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Laccase and catecholoxidase activities contribute to innate immunity in slipper limpets, *Crepidula fornicata*

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Abstract

The slipper limpet *Crepidula fornicata* is an invasive, non-native, marine species found throughout the coastal waters of southern England and Wales, UK. These limpets are considered to blight commercial shellfish banks, notably oysters, yet little is known about their disease-carrying capacity or their immunobiology. To address the latter, we isolated haemolymph (blood) from limpets and tested for the presence of the immune-enzyme phenoloxidase. Invertebrate phenoloxidases produce melanic polymers from simple phenolic substrates, which are deployed in the presence of pathogens because of their potent microbicidal and microbiostatic properties. We used a series of established substrates (e.g., tyrosine, hydroquinone) and inhibitors (e.g., 4-hexylresorcinol, benzoic acid) to target three distinct enzymes: laccase (*para*-diphenoloxidase), catecholoxidase (*ortho*-diphenoloxidase) and tyrosinase (monophenoloxidase). We confirmed laccase and catecholoxidase activities and characterised their kinetic properties across temperature and pH gradients (5 – 70°C and 5 – 10, respectively). Crucially, we demonstrated that products derived from such laccase and catecholoxidase activities reduced significantly the numbers of colony-forming units of both Gram-positive and Gram-negative bacteria *in vitro.* We further screened limpet tissues for signs of melanin using wax histology, and found cells replete with eumelanin-like pigments and lipofuscin in the digestive gland, connective tissues, barrier epithelia and gills. Our data represent the first account of enzyme-based antibacterial defences, notably laccase, in *C. fornicata*.

Keywords:
Phenoloxidase; Innate immunity; Gastropod; Melanogenesis; Invasive species; Lipofuscin; Haemocyanin

Abbreviations:

ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); **CO**, catecholoxidase; **CTAB**, Cetrimonium bromide; **DHPPA**, 3,5-Dihydroxyphenylpropionoic acid; **L-DOPA**, L-3,4-Dihydroxyphenylalanine; **EDTA**, ethylenediaminetetraacetic acid; **4-HA**, 4-hydroxyanisole; **4-HR**, 4-hexylresorcinol; **PO**, phenoloxidase; **PPD**, *para*-Phenylenediamine; **PTU**, phenylthiourea; **Syringaldazine**, 4-Hydroxy-3,5-dimethoxybenzaldehyde azine; **TY**, tyrosinase

1. Introduction

An indispensable innate immune defence strategy of invertebrates is the use of phenoloxidase (PO) enzymes in the haemolymph and solid tissues to trigger melanin synthesis (Smith and Soderhall, 1991; Cerenius *et al.,* 2008). The catalytic steps involved in converting simple phenolic substrates (e.g., tyrosine, dopamine) into pigment precursors (quinones), and ultimately melanin, generate antimicrobial by-products in the form of reactive oxygen/nitrogen species as well as semi-quinone intermediates (Zhao *et al.,* 2007 and 2011; Cerenius *et al.,* 2010a; Xing *et al.,* 2012; Coates and Talbot, 2018). Often, the term phenoloxidase (PO) is used interchangeably to represent several distinct copper-containing enzymes: tyrosinase (EC 1.14.18.1), catecholoxidase (EC 1.10.3.1) and laccase (EC 1.10.3.2). Substrate and inhibitor specificities can be employed to discriminate between these phenoloxidases (POs). Tyrosinase catalyses the *ortho*-hydroxylation of monophenols (e.g., L-tyrosine) into *ortho*-diphenols (e.g., L-DOPA), and the two-electron oxidation of *o*-diphenols into *o*-quinones (e.g., DOPAchrome). Catecholoxidase performs the second reaction only, whereas laccase carries out the single-electron oxidation of both *ortho* and *para*-diphenols amongst other substrates (e.g., *para*-diamines; Reiss *et al.,* 2013; Whitten and Coates, 2017). The differences in catalysis can be attributed to their active sites; laccase contains a mononuclear (type1) copper site as well as a trinuclear copper cluster, whereas tyrosinase and catecholoxidase contain a dinucelar (type 3) copper site (Solomon *et al.,* 2014). Such structural features of laccase facilitate its wide catalytic potential.

Once pathogens breach the physical barriers of the exoskeleton or integument, they are recognised in the haemolymph by circulating haemocytes equipped with pathogen recognition receptors that stimulate the proteolytic, prophenoloxidase activation

77 cascade amongst other acute phase effectors (Cerenius *et al.,* 2010b). Melanic
78 polymers are generated and used to immobilise pathogens and facilitate their
79 destruction – usually in concert with haemocyte encapsulation and nodulation. Beyond
80 innate immunity, phenoloxidases contribute to developmental morphogenesis, cuticle
81 hardening and sclerotization post-ecdysis, and assist in clot development at wound
82 sites (haemostasis; Bidla *et al.,* 2009; Eleftherianos and Revenis, 2011). Melanin-
83 mediated defences have been studied extensively in insects (reviewed by González-
84 Santoyo and Córdoba-Aguilar, 2012), crustaceans (reviewed by Cerenius *et al.,* 2008),
85 and to a lesser extent, bivalves (Zhou *et al.,* 2012; reviewed by Luna-Acosta *et al.,*
86 2017). Conversely, such experimental evidence for a proPO cascade or tyrosinase is
87 lacking for gastropods – an exception being the well-characterised (inducible)
88 phenoloxidase activity of the oxygen-transport protein haemocyanin (Siddiqui *et al.,*
89 2006; Dolashki *et al.,* 2011; Raynova *et al.,* 2013; Coates and Nairn, 2014; Coates
90 and Costa-Paiva, 2020). Like the vast majority of invertebrates studied thus far, the
91 gastropod innate immune repertoire consists of physical barriers (exoskeleton),
92 cellular (haemocyte) and humoral (soluble) defences (Loker, 2010). To the best of our
93 knowledge, in-depth biochemical characterisations of gastropod phenoloxidase(s)
94 have been performed on the commercially important abalone genus *Haliotis* (Le Bris
95 *et al.,* 2014) and medically important snail genus *Biomphalaria* (Le Clec'h *et al.,* 2016).
96 In both instances, laccase-type phenoloxidase was the dominant form of activity
97 recorded.

98 The slipper limpet *Crepidula fornicata* (Linnaeus, 1758) is an invasive, non-native,
99 marine gastropod in the Calyptraeidae family. It is native to the east coast of the United
100 States of America but is now a pertinent example of an introduced species that can
101 influence its non-native range (Orton, 1926; Cole and Baird, 1953; McNeill *et al.,* 2010;
102 Bohn *et al.,* 2012). Slipper limpets were introduced accidently to European coastal
103 waters at the end of the 19th century, most likely with shipments of *Crassostrea*
104 *virginica* being imported for the establishment of aquaculture (Blanchard, 1997). These
105 limpets can be found in large numbers in most oyster production areas in England and
106 Wales, and are implicated in having a major negative impact on native bivalves,
107 especially the European flat oyster *Ostrea edulis* (Hayer *et al.,* 2019). In shallow bays,
108 *C. fornicata* can smother the sediment forming beds with several thousand individuals
109 per m^2. Dense populations of *C. fornicata* can trap suspended silt, faeces and pseudo-

faeces altering the composition and structure of the seabed (Chauvaud *et al.,* 2000). Despite the sizeable volume of literature dedicated to the ecology of slipper limpets and their interactions with shellfish of commercial value, there remains a paucity of knowledge on their disease profiles, immunobiology or haemolymph biochemistry.

To address the current knowledge gap, the overall aim of this study was to examine the haemolymph of *C. fornicata* for the presence of the immune enzyme, phenoloxidase. First, we used a combination of general and specific substrates and inhibitors to discriminate between putative phenoloxidases (monophenolase, *para*- and *ortho*-diphenolase). Second, we assessed the antiseptic properties of enzyme-catalysed reaction products toward Gram-positive/negative bacteria, and third, we inspected limpet tissues for evidence of melanin using a histological approach.

2. Materials and Methods

All chemicals/reagents used were of the highest purity available from Sigma-Aldrich (Dorset, UK) at the time of purchase.

2.1 Experimental animals

Field sampling and collection of live adult *C. fornicata* stacks (Figure 1A) took place in the low intertidal zone (~0.8-1.5m above chart datum) at Mumbles Beach, Swansea, South Wales, UK (51.571882, -3.987040). Samples were returned to the laboratory and processed immediately. Individuals were separated from stacks and cleaned of epibionts.

2.2 Isolation and preparation of haemolymph

Haemolymph was isolated from the animals by first removing the tissue mass from the shell using a blunt-ended probe and allowing the haemolymph to pool in the shell cavity (Figure 1B). The haemolymph was collected using a 22-gauge hypodermic needle fitted to a 1 mL sterile syringe. Haemolymph samples were combined from 3 to 5 limpets per replicate and centrifuged at 1000 x *g* for 5 min at 4°C to separate the haemocyte fraction. The cell-free supernatant was retained, stored at 4°C, and used in enzyme assays within 1 – 2 days (no deterioration was observed for this duration).

142

143 *2.3 Protein determination of the haemolymph*

144 The total protein content of the *C. fornicata* acellular fraction of haemolymph was
145 quantitated by the Biuret method (Gornall *et al.,* 1949), using egg albumin (0 – 20 mg
146 mL^{-1}) as a protein standard.

147

148 *2.4 Assay for phenoloxidase-like activities*

149 Phenoloxidase activities were assayed spectrophometrically in 96-well microplates
150 (Greiner 96-F-bottom) or 1 mL cuvettes using a BMG LABTECH SPECTROstar Nano
151 equipped with a cuvette port and microplate reader. Each assay consisted of 100 mM
152 sodium phosphate (NaPi) buffer pH7.4 and 1 $mgmL^{-1}$ haemolymph protein (pre-
153 incubated at room temperature (~20^{o}C) for 5 minutes). Substrates were added at
154 varying concentrations (listed in Table 1) to initiate the reaction and run for 10 minutes
155 (initial assays with representatives from all substrate types were run for 40 minutes,
156 but rates of product accumulation slowed after 10 minutes). All assays were performed
157 in triplicate (three technical replicates per biological replicate) at 20^{o}C. Results were
158 systematically corrected for non-enzymatic autoxidation of each substrate in the
159 absence of cell-free haemolymph. Enzymatic activities were recorded and converted
160 to units [U: µmol per minute per mg (protein)] using the following absorption
161 coefficients and wavelengths: 36,000 M^{-1} cm^{-1} for $ABTS^{+}$ (oxidised ABTS, A420 nm),
162 65,000 M^{-1} cm^{-1} for syringaldazine^{+} (oxidised syringaldazine, A525 nm), 1,370 M^{-1} cm^{-1}
163 for benzoquinone (oxidised hydroquinone, A390 nm), 1,910 M^{-1} cm^{-1} for PPD^{+}
164 (oxidised *p*-Phenylenediamine, A520 nm), and 3,600 M^{-1} cm^{-1} for DOPAchrome and
165 its derivatives (oxidised $_{L}$-DOPA, dopamine and caffeic acid, A492 nm).

166

167 *2.5 Inhibition of phenoloxidase-like activities*

168 Assays were prepared as described above; however, haemolymph protein (1 $mgmL^{-1}$)
169 was pre-incubated with an inhibitor for 5 minutes prior to the addition of substrate,
170 either hydroquinone (5 mM) or dopamine (5 mM). The inhibitors benzoic acid, citric
171 acid, cetrimonium bromide (CTAB), ethylenediaminetetraacetic acid (EDTA), 4-
172 hexylresorcinal (4-HR), and phenylthiourea (PTU) were used across the concentration
173 range 0.1 – 1 mM. Each combination of substrate and inhibitor was carried out in
174 triplicate on three independent occasions. Inhibition data are expressed as the

175 percentage reduction in enzymatic activity when compared to control values (i.e.,
176 substrate only).
177
178 *2.6 Influence of pH and temperature on phenoloxidase-like activities*

179 Assay mixtures were prepared as stated above (section 2.4), with 1 mgmL^{-1} protein, 5
180 mM of substrate (ABTS, dopamine or hydroquinone) in NaPi pH7.4, and incubated at
181 20^{o}C for 10 minutes prior to product quantification (Table 1). To find the optimum
182 temperature of all three enzyme-ligand combinations, reactions were run between 5^{o}C
183 and 70^{o}C. To find the optimum pH, the NaPi buffer was adjusted to values ranging
184 from 5 to 10 (in increments of 0.5).
185
186 To gain insight into the haemolymph pH of *C. fornicata in situ*, 141 fresh limpets were
187 collected in March 2019. Haemolymph was isolated from every animal (as described
188 in section 2.2) and screened using Mquant® Universal pH indicator strips.
189
190 *2.7 Bacterial culture and antibacterial assays*

191 Laboratory strains of Gram-positive (B*acillus megaterium, B. subtilis, Micrococcus*
192 *luteus*) and Gram-negative (*Escherichia coli* K12, *Pantoea agglomerans*) bacteria
193 were sourced from Blades Biological Ltd (Kent, UK). Single colonies were picked from
194 nutrient agar (Thermo Scientific) and cultured overnight in liquid medium at 37^{o}C,
195 except *P. agglomerans*, which was grown at 30^{o}C. Optical density values were
196 recorded using a V-1200 spectrophotometer. Once bacterial suspensions reached an
197 OD_{600} value of 1, cells were pelleted via centrifugation at 1000 *x* g for 5 min (room
198 temperature), washed twice in NaPi pH 7.4, and diluted in the same buffer to yield 1
199 x10^{6} colony forming units (CFUs) per mL.
200
201 Upon completion of phenoloxidase assays using 5 mM of substrate ($_{L}$-DOPA,
202 dopamine, hydroquinone), reaction volumes were centrifuged at 4000 *x* g for 5 minutes
203 (room temperature) using Amicon Ultra Filter Units (Millipore) with a 10 kDa molecular
204 weight cut-off to remove any potential laccase or catecholoxidase enzymes. Reaction
205 filtrates (100 µL) were mixed with bacterial suspensions in a 1:1 ratio and incubated
206 at room temperature for 1 hour. Following incubation, samples were diluted serially in
207 NaPi pH 7.4 so that ~200 CFUs were spread onto nutrient agar and allowed to grow

at 30°C (*P. agglomerans*) or 37°C (all other bacteria) for ≤48 hours. Control assays in the absence of substrate, and in the presence of an inhibitor (1 mM PTU), were run to attribute antibacterial activity to laccase- and catecholoxidase-derived products only.

2.8 Histology of Crepidula fornicata *soft tissues*

Whole tissue histology of *C. fornicata* was used to screen a subset (*n* = 10) for signs of tissue pigmentation, namely melanin. Intact tissues were separated from limpet shells using a blunt-ended probe, submerged in Davidson's seawater fixative (Hopwood, 1996) for 24 hours, and washed in dH_2O prior to storage in 70% ethanol. Samples were dehydrated using an ethanol series, 70%, 80% and 90% for 1 hour each, followed by 3x 1 hour in 100% ethanol. These samples were washed twice in HistoClear/HistoChoice for 1 hour each prior to immersion in paraffin wax: HistoChoice (1:1) for 1 hour. Embedded samples were cut into sections 5 – 7 µm in thickness (using a Leica RM2245 microtome), adhered to glass slides using egg albumin (~1% w/v), and dried for 24 hours. Slides were stained using Cole's haematoxylin and eosin. Stained slides were inspected and imaged using an Olympus BX41 microscope.

2.9 Data handling

All values reported here represent the mean ± standard error. Enzyme assays were performed in triplicate on three independent occasions. Michaelis-Menten non-linear regression and Lineweaver-Burk plots were used to calculate K_M and V_{max} values. Antibacterial assays were also performed in triplicate on three independent occasions, with data being analysed using 2-way ANOVA and Tukey's multiple comparison (post-hoc) tests. Statistical differences were considered significant when $P \leq 0.05$. Data analyses and visualisations were performed in GraphPad PRISM v7. Histology images were adjusted for contrast and colour balance only.

3. Results

3.1 Characterising phenoloxidase-like activities in the haemolymph of Crepidula fornicata

Using a broad series of known phenoloxidase substrates, we confirmed enzymatic activity in the presence of three *ortho*-diphenols, one *para*-diphenol, two methoxy-

containing phenols, and one non-phenolic *para*-diamine (Figure 2, Supplementary Figure 1). At concentrations ≤10 mM for caffeic acid, dopamine and L-DOPA, ≤15 mM for hydroquinone, ≤20 mM for ABTS and *p*-phenylenediamine, and ≤50 mM for syringaldazine, kinetic data were calculated using the Michaelis-Menten equation and Lineweaver-Burk intercepts (Table 2). Goodness of fit values (R^2) for all regressions ranged from 0.74 – 0.96. The Michaelis constant K_M for all three *ortho*-diphenols was <1.5 mM, with L-DOPA being the lowest at 0.26 mM, which suggests it is the preferred substrate *in vivo*. Hydroquinone (*p*-diphenol) had a similarly low K_M value of 2.05 mM, however, its maximum velocity (V_{max}) of ~4.4 U was 3-fold higher than L-DOPA and 1.8-fold higher than dopamine (1.4 U and 2.5 U, respectively; Figure 2, Table 2). The highest V_{max} value of 5.7 U was recorded for the exogenous substrate ABTS (a methoxy-containing phenol), but this was accompanied by the highest K_M value of 21 mM – indicating the enzyme-ligand complex is not stable. Under our experimental conditions, we did not observe any measurable activity in the presence of three common monophenols (4-hydroxyanisole, tyramine, L-tyrosine) or a single *meta*-diphenol (DHPPA) using concentrations from 0.1 mM to >25 mM. Additionally, the use of sodium dodecyl sulphate (SDS) at concentrations in excess of critical micelle formation (~3.5 mM) did not enhance enzymatic activity of the haemolymph protein (data not presented).

Enzyme-catalysed turnover of substrates was assessed further using a series of known phenoloxidase inhibitors (Table 3). Citric acid and benzoic acid are non-specific inhibitors of PO activity, and concentrations in excess of 0.1 mM thwarted product formation by 71 – 100%, regardless of the substrate used. As the active sites of POs use copper to facilitate catalysis, the metal chelator EDTA decreased dopamine oxidation by 86 – 100% and hydroquinone oxidation by 72 – 100% (Table 3). Using the laccase-specific inhibitor CTAB, and the laccase-specific substrate hydroquinone, activity diminished by 100%. However, using CTAB at the highest concentration of 1 mM in the presence of dopamine, did not eliminate all enzyme activity (~10% left) – indicating the presence of a second phenoloxidase. Using the tyrosinase- and catecholoxidase-specific inhibitor 4-hexylresorcinol (at 0.5 and 1 mM), enzyme activity decreased by >80%. 4-Hexylresorcinol had little impact when hydroquinone replaced dopamine, with 90% of enzyme activity remaining intact (Table 3).

To gain insight into endogenous conditions, we collected fresh limpets, isolated the haemolymph, and measured the pH. Values ranged from 7 – 9 with an average pH of 7.5 ± 0.15 (n = 141). Following this, we selected representatives of the three substrate classes with the highest V_{max} values, ABTS (5.7 U), dopamine (2.5 U) and hydroquinone (4.4 U; Table 2), and determined activity across a pH (5 – 10) gradient *in vitro* (Figure 3A). Maximum levels of ABTS oxidation occurred at pH 5.5, whereas the enzymatic turnover of dopamine and hydroquinone (into dopaminechrome and benzoquinone) were highest at pH 7 and 8, respectively. Subjecting the haemolymph samples to increasing temperatures from 5 – 70°C, revealed temperature optima of 35°C for dopamine and 45°C for hydroquinone (Figure 3B). Under these conditions, there were no substantial differences in product formation from hydroquinone between temperatures 35 and 50°C (89 – 100% inclusive).

3.2 Antibacterial potency of enzymatic reaction products

Using both *ortho* and *para* isomers of diphenols (dopamine, L-DOPA and hydroquinone) at a standardised concentration of 5 mM, we tested the antibacterial properties of their respective oxidised quinone (by)products (dopaminechrome, DOPAchrome, benzoquinone). Overall, the exposure of bacteria to these enzyme-derived products led to significant reductions in CFUs; $F_{(3, 40)} = 254.7$, $P < 0.0001$ (Figure 4). The majority of variation within the data, 87%, can be attributed to the type of substrate used. Gram-negative bacteria were sensitive to all reaction products, in particular, oxidised hydroquinone (i.e., benzoquinone) was highly effective against *P. agglomerans* – reducing CFUs by 95%. Conversely, Gram-positive bacteria were less sensitive to reaction products, e.g., oxidised L-DOPA (i.e., DOPAchrome) led to the smallest decline of 24% when exposed to *B. subtilis*. With that said, microbial target was determined to be a significant factor ($F_{(4, 40)} = 7.03$, $P = 0.002$) and accounts for 3.2% of the variation within the data. The bactericidal potency of diphenols can be ranked hydroquinone>dopamine>L-DOPA, and after 1-hour incubation each one caused sufficient damage to prevent replication, immobilise and/or kill the microbes. Although the use of L-DOPA did lead to decreases in *B. megaterium* and *B subtilis* CFUs, neither were significantly different to the respective controls ($P = 0.099$ and $P = 0.335$; Supplementary Figure 2).

3.3 Histological observations of Crepidula fornicata *tissues*

Using wax (H & E) histology, discrete brown/black pigmentation (eumelanin) was observed in the lining of the gill tissue, barrier epithelium, connective tissue, and border cells of the foot musculature (Figure 5). These melanic-deposits accumulated at the apical surface of epithelial cells (Figure 5D), but did not appear pathologic (no signs of infection or trauma). The cellular arrangement is uniform and there is no clear sign of a host response, e.g., haemocyte infiltration or encapsulation, to accompany the melanisation (which can be found in compromised tissues of invertebrates). Interestingly, yellowish pigmentation reminiscent of the lysosomal degradation product, lipofuscin, was visible in the digestive gland intra- and inter-tubular structures (Figure 5B), as well as connective tissue (Figure 5E and 5F). Lipofuscin tends to accumulate close to the nuclei of cells, which is evident here, and can sometimes appear brown due to the high levels of melanin resulting from oxidoreductase activity (Figure 5B and 5F).

4. Discussion

Herein, we compile strong evidence that proteins present in the acellular haemolymph of *C. fornicata* display phenoloxidase-like activities. The haemolymph tested negative for tyrosinase (monophenolase) activity and also appeared incapable of oxidising the *meta*-diphenol DHPPA. The low Michaelis' constant (K_M) values for both laccase-type (*para*) and catecholoxidase-type (*ortho*) substrates suggested the enzyme-ligand interactions were stable (Table 2), except for the methoxy-containing phenols (syringaldazine and ABTS) with calculated values in excess of 20 mM. The oxidation of general *o*-diphenols (e.g., dopamine) and the more-specific *p*-diphenol (hydroquinone) were inhibited by the metal chelator EDTA, and in doing so, confirmed the activities to be derived from metalloenzymes – as seen in *C. gigas* (Luna-Acosta *et al.,* 2010). In the presence of hydroquinone, the laccase specific inhibitor CTAB prevented all product formation. However, in the presence of dopamine, CTAB inhibited activity by a maximum of 91%. Moreover, the highest concentration of the tyrosinase/catecholoxidase-specific inhibitor 4-hexylresorcinol (1 mM) hindered activity by ~80% and ~10% in the presence of dopamine and hydroquinone, respectively (Table 3). These data endorse the presence of two independent diphenoloxidases within *C. fornicata* haemolymph, namely laccase and

340 catecholoxidase. Regarding the aromatic amine, *p*-phenylenediamine, we obtained a
341 K_M value of 2.01 mM, which is in line with those published for *Biomphalaria* sp., 1.19
342 to 1.45 mM (Le Clec'h *et al.,* 2016). Using similar assay conditions, Le Bris *et al.* (2014)
343 reported a much higher K_M value of 13.5 mM for *Haliotis tuberculata*, and Luna-Acosta
344 *et al.* (2011) recorded the highest at 45 mM when studying *C. gigas*. We observed pH
345 optima at 7 and 8, and temperature optima at 35°C and 45°C *in vitro* for dopamine and
346 hydroquinone, respectively (Figure 3). The *ex vivo* pH of *C. fornicata* haemolymph
347 varied between 7 and 9, which suggests it is more suited for hydroquinone oxidation
348 (Figure 3). These data fall within the reported ranges for both laccase-type and
349 catecholoxidase-type enzymes. For example, when using *p*-phenylenediamine, the
350 pH maximum was 8.5 for *Biomphalaria sp.* (Le Clec'h *et al.,* 2016), 8.2 for *H.*
351 *tuberculata* (Le Bris *et al.,* 2014), and 8.4 for *Venerupis philippinarum* (Le Bris *et al.,*
352 2013). When using an *o*-diphenol, optimal activity was achieved at pH 8 for *Saccostrea*
353 *glomerata* (Aladaileh *et al.,* 2007), pH 6 – 7.5 for *C. virginica* (Jordan and Deaton,
354 2005) and *Chlamys farreri* (Sun and Li, 1999).
355
356 Previously, Pires *et al.* (2000) detected three catecholamines – dopamine, $_L$-DOPA
357 and norepinephrine – in *C. fornicata* larvae and juveniles (using high performance
358 liquid chromatography). Inhibition of tyrosine hydroxylase and dopamine-β-
359 hydroxylase using α-methyl-DL-m-tyrosine and diethyldithiocarbamate reduced levels
360 of catecholamines by 20 – 50%, and interfered with morphogenesis. Herein, we
361 calculated low K_M values <1.5 mM for two of the catecholamines mentioned above
362 (Figure 2, Table 2). We posit that $_L$-DOPA and dopamine are endogenous substrates
363 of phenoloxidase(s) in *C. fornicata* adults.
364
365 Whilst bioprospecting molluscs for antiseptic compounds, Defer *et al.* (2009) prepared
366 some acidic extracts of *C. fornicata* tissues and recorded antibacterial activity against
367 *M. luteus* (Gram-positive) and *Listonella anguillarum* (Gram-negative), and virustatic
368 properties toward *Herpes simplex virus* type 1 (viral replication was reduced by 40%
369 when compared to the control). We also describe anti-infective properties of *C.*
370 *fornicata* haemolymph (Figure 4), yet importantly, our evidence implies the mechanism
371 of action is of enzymatic origin. The following points contend that CFU declines were
372 due to a combination of the noxious intermediates of laccase and/or catecholoxidase

373 reaction products: (1) in the absence of any substrate and in the presence of the
374 phenoloxidase inhibitor PTU, CFU numbers were in line with controls (>97%); (2) in
375 the absence of haemolymph protein, no measurable antibacterial activity was
376 observed; (3) using a 10 kDa filter to remove potential phenoloxidase(s) from the
377 reactions mixtures prior to microbial exposure reduced the likelihood of proteinaceous
378 macromolecules interacting directly with the targets. The penultimate step of the
379 eumelanin synthesis pathway is 5,6-Dihydroxyindole (DHI) formation, which can
380 happen spontaneously or enzymatically from DOPA-derivatives, and is known to have
381 direct antimicrobial activity (Zhao *et al.,* 2007). DOPAchromes themselves are
382 unstable, as are the cytotoxic oxidising and nitrosative radicals produced during
383 phenol oxidation (Coates and Nairn, 2014).
384
385 Traditionally, laccases have not been considered part of the invertebrate innate
386 immune system, despite their capacity to metabolise melanin precursors, i.e., phenols.
387 First, Luna-Acosta *et al.* (2011) noted restricted growth (>30%) of the marine
388 pathogens *Vibrio splendidus* LGP32 and *Vibrio aestuarianus* 02/41 after treatment
389 with *C. gigas* haemocyte lysate supernatant and two substrates, *p*-phenylenediamine
390 and L-DOPA. The anti-vibrio properties were thwarted by the addition of the
391 phenoloxidase inhibitor, PTU. Our data complement these earlier observations. The
392 reaction products derived from hydroquinone and dopamine oxidation were highly
393 effective against all microbes tested (Figure 4) but were indistinguishable from controls
394 when PTU was added. In contrast, L-DOPA oxidised (by)products were not as effective
395 against Gram-positive bacteria, notably *Bacillus* sp. Similar measurements were taken
396 with regards the relatively weak antimicrobial activity of crayfish phenoloxidase and
397 horseshoe crab haemocyanin-derived phenoloxidase when L-DOPA was used
398 compared to other diphenols (e.g., 4-*tert*-butylcatechol) at the same concentration
399 (Cerenius *et al.,* 2010a, Coates and Talbot, 2018). Recently, Shi *et al.* (2017)
400 challenged Pacific white shrimp *P. vannamei* with *Vibrio parahaemolyticus*, *M.*
401 *lysodeikticus* and white spot syndrome virus (WSSV) and noted increased expression
402 of laccase-specific mRNAs. In a separate experiment, the authors silenced the laccase
403 gene using dsRNA, which increased shrimp susceptibility to both bacterial types, and
404 caused >20% higher mortality. In a subsequent study, Chen *et al.* (2020) identified a
405 second laccase gene (*LvLac*2) from *P. vannamei* within the epidermal layers of the
406 carapace that was also linked to immune activity. Injection of shrimp with WSSV or *V.*

407 *alginolyticus* led to increased expression of the *LvLac*2 gene, and the oxidative stress-
408 associated transcription factor NF-E2. Additionally, injection of dsRNA for *LvLac*2
409 reduced the survivorship of shrimp when challenged with WSSV. A notable side-effect
410 of eliminating laccase gene expression was an increase in tissue damage found in the
411 hepatopancreas of shrimp immune-stimulated with β-glucans. The authors concluded
412 that it was caused by oxidative damage in the absence of laccase, and that laccase
413 likely has multiple functions.
414
415 Phenoloxidases are distributed widely amongst metazoans, microbes and plants.
416 Their roles differ depending on the organism, for example: plant polyphenoloxidases
417 and arthropod tyrosinases are involved in host counter-responses to disease-causing
418 agents, while fungal laccases act as enzymatic antioxidants/detoxicants and assist in
419 lignocellulose degradation (Baldrian, 2006; Cerenius *et al.,* 2008; Janusz *et al.,* 2020).
420 Our histological screen of *C. fornicata* solid tissues revealed the presence of melanin
421 and lipofuscin-like pigments across diverse tissue types. In previous work by Tiley *et*
422 *al.* (2018 and 2019), brown inclusion bodies – bulbous or conical in shape – were
423 characterised in the digestive gland of another gastropod, the queen conch *Lobatus*
424 *gigas*. Using a combination of techniques, including histochemical staining and
425 electron microscopy, these were confirmed to be aggregates of melanin, iron,
426 glycoproteins and mucopolysaccharides. In line with our observations of slipper limpet
427 tissues, Tiely *et al.* (2018, 2019) did not find any evidence of damage, inflammation or
428 infection (e.g., apicomplexan parasites), however, they did observe such pigmented
429 deposits in several other areas, including ganglia. These studies may go some way to
430 explain the presence of lipofuscin – a lysosomal degradation product in the digestive
431 gland and connective tissues of *C. fornicata* (Figure 5). Lipochrome in the form of small
432 yellow aggregates can be considered stage 1 lipofuscin, which can go on to form
433 immature (stage 2) brown bodies. These brown bodies are often associated with
434 pathogen clearance, mineral storage and cellular senescence, and the darker
435 pigmentation can be attributed to melanin accumulation form oxidation reactions
436 (Valembois *et al.,* 1994).
437
438 The published genomes of several bivalves, *C. gigas* (Zhang *et al.,* 2012), *C. farreri*
439 (Li *et al.,* 2017) and *Pinctada fucata martensii* (Du *et al.,* 2017), revealed major gene

expansion (sub/neo-functionalisation) events for phenoloxidases, notably tyrosinases and laccases. Moreover, expression of laccase and tyrosinase-like protein mRNAs were up-regulated in regions such as the mantle and digestive gland, which further implies multiple roles in development, detoxification and defence. Interestingly, the expression of at least two laccase genes has been recorded in the epithelium, muscle, intestine, stomach, hepatopancreas, gill, haemocytes, nerve tissue and heart of penaeid shrimp (Shi *et al.,* 2017; Chen *et al.,* 2020).

5. Conclusion

We establish that enzymes present in the haemolymph of the invasive gastropod *C. fornicata* can accept diphenolic substrates and convert them into quinones (melanin precursors) in a manner similar to laccases (EC 1.10.3.2) and/or catecholoxidases (EC 1.10.3.1). The resulting (by)products are cytotoxic and possess broad-spectrum antibacterial properties. The capacity of this gastropod to generate melanin is evidenced further by the distribution of this pigment across many tissues. Taken together, we form the opinion that two constitutive phenoloxidases contribute to biological defences in *C. fornicata.*

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Author contributions

C.J.C. conceived and designed the experiments. All authors performed experiments and/or processed samples. E.A.Q. and C.J.C. collated and analysed data. C.J.C. drafted the text. C.J.C. revised the manuscript with input from E.A.Q., A.F.R.

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669 **Table 1** Substrate parameters used to discriminate between phenoloxidase activities

Specificity	**Substrate**	**Molecular weight**	**Concentration range (mM)**	**Wavelength** [product detection]
Laccase	PPD	108.14	0.1 - 10	520
	Syringaldazine	360.36	0.05 - 50	525
	ABTS	548.58	0.01 - 20	420
	Hydroquinone	110.11	0.1 - 15	390
Non-specific	Caffeic acid	180.16	1 - 10	492
	L-DOPA	197.19	0.1 - 5	492
	DHPPA	182.17	0.25 - 80	492
	Dopamine	189.64	0.1 - 10	492
Tyrosinase	L-Tyrosine	181.19	0.01 - 25	492
	4-HA	124.14	0.1 - 30	492
	Tyramine	137.18	0.1 - 30	492

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679 **Table 2** Kinetic properties of laccase and catecholoxidase activities

Substrate	**Substrate class**	**Enzyme**	**K_M** (mM)	**V_{max}** (μmol min^{-1} mg^{-1})	**R^2**
ABTS	methoxy-phenol	Laccase	21.1 ± 4.82	5.71 ± 0.81	0.96
Hydroquinone	*para*-diphenol	Laccase	2.05 ± 0.38	4.37 ± 0.26	0.79
p-Phenylenediamine	non-phenolic	Laccase	2.01 ± 0.44	1.73 ± 0.13	0.93
Syringaldazine	methoxy-phenol	Laccase	21.2 ± 8.3	4.51 ± 0.74	0.82
Caffeic acid	*ortho*-diphenol	Non-specific	1.11 ± 0.43	1.63 ± 0.62	0.74
DHPPA	*meta*-diphenol	Non-specific	-	-	-
L-DOPA	*ortho*-diphenol	Non-specific	0.26 ± 0.07	1.4 ± 0.08	0.82
Dopamine	*ortho*-diphenol	Non-specific	1.21 ± 0.32	2.51 ± 0.18	0.85
4-Hydroxyanisole	mono-phenol	Tyrosinase	-	-	-
Tyramine	mono-phenol	Tyrosinase	-	-	-
L-Tyrosine	mono-phenol	Tyrosinase	-	-	-

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692 **Table 3** Inhibition of laccase and catecholoxidase activities

Inhibitors	Enzyme target	Inhibitor conc.	Inhibition (%)	
			Dopamine#	Hydroquinone#
Benzoic acid	Non-specific	0.1 mM	95.9	76.9
		0.5 mM	98.7	84.1
		1 mM	100	100
Citric acid	Non-specific	0.1 mM	87.4	71.3
		0.5 mM	88.9	78.9
		1 mM	100	100
CTAB	Laccase	0.1 mM	67	98
		0.5 mM	84	100
		1 mM	90.7	100
EDTA	Non-specific	0.1 mM	85.6	72.3
		0.5 mM	92.6	97.6
		1 mM	100	100
4-hexylresorcinol	Catecholoxidase & Tyrosinase	0.1 mM	57	-
		0.5 mM	84	-
		1 mM	82.8	10.3
PTU	Non-specific	0.1 mM	84.1	93.5
		0.5 mM	89.7	98.7
		1 mM	90	100

693 **#**, substrates were used at a standard concentration of 5 mM for all inhibition assays
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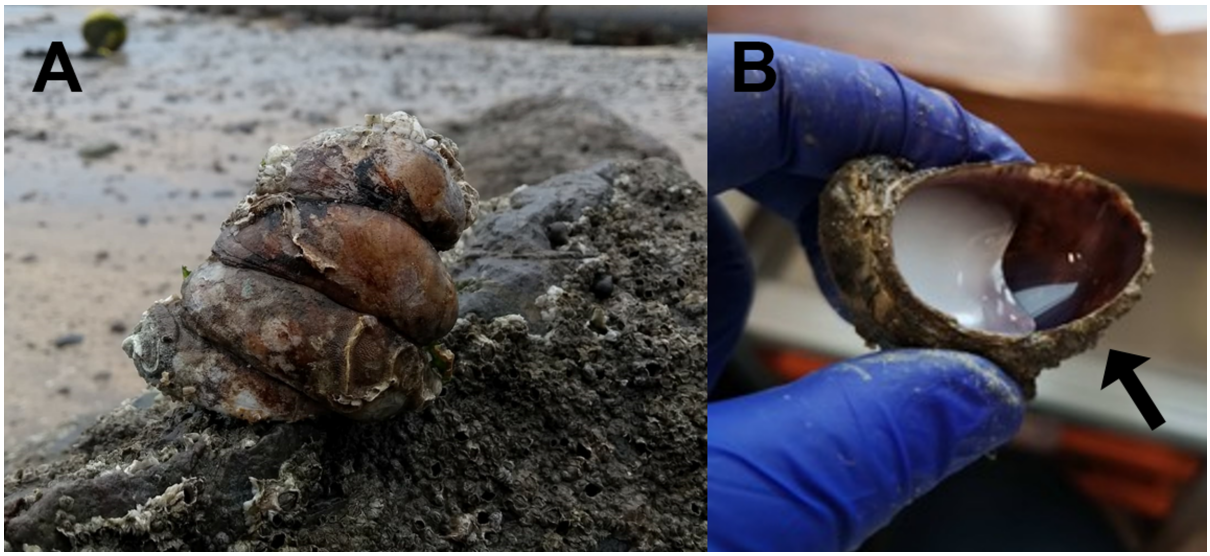

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703 **Figure 1**. **Typical stack formation of *Crepidula fornicata* (A) and accessibility of**
704 **haemolymph after (solid) tissue removal (B).** Black arrow points to pooled
705 haemolymph at the aperture of the shell.
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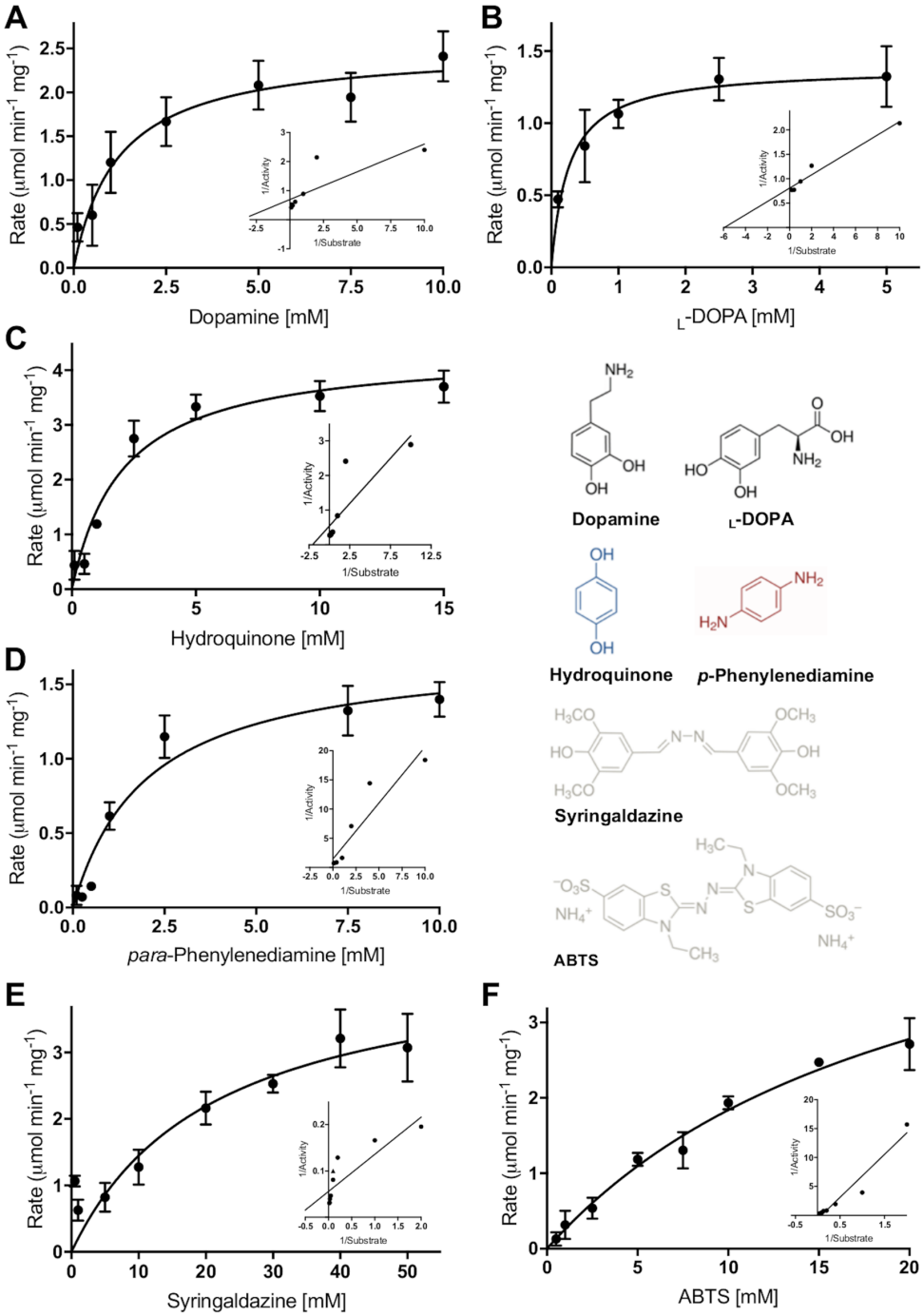

Figure 2. **Laccase and catecholoxidase activities of *Crepidula fornicata* haemolymph protein in the presence of diverse substrates *in vitro***. Protein (1 mg mL^{-1}) was incubated in the presence of each substrate for 10 minutes. Products derived from the enzymatic oxidation of substrate were observed across several wavelengths (listed in Table 1). Values represent the mean ± standard error (*n* = 3 biological replicates made-up of 3 technical replicates each). Enzyme-substrate kinetics were calculated in GraphPad PRISM v7 using Michaelis-Menten non-linear regression. Each panel also contains the respective double-reciprocal (Lineweaver-Burk) plot. Inset – chemical structures of *ortho*-diphenols (coloured black), *para*-diphenol (coloured blue), phenols with methoxy groups (coloured grey), and a non-phenolic substrate (coloured red).

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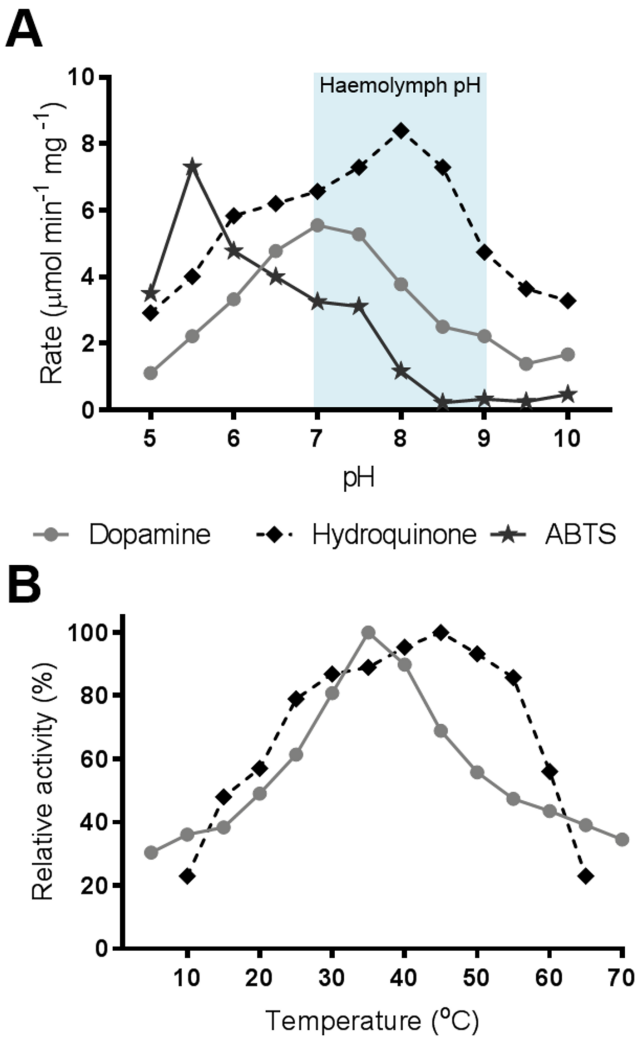

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724 **Figure 3**. **Effect of pH and temperature on laccase and catecholoxidase activities**
725 **in the haemolymph of *Crepidula fornicata*.** Protein (1 mg mL^{-1}) was incubated in
726 the presence of either substrate for 10 minutes across the pH range 5 – 10 (**A**) and
727 the temperature range 5 – 70°C (**B**). Activity (rate) was measured as the amount of
728 product formed from the oxidation of dopamine (into dopachrome), hydroquinone (into
729 benzoquinone), and ABTS (into $ABTS^{+}$). In (**A**), the pH range of fresh (*ex vivo*) limpet
730 haemolymph (*n* = 141) is shaded blue. In (**B**), values are expressed as a percentage
731 of the mean maximum value for dopamine (35°C) and hydroquinone (45°C).
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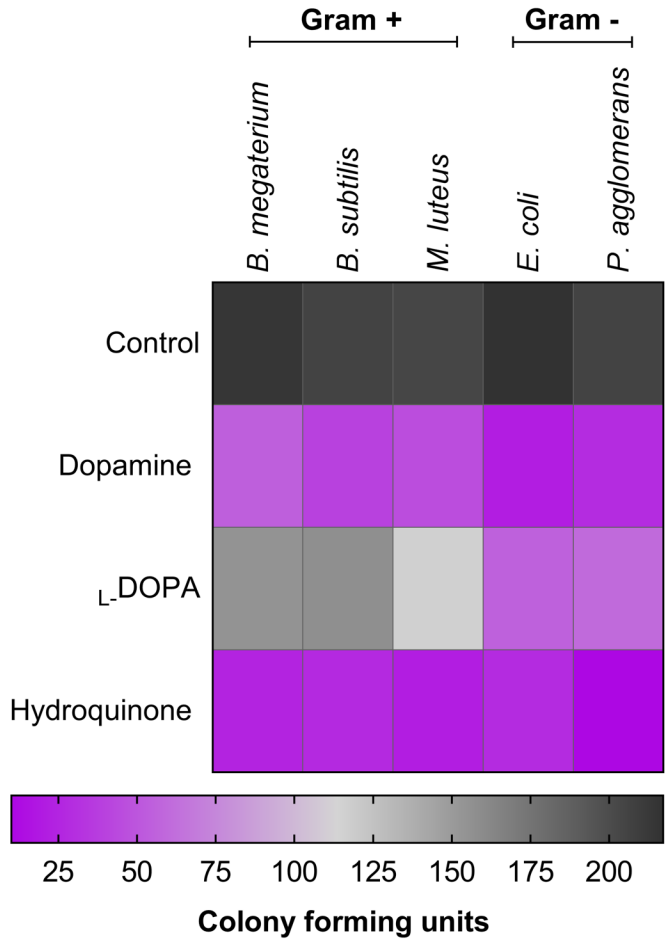

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735 **Figure 4**. **Antibacterial effects of laccase- and catecholoxidase-derived reaction**
736 **products *in vitro***. Cell-free haemolymph protein (1 mg mL^{-1}) from *Crepidula fornicata*
737 was incubated with *ortho*-diphenolic (dopamine, L-DOPA) and *para*-diphenolic
738 (hydroquinone) substrates for 10 minutes. Post-incubation, proteins were filtered (>10
739 kDa cut-off) using centrifugation, and the subsequent reaction mixtures containing the
740 oxidised products were incubated with Gram-positive (*B. megaterium, B. subtilis, M.*
741 *luteus*) and Gram-negative (*E. coli, P. agglomerans*) bacteria. The heat map depicts
742 the mean number of colony forming units for treated microbes (n = 3) and controls
743 (substrates were omitted).
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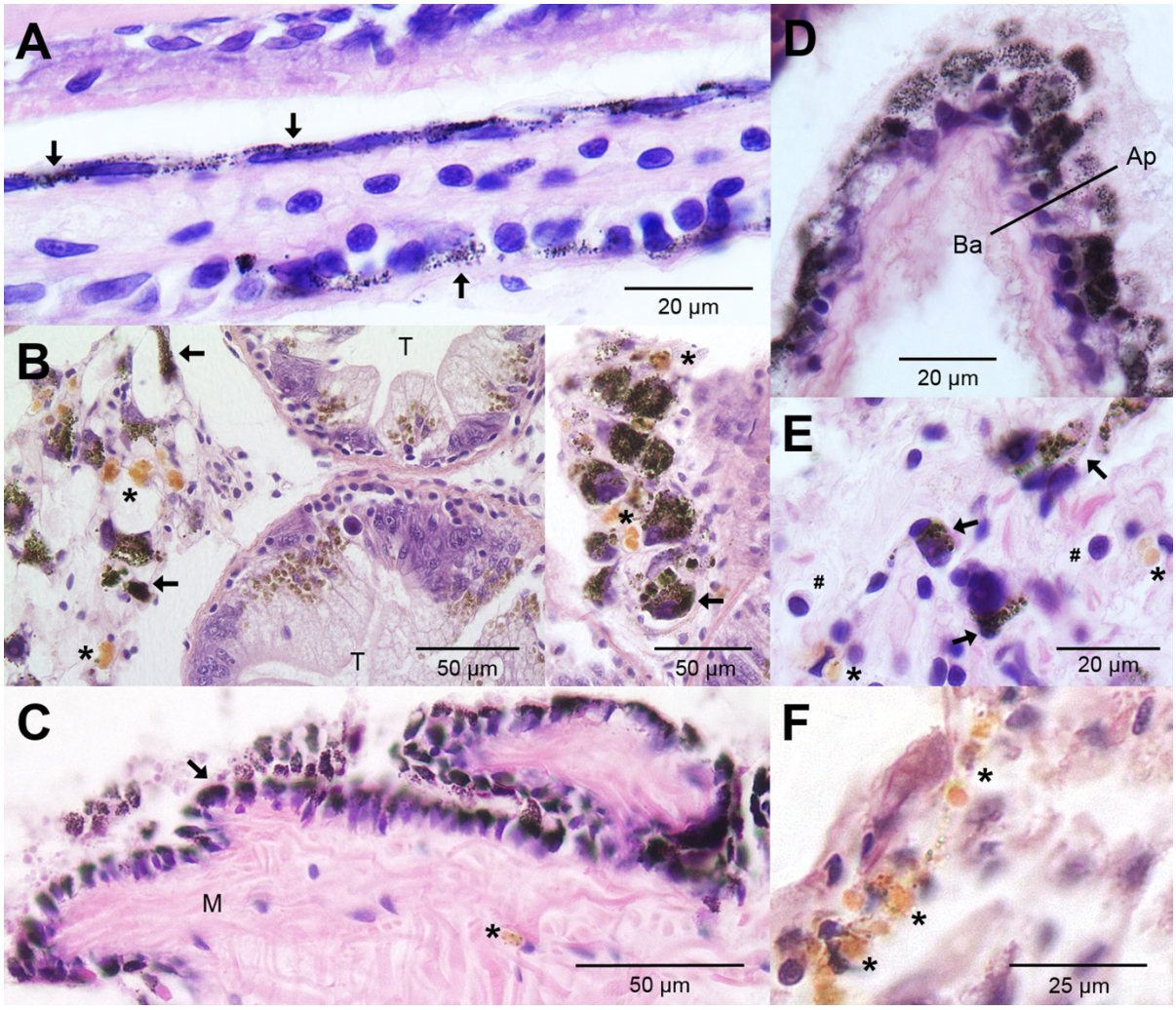
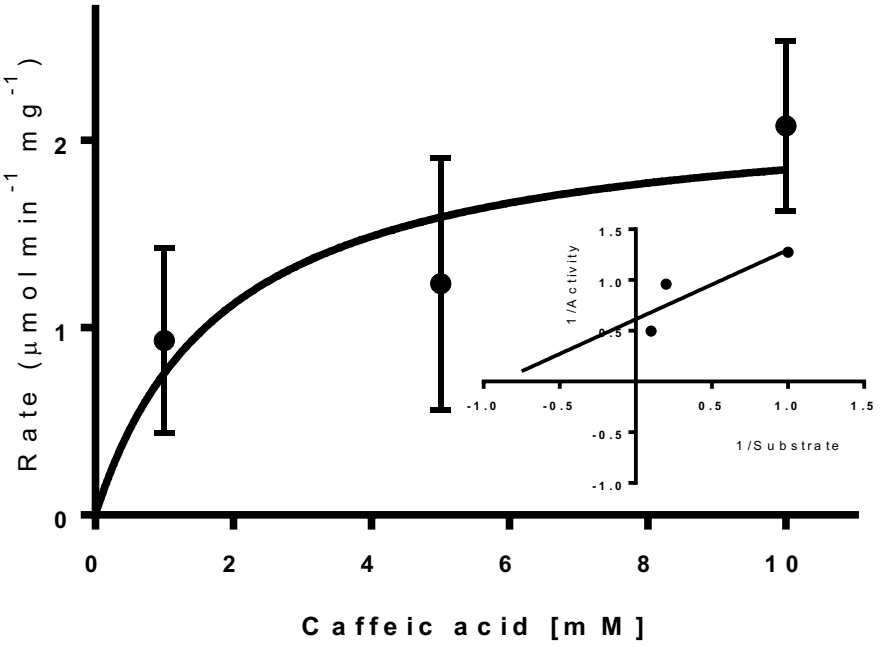


Figure 5. **Tissue histology of *Crepidula fornicata*.** Photomicrographs depict transverse sections of gill tissue (A), the digestive gland (B), the foot musculature (C), barrier epithelium (D), and connective tissues (E and F). In all images, arrows point to melanin deposits within a variety of cell types, and, each asterisk (*) indicates lipofuscin-like material. Ap, apical; Ba, basal; M, muscle; T, tubule. In (E), a hashtag (#) denotes the presence of a haemocyte.



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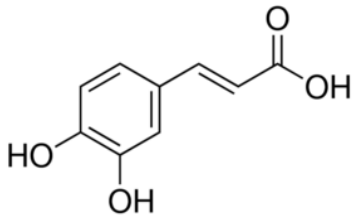

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763 **Supplementary Figure 1 Catecholoxidase activity of *Crepidula fornicata***
764 **haemolymph protein in the presence of caffeic acid *in vitro***. Protein (1 mg mL^{-1})
765 was incubated in the presence of substrate for 10 minutes. Products derived from the
766 enzymatic oxidation of substrate were observed at 492 nm. Values represent the mean
767 ± standard error *(n* = 3 biological replicates made-up of 3 technical replicates each).
768 Enzyme-substrate kinetics were calculated in GraphPad PRISM v7 using Michaelis-
769 Menten non-linear regression. The panel also contains the respective double
770 reciprocal (Lineweaver-Burk) plot. Inset – chemical structure caffeic acid.
771 Non-linear regression, R^2 = 0.38.
772 Double-reciprocal plot, R^2 = 0.74.
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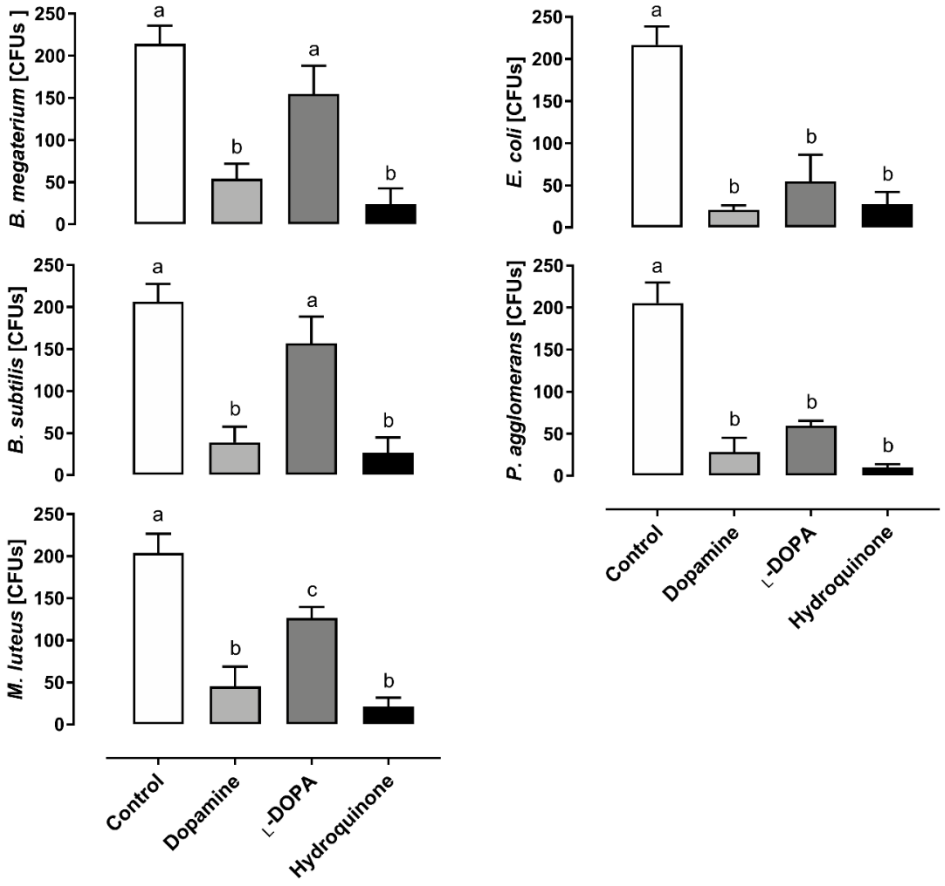

Supplementary Figure 2 Antibacterial effects of laccase- and catecholoxidase-derived reaction products *in vitro*. Cell-free haemolymph protein (1 mg mL^{-1}) from *Crepidula fornicata* was incubated with *ortho*-diphenolic (dopamine, L-DOPA) and *para*-diphenolic (hydroquinone) substrates for 10 minutes. Post-incubation, proteins were filtered (>10 KDa cut-off) using centrifugation, and the subsequent reaction mixtures containing the oxidised products were incubated with Gram-positive (*B. megaterium, B. subtilis, M. luteus*) and Gram-negative (*E. coli, P. agglomerans*) bacteria. Unshared letters represent significant differences ($P \leq 0.05$) – determined by Tukey's multiple comparisons (*post hoc*).