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**Deciphering shell proteome within different Baltic populations of mytilid mussels illustrates important local variability and potential consequences in the context of changing marine conditions.**

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# 1 **1. Introduction**

2 The mollusc shell, primarily comprising calcium carbonate ( $\text{CaCO}_3$ ) and an organic matrix of  
3 proteins, carbohydrates etc., exhibits outstanding mechanical properties. Shell formation in-  
4 volves genetically controlled secretion and export of mineral precursors and organic mole-  
5 cules. In bivalves, this process involves the development of an organic framework, comprised  
6 of proteins, glycoproteins, lipids, carbohydrates, which act as a template for the nucleation of  
7  $\text{CaCO}_3$  minerals (Lowenstam, 1981). The different  $\text{CaCO}_3$  polymorphs and their associated  
8 organic compounds are arranged in distinctive patterns, resulting in a vast number of shell  
9 microstructures (Carter, 1980). While calcification rate is governed by the calcium carbonate  
10 saturation state at the site of shell formation (Morse et al., 2007; Waldbusser et al., 2015),  
11 shell organic compounds, typically called shell matrix proteins (SMPs), favour mineral pre-  
12 cipitation, even under low calcium carbonate saturation state conditions (Evans, 2017;  
13 Thomsen et al., 2017).

14 In molluscs, SMPs are secreted by the outer mantle epithelial layer and their secretion is both  
15 temporal and spatial during the development of the shell (Evans, 2019). Proteins such as silk-  
16 fibroin along with beta-chitin polysaccharide forms a gel-like phase and contributes to con-  
17 trolled nucleation (Addadi et al., 2006; Perovic et al., 2014).  $\text{CaCO}_3$  shell microstructures are  
18 classified based on their morphology, and include prismatic, spherulitic, cross-lamellar, ho-  
19 mogenous and nacreous forms (McDougall et al., 2018; Chateigner et al., 2000). A large  
20 number of SMPs are found to be specific to aragonite such as moderately acidic MSI60, N14,  
21 AP7, and basic lustrin A, perlucin, N19, for examples (Marin et al., 2008). Specific protein  
22 domains such as VWA, IGF-BP, C-type lectin domains are often found in these SMPs. Simi-  
23 larly, Aspein, prismaticin-14, P43 etc., are calcite specific and are enriched in acidic amino ac-  
24 ids (extremely acidic) and disposed to bind to metal ions such as calcium. In *M. galloprovin-*  
25 *cialis*, SMPs such as calponin-like 8, BSMP like, Fibronectin-like, GA-rich etc., were identi-

26 fied in the nacreous layer and calponin-like-16, chitin synthase-like, Gigasin-like, GFS-rich  
27 etc., in the fibrous prismatic layer. Also, calponin-like 6, cathepsin-like-1, Fibronectin-like-1,  
28 RKA-rich, etc., were found in both the layers (Gao et al., 2015). One striking feature of mol-  
29 luscan SMPs is the occurrence of repetitive, low complex domains (Jackson et al. 2010) hav-  
30 ing low amino-acid diversity conferring them strong static conformations or flexible regions  
31 lacking well-defined folding structures (Coletta A et al., 2010). The SMPs not only control  
32 biomineralization but also confer the shells unique properties such as ductility, or fracture  
33 resistance etc.

34 The Baltic Sea is semi-enclosed and one of the largest brackish water bodies of the planet.  
35 Post glacially, the Baltic underwent dramatic changes from marine to fresh and finally to  
36 brackish water. This was due to limited water exchanges with the North Sea and also a rela-  
37 tively large fresh water supply ( $\approx 15000 \text{ m}^3 \text{ s}^{-1}$ ) which has resulted in a salinity gradient from  
38 high salinity in the west to low salinity in the east with an average salinity of 7.4 psu (Gus-  
39 tafsson & Westman, 2002). As a result of the post-glacial history and brackish water envi-  
40 ronment, biodiversity and species richness is low compared to the North Sea, yet in terms of  
41 biomass, they are comparable (Ojaveer et al., 2010). Low salinity and low total alkalinity  
42 (seawater pH buffering capacity) leads to unfavorable conditions for calcifying organisms due  
43 to under saturated seawater conditions for calcium carbonate (Tyrrell et al., 2008, Müller et  
44 al., 2016; Sanders et al., 2018). Despite suboptimal conditions for calcification and the physi-  
45 ological stress associated with low salinity, marine calcifying *Mytilid* mussels are extensive in  
46 many benthic ecosystems and are key organisms for nutrient recycling and supporting biodi-  
47 versity (Vuorinen et al., 2002, Norling & Kautsky, 2008). Baltic Sea forms a hybrid zone be-  
48 tween *Mytilus edulis* and *Mytilus trossulus* (Väinölä & Strelkov, 2011) with *M. edulis* allele  
49 frequencies being higher at high salinity and *M. trossulus* allele frequencies being higher at  
50 low salinities in the Central and Eastern Baltic (Stuckas et al., 2009). Gene introgression is so



51 extensive between both species that pure individuals of either species are not present at all  
52 and genetic divergence from neighboring North Sea populations is high (Johannesson & An-  
53 dré, 2006, Stuckas et al., 2017). This noticeable genetic divergence suggests local adaptation  
54 of Baltic *Mytilid* (referred as Baltic mussels) populations to low salinity and recent findings  
55 suggest that Baltic populations from low salinity (7 psu) are more tolerant of low seawater  
56  $[Ca^{2+}]$  (characteristics of extremely low salinities) compared to populations from higher salin-  
57 ity (16 psu) (Thomsen et al., 2017). Baltic blue mussels calcify slowly and attain a size which  
58 is one third that of North Sea mussels with thin and elongate shells (Kautsky, 1982).

59 In fact, Baltic blue mussels grow to larger sizes when transplanted to marine waters, empha-  
60 sizing that calcification rates and maximum shell size are independent from genetic make-up  
61 (Kautsky, Johannesson & Tedengren, 1990). One of the most prominent features of the Baltic  
62 is, the number of mussel predators such as the starfish *Asterias rubens* and the crab *Carcinus*  
63 *maenas* are comparatively absent in the eastern parts of Baltic where the salinity is less than  
64 10 psu. This lack of predation pressure may have additionally led to a reduction in morpho-  
65 logical defenses such as weak adductor muscles and thinner shells (Reimer & Harms-  
66 Ringdahl, 2001).

67 To understand how Baltic blue mussels can form shells under the extremely low calcium car-  
68 bonate saturation states, it is crucial to deduce the molecular mechanisms underpinning shell  
69 formation, especially the fate of SMPs, which are key regulators of the biomineralization pro-  
70 cess. Many studies under controlled conditions have shown that variations in pH, temperature  
71 and salinity affect the shell formation process, growth, survival etc., in bivalves (Mackenzie et  
72 al., 2014). In fact, these factors predominately alter metabolic processes and energy partition-  
73 ing to growth and calcification (Sanders et al., 2018). However, valuable clues offered by the  
74 SMPs to understand the molecular mechanisms of adaptation of the calcifiers in the context of  
75 changing marine conditions have not received any attention yet. In this work, we investigated

76 the modulation of the SMPs and how it alters the physical and biochemical properties of the  
77 shells in Baltic blue mussels living in brackish water with respect to mussels inhabiting fully  
78 marine North Sea.

## 79 **2. Materials and methods:**

### 80 **2.1. Sample collection**

81 Information about sample collection is described in detail in a recent publication (Telesca *et*  
82 *al.*, 2019). Briefly, Sub-tidal populations of adult Baltic *Mytilus* spp. (shell length: 30-40 mm,  
83 age: 2-4 years) were collected in July-August 2015 from Sylt (German North Sea coast:  
84 54°54' N 8°20' E), Usedom (German Baltic Sea coast: 53°56' N 14°05' E) and Nynäshamn  
85 (Swedish east coast: 53°56' N 14°05' E) (Figure 1a). Salinity is relatively stable in the central  
86 Baltic Sea and not significantly different between the 2 sites (approximately 5.3 and 5.9 psu  
87 for Usedom and Nynäshamn, respectively) but relatively very low compared to North Sea site  
88 (Sylt, salinity is approximately 29.3 psu). Based on water samples and measurements taken at  
89 the time of sampling, pH and temperature were not largely different (although both parame-  
90 ters are much more variable at both Baltic sites compared to the North Sea site, Sylt) between  
91 Baltic sites. Carbonate chemistry (pH, CO<sub>2</sub> and calcium carbonate saturation state) is more  
92 stable in the North Sea due to the higher total alkalinity and a higher buffering capacity to pH  
93 change. Age determination was an estimate based on growth rates for each population and  
94 shell lengths of samples (See Vuorinen *et al.* 2002). Shell length was kept constant between  
95 sampled populations to enable direct comparison. Soft tissue was removed immediately from  
96 the shells using a scalpel and shells were air dried at room temperature for a minimum of 2  
97 weeks before analysis.

### 98 **2.2. Shell mechanical tests**

99 The fracture force of the left valve of the specimens (n=6) from each location was tested us-  
100 ing a simple compression method. All shell valves were placed on a steel block on the stage  
101 of the force stand (Instron 5543) in an identical orientation (shell length along the horizontal  
102 axis, outer shell surface facing upwards). Mechanical tests were carried out using a second  
103 metal block, fixed on the load frame of the force stand, which was lowered onto the valve at a  
104 speed of 0.3 mm min<sup>-1</sup> (simple compression test) until fracture occurred. Displacement and  
105 force were recorded continuously using a 100N load cell at a frequency of 10 Hz.

### 106 **2.3. FTIR analysis for mineral composition**

107 0.5 mg of the shell powder for each location (n=5) was analyzed using an ATR-FTIR Vertex  
108 70 spectrometer (Bruker). The samples were measured using a Single Reflection diamond  
109 ATR system (Golden Gate, Specac). Each spectrum comprised 64 scans at 4 cm<sup>-1</sup> resolutions.  
110 The peak values such as 711 cm<sup>-1</sup>, 700 cm<sup>-1</sup>, 856-875 cm<sup>-1</sup>, 1401-1754 cm<sup>-1</sup>, 1785-1793 cm<sup>-1</sup>  
111 were used to distinguish between calcite and aragonite polymorphs. Loftus and coworkers  
112 have demonstrated a method to establish calcite and aragonite ratio in shell samples using  
113 specific FTIR ATR absorbance peak height ratio (Loftus *et al.*, 2015). We used 875/1785 cm<sup>-1</sup>  
114 <sup>1</sup> (C-O v2 - out-of plane- bending in calcite / combination of stretching vibrations v1 and v4  
115 in aragonite respectively) and 1793/1785 cm<sup>-1</sup> (combined v1 and v4 stretching vibrations in  
116 calcite vs aragonite) peak ratios to consistently provide validated estimates of a similar mix of  
117 the two polymorphs in the samples.

### 118 **2.4. Scanning electron microscopy**

119 Three mussel shells with same shell size from each location were mechanically fragmented.  
120 Prior to analysis, the freshly fractured transections were gold-coated. Scanning electron mi-  
121 croscopy was carried out using a Hitachi SU3500 (Hitachi, France). In order to avoid parallax  
122 error resulting from irregular fractured surface, the angle of the electron beams was tilted ac-

123 cordingly. The periostracum and shell thickness measurements were performed in the area  
124 where the thickness of nacre and prism were 1:1 ratio. This was used to frame a “region of  
125 interest” and five measurements were made at different positions spread over each frame (re-  
126 gion of interest) for each individual (total measurement for each site =15).

## 127 **2.5. Sample preparation for total organic content and proteomics:**

128 Five individual shells collected from each location were washed separately with 5-10% sodi-  
129 um hypochlorite (NaOCl) to remove surface organic impurities and periostraca. Shells were  
130 then washed with milli-Q® water and air-dried. The shells were then cut into small pieces and  
131 polished with a dremel drill. Before pulverizing the shells to a powder, they were washed  
132 briefly with NaOCl and milli-Q® water and air-dried. Powdered shell fragments were graded  
133 using 250-µm mesh. 400 mg of shell powder was decalcified using 5% cold acetic acid (5 ml)  
134 for an hour and 10% cold acetic acid (5 ml) overnight to ensure complete dissolution of Ca-  
135 CO<sub>3</sub>. The homogenate was centrifuged (14,000 rpm, 20 mins, 4°C) to separate the acid solu-  
136 ble (ASM) supernatant and the acid insoluble matrix (AIM) pellet. The AIM was washed with  
137 milli-Q® water several times to remove all traces of the acid and freeze-dried. The ASM was  
138 washed with milli-Q® water on a 10 kDa filter (Sartorius, VIVASPIN 20) to remove the acid  
139 and freeze-dried. The dry samples (ASM and AIM) were weighed to estimate the total  
140 organic content.

141 For proteomics, both dried AIM and ASM were denatured using 30 µL of 8 M urea and then  
142 15 mM dithiothreitol (DTT) in 100 mM triethyl ammonium bicarbonate (TEAB), both the  
143 steps were carried out at 37°C for 1h each. Alkylation was done using 20µL of 15 mM iodoa-  
144 cetamide in the dark and at room temperature for 1h. The resulting solution was diluted with  
145 100 mM ammonium bicarbonate to reduce the concentration of urea to 2M and digestion was  
146 carried out by adding 10 µg of trypsin (Sigma-Aldrich, France) to the AIM and ASM samples  
147 and incubated at 37°C overnight. The resulting digested peptides were acidified with 10 µL of

148 10% formic acid and desalted using 4mm Empore™ SPE (Sigma-Aldrich, France) cartridges.  
149 Desalted peptides from both ASM and AIM fractions from each sample were mixed into a  
150 single sample and quantified using a Bicinchoninic acid kit (Sigma-Aldrich, France) to de-  
151 termine total protein content.

152 For mass spectrometry analysis, peptides were concentrated on a C<sub>18</sub> cartridge (Acclaim  
153 PepMap100, 5µm particles, 300µm i.d. x 5 mm, Thermo Scientific) at 15 µl/min flow rate in,  
154 0.05% aq. TFA: ACN (98:2, v/v) (buffer A) for 5 minutes followed by elution to a C<sub>18</sub> col-  
155 umn (Acclaim PepMap100, C<sub>18</sub>, 3 µm particles, 75 µm i.d. x 50 cm length, ThermoFisher  
156 Scientific) at a flow rate of 220 nL.min<sup>-1</sup>. Peptides were then separated using buffer B  
157 (ACN/0.1% and aq. FA (90:10 v/v)) with the following gradient: 2-40% buffer B for 170 min,  
158 then 40-50% buffer B for 10min. The eluted peptides were analyzed by a nano-ESI quadru-  
159 pole-Orbitrap mass spectrometer (Q Exactive, Thermo Fisher Scientific) with the following  
160 parameters: one full MS acquisition (Resolution 70,000 at m/z 200, Automatic gain control  
161 target value 1e6, max. injection time 250 ms), followed by 10 MS/MS (TOP 10, CID) with  
162 isolation window 2 m/z, fixed first mass 100 m/z, resolution 17,500 at m/z 200, AGC target  
163 value 5.10<sup>4</sup> counts, maximum injection time 120 ms, CE 30 and dynamic exclusion of 60 s.

## 164 **2.6. Proteomic data analysis**

165 The MS/MS spectra obtained from the five individual samples from the three locations were  
166 searched against a mantle transcriptome database of *M. edulis* and *M. trossulus* published  
167 recently (Knöbel, L et al., 2020) using an in-house Mascot server (Matrix Science, London,  
168 UK; version 2.4.1). The database search was carried out using following parameters: missed  
169 cleavage = 1, carbamido-methylation of cysteine as fixed modification and oxidation of me-  
170 thionine, de-amidation of aspartic acid and asparagine as variable modifications, mass toler-  
171 ance of MS and MS/MS experiments as 20 ppm and 0.5 Da. Scaffold software (Proteome  
172 Software Inc., Portland, USA; version 3.6.5) was used to validate and group the matched pro-

173 teins. The following criteria were set for each sequence scaffold for validating the proteins:  
174 peptide identification probability greater than 95% as specified by Peptide Prophet algorithm  
175 and protein identification probability greater than 95% as specified by Protein Prophet algo-  
176 rithm with minimum two unique peptides (containing at least eight amino acids) matched.  
177 BLAST2GO tool (Omicsbox, Bio Bam, Valencia, Spain; version 4.0.7) was used to carry out  
178 sequence similarity protein searches against NCBI protein database. In addition to in-built  
179 InterPro results from BLAST2GO, Conserved Domain Database searches  
180 (<https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>) were performed to predict the con-  
181 served protein domains in the target protein sequences.

182 A label free semi-quantitative approach was performed in PEAKS studio software (Bioinfor-  
183 matics Solutions Inc., Waterloo, Canada; version 7) to identify differentially expressed pro-  
184 teins. The differential expression protein patterns were based on log<sub>2</sub> ratio to average signal  
185 intensity area across samples. The results were validated using the following parameters: [1]  
186 The peptide significance threshold  $\geq 10 \times 10 \log P$  and protein significant threshold  $\geq$   
187  $20 \times 10 \log P$ . [2] The MS signal filter was set to  $\geq 10^6$  average intensity area and  $\geq 0.5$  quality  
188 of the signal. [3] Mass error tolerance is 20 ppm. [4] Confident sample number  $\geq 3$ . [5] Pro-  
189 tein fold change  $\geq 2$ . Normalization was done using TIC. FDR threshold is 1%. The reference  
190 sample for relative quantification was auto-detected by the software based on the sample pro-  
191 file.

## 192 **2.7. Statistical analysis**

193 Differences in the gross shell features from different locations related to fracture force, FTIR  
194 and shell traits were analyzed using Kruskal-Wallis non-parametric rank test to determine  
195 whether there were significant differences among the shells from different locations. This test  
196 was chosen as the need for the data to follow normal distribution is not required but the as-  
197 sumption of independent observations was met. Null hypothesis of equal mean ranks was

198 considered and the low  $p$ -value allowed to reject the null hypothesis. PCA analysis was per-  
199 formed as multivariate analysis to fit shell traits (thickness and organic content) covariance  
200 matrix into orthogonal decomposition according independent loadings for further exploratory  
201 data analysis.

202 Statistical tests were carried out using R v3.6.0 (R: A language and environment for statistical  
203 computing. R Foundation for Statistical Computing, Vienna, Austria. URL [https://www.R-](https://www.R-project.org/)  
204 [project.org/](https://www.R-project.org/)] using the *Kruskal.test()* from the native package ‘stats’. The graphical results  
205 were generated using the package ‘lattice’.

206

### 207 **3. Results :**

#### 208 **3.1. Shell mechanical test**

209 Shell fracture tests waere carried out to find if significant differences exist between the three  
210 populations, reflecting shell resistance. Differences in shell length ( $n=7$ ) were not observed to  
211 be significantly different among the three sites (Fig.1b, Kruskal-Wallis chi-squared= 0.28,  
212  $p=0.86$ ) excluding it as contributing to shell fracture results. Breaking load ( $F_{\max}$ ) indicated  
213 that Baltic blue mussels (Nynäshamn and Usedom) exhibit lower shell-fracture resistance  
214 than North Sea (Sylt) mussels (Kruskal-Wallis chi-squared =8.43,  $p = 0.01$ , Supplementary  
215 Data 1).

#### 216 **3.2. Mineral composition**

217 *Crassostrea gigas* (calcite) and *Mya truncata* (aragonite) shell powders were used as a refer-  
218 ence in FTIR-ATR analysis to measure the absorbance peak profiles of calcite and aragonite  
219  $\text{CaCO}_3$  polymorphs in *Mytilus* shells (Figure 1c, d). The absorbance peaks measured from the  
220 Baltic and North Sea mussel shells were identical to both calcite and aragonite profiles of the  
221 reference species, confirming the presence of both polymorphs. Significant variation in cal-

222 cite/aragonite ratio of the shells from different sites could potentially contribute to difference  
223 in shell mechanical properties. However, absorbance ratios for 875/1785  $\text{cm}^{-1}$  showed no dif-  
224 ference in the calcite/aragonite ratio (Kruskal-Wallis chi-squared = 0.78,  $p= 0.67$ ) with re-  
225 spect to different locations (Figure 1e, f). Similar results were obtained for absorption ratio  
226 1793/1785  $\text{cm}^{-1}$  (kruskal-wallis chi-squared = 0.86,  $p= 0.65$ , Figure 1e, f).

### 227 **3.3. Shell traits (periostracum, shell thickness, organic content and microstructure)**

228 Shell morphometric patterns of  $n=3$  equally sized animals collected from North Sea and Bal-  
229 tic Sea were analyzed. For each shell, data from five different points on the shells were col-  
230 lected. Shell thickness varied significantly among the three sites (Kruskal-Wallis chi-squared  
231 = 35.27,  $p= 2.19\text{e}^{-8}$ ) and periostracum thickness also showed the same trend (kruskal-wallis  
232 chi-squared = 30.42,  $p= 2.47\text{e}^{-7}$ ; supplementary Data 2). Plotting periostracum/shell thickness  
233 ratio (as %) separates the shells according to their location (supplementary figure 1a) and  
234 same trend is observed by plotting mineral content of the shells.

235 The organic content obtained after decalcification of the shells (periostracum being removed  
236 during shell cleaning process) varied significantly among the three sites (Kruskal-Wallis chi-  
237 squared = 5.06,  $p= 0.07$ , supplementary Data 3). Higher organic contents were found in  
238 Nynäshamn shells followed by Usedom and Sylt shells (Supplementary Figure 1b). It can be  
239 seen that the shell traits from different locations vary significantly as a principal component  
240 analysis of these traits clearly revealed clustering of the mussels into three different groups  
241 (supplementary Figure 1c). The first component gets a clear opposition in scores projection  
242 between Sylt mussel shells on one part Nynäshamn and Usedom mussel shells on the other  
243 part explained by a contrast between the mineralized layer thickness and the organic content.  
244 The second component gets a clear opposition in scores projection between Usedom mussel



245 shells on one part and Nynäshamn and Sylt mussel shells on the other part, which is mainly  
246 explained by the variable ‘periostracum thickness’.

247 Scanning electron microscopy images of Baltic blue mussel shells indicates altered in the mi-  
248 crostructure as evidenced by relatively thin aragonite tablets and irregular calcite prisms com-  
249 pared to shells of North Sea mussels (Figure 2).

### 250 **3.4. Shell matrix proteins**

251 To identify the shell matrix proteins presents in the shells of *Mytