. Symbols/abbreviations: ***, statistical significance <0.001, ns: not significant.

auto-oxidation of L-tyrosine because the OD in tests in which haemolymph was replaced with water (i.e. controls) showed high and irregular variation over time. However, snail haemolymph did not show any tyrosinase activity (see the Results section), which is why the results could not be confounded by auto-oxidation. In the case of L-dopa and PPD, we estimated the proportion of reactions that may arise due to auto-oxidation (i.e. the average increase in OD in tests with water/average increase in OD in tests with haemolymph).

Statistical analyses

To estimate the substrate specificity of *L. stagnalis* PO-like activity, we first analysed the variation in the strength of the reactions in tests conducted without DETC. We did this using the analysis of variance (ANOVA) for correlated samples (i.e. within-subjects ANOVA). We used the strength of the reactions with different substrates as a within-subject factor because all three substrates were tested using the same experimental snails. In the analysis, we corrected the degrees of freedom using the Greenhouse–Geisser correction because of the lack of sphericity in the data. After that, we compared the strength of the reactions between the tests conducted with and without DETC separately for each substrate using paired-sample *t*-tests. Furthermore, we tested if the changes in OD differed from zero separately for the tests conducted with and without DETC on each substrate type using one-sample *t*-tests.

RESULTS

The strength of the oxidative reactions detected when estimating the PO-like activity of snail haemolymph using the three different substrates L-dopa, PPD and L-tyrosine showed large differences (tests without DETC) (ANOVA for correlated samples with Greenhouse–Geisser correction: $F_{1.494,61.258} = 339.246$, $P < 0.001$). The reactions were strongest when measured using L-dopa (Fig. 1; 1.6% of the reaction arose from auto-oxidation). The use of the PO inhibitor DETC did not completely inhibit the oxidation of L-dopa [one-sample *t*-test (reference value = 0): $t_{41} = 7.713$, $P < 0.001$], but reduced its strength by 88.4% compared to the tests without DETC (paired-sample *t*-test: $t_{41} = 31.116$, $P < 0.001$; Fig. 1; no auto-oxidation in reactions with DETC). This suggests that in *Lymanea stagnalis*, nonPO enzymes may also oxidize L-dopa, although their contribution is smaller than that of POs. The use of PPD as a substrate led to the second strongest reactions; these were 39.5% weaker compared to the tests using L-dopa (Fig. 1; 8.0% of the reaction without DETC arose from auto-oxidation). Additionally, DETC led to almost complete inhibition of the oxidation of PPD (paired-sample *t*-test: $t_{41} = 14.725$, $P < 0.001$; Fig. 1; 94.3% of the reaction with DETC was due to auto-oxidation). This indicates laccase-like activity in *L. stagnalis* haemolymph, and that the measurements were not confounded by nonPO enzymes. The use of L-tyrosine as a substrate did not lead to oxidative reactions [one-sample *t*-test for tests without DETC (reference value = 0): $t_{41} = -1.135$, $P = 0.263$; Fig. 1], suggesting that *L. stagnalis* snails do not express tyrosinase activity.

DISCUSSION

The freshwater snail *Lymnaea stagnalis* is a commonly used model organism in ecological immunology (reviewed by Fodor *et al.*, 2020). In common with many other invertebrates (e.g. Adamo, 2004; Moret & Schmid-Hempel, 2009; Joop *et al.*, 2014), research conducted using *L. stagnalis* utilizes L-dopa (see Seppälä & Leicht, 2013) as a nonspecific substrate for an oxidative reaction induced by PO enzymes from different families (Thurston, 1994; Rescigno *et al.*, 1998; Dittmer & Kanost, 2010; Gorman *et al.*, 2012). However, understanding the relative importance of different PO enzymes in the reaction would be beneficial. First, if a certain PO enzyme dominates the reaction, then measurements could be optimized using an enzyme-specific substrate. Second, if multiple PO enzymes contribute to the reaction, then the L-dopa assay could be a useful composite measure for overall PO activity. In this case, however, the possible role of other factors that oxidize L-dopa (e.g. haemocyanins) should be considered.

By testing the substrate specificity of PO-like activity in *L. stagnalis* haemolymph, we demonstrated that there are strong oxidative reactions when the enzyme-specific substrate PPD is used. Because the PO inhibitor DETC suppressed this reaction almost entirely, our result indicates laccase-like activity in *L. stagnalis*. Laccases have also been detected in a recent RNA-seq study that sequenced the transcriptomes of *L. stagnalis* snails exposed to different immune elicitors and changes in environmental conditions (Seppälä *et al.*, 2021). In that study, the transcription of laccases increased when the snails were exposed to bacteria and trematode-infected gonad tissue taken from another snail, and it also varied among the examined individuals (Seppälä *et al.*, 2021). Together, these findings indicate high suitability and relevance for examining haemolymph laccase-like activity in future ecoimmunological research conducted at the phenotypic level. However, auto-oxidation of PPD (8% of the total reaction in this study) needs to be considered using non-haemolymph controls in the assay.

The use of another enzyme-specific substrate, L-tyrosine, did not lead to an oxidative reaction. This result suggests that tyrosinases do not contribute to the PO-like activity of *L. stagnalis* haemolymph. Our finding is surprising because tyrosinases have been detected in *L. stagnalis* immune transcriptomes (Seppälä *et al.*, 2021). The transcription of tyrosinases also shows high among-individual variation, thus suggesting their potential relevance for ecoimmunological research (Seppälä *et al.*, 2021). The discrepancy between the transcriptome and phenotypic levels could have arisen for at least two reasons. First, *L. stagnalis* immune transcriptomes were conducted on homogenized whole-body tissues rather than haemolymph. Thus, tyrosinases could be expressed in tissues that were not examined in this study. Alternatively, although transcribed, the expression of tyrosinases may stop post transcription in *L. stagnalis*. In addition to transcription, the gene expression pathway includes translation, protein-folding and subunit aggregation (see, e.g. Alberts *et al.*, 2015), which are crucial steps in determining organismal phenotypes. In fact, although mRNA and protein levels for specific genes and their products are often substantially positively correlated with each other, this is not always the case (reviewed by Buccitelli & Selbach, 2020). Gene transcription is increasingly used in ecoimmunology as a substitute for phenotypic measures (e.g. Barribeau *et al.*, 2014; Brunner, Schmid-Hempel & Barribeau, 2014; Ferro *et al.*, 2017; Greenwood *et al.*, 2017), but our result suggests that variation in the transcription of immune genes may not always reflect host immune capacity.

Despite the enzyme-specific reaction to PPD indicating laccase-like activity in *L. stagnalis*, the nonspecific substrate L-dopa showed the strongest oxidative reaction in this study. This finding suggests that enzymes other than laccases also contribute to the reaction. These may include, for example, PO enzymes from the family catecholases, as well as other enzymes that oxidize L-dopa, such as the haemocyanins (reviewed by Cerenius & Soderhäll, 2021), which are known also from *L. stagnalis* (Schäfer *et al.*, 2019). Because the PO inhibitor DETC did not entirely suppress the oxidation of L-dopa, nonPO enzymes are likely to explain part of the reaction. These findings suggest that the use of L-dopa as a substrate in a PO assay could provide a composite measure for the activity of different PO enzyme families. Still, the measurement may be confounded by the contribution of other enzymes, on average >10%. However, the auto-oxidation of L-dopa was weak (1.6% of the reaction). Our results on the oxidation of L-dopa call for further examination/characterization of the enzymes contributing to the reaction at the protein level and/or for the use of PO inhibitors that are specific to certain enzyme families (e.g. Luna-Acosta *et al.*, 2010).

In other mollusc species in which the substrate specificity of PO activity has been examined, laccase activity, as indicated by the oxidation of PPD, has been detected in two *Biomphalaria* species (Le Clec'h *et al.*, 2016), *Crassostrea gigas* (Luna-Acosta *et al.*, 2010), *Crepidula fornicata* (Quinn *et al.*, 2020), *Haliotis tuberculata* (Le Bris *et al.*, 2014) and *Venerupis philippinarum* (Le Bris *et al.*, 2013). Additionally, catecholase activity and weak tyrosinase activity have been detected in *C. fornicata* (Quinn *et al.*, 2020) and *V. philippinarum* (Le Bris *et al.*, 2013), respectively. These findings suggest that laccases play an important role in molluscan PO activity. However, differences in the contribution of various enzymes to PO activity still exist among species. For example, in *Biomphalaria*, PO activity is likely driven mainly by laccases because the oxidation of the nonspecific substrate L-dopa is much weaker compared to PPD, which is specific to laccases (Le Clec'h *et al.*, 2016). In this study, however, *L. stagnalis* haemolymph showed stronger oxidation of nonspecific L-dopa than of PPD. Because laccases may oxidize L-dopa less efficiently than PPD, our result suggests broader diversity of enzymes oxidizing L-dopa in *L. stagnalis* compared to *Biomphalaria* species. The difference between the taxa could arise, for example, because haemocyanin, which is highly abundant in *L. stagnalis* but not in *Biomphalaria*, which uses haemoglobin to transfer oxygen (Jones, 1964). This result and the mismatch between the observations on tyrosinases at phenotypic and transcriptome levels call for a broad comparative investigation of PO enzymes at different levels (e.g. transcriptome, proteome and phenotype) across species in the phylum Mollusca.

To conclude, our results using substrates specific to oxidation by certain PO enzymes showed strong laccase-like but no tyrosinase-like activity in *L. stagnalis* haemolymph. These findings reflect a typical pattern in molluscs (see Luna-Acosta *et al.*, 2010; Le Bris *et al.*, 2013, 2014; Le Clec'h *et al.*, 2016; Quinn *et al.*, 2020) and support the use of enzyme-specific assays in future ecoimmunological studies. However, we detected the strongest oxidative reactions when a nonspecific substrate was used. The reason for this is unknown, which calls for further investigations on other PO enzymes (i.e. catecholases) and other proteins with oxidative potential in *L. stagnalis*. Because the lack of tyrosinase-like activity in this study does not align with a recent RNA-seq study in which tyrosinases were detected (Seppälä *et al.*, 2021), further examinations of *L. stagnalis* PO-like activity should be conducted at the protein level. More generally, our findings encourage investigating the role of different PO enzymes in determining PO activity across ecoimmunological model species to strengthen research through improved PO assays.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Molluscan Studies* online.

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AUTHORS' CONTRIBUTIONS

O.S. conceptualized the study, O.S. and T.S. planned the experiment, T.S. collected the data, O.S. and T.S. analysed the data and O.S. wrote the manuscript with T.S. contributing to the text.

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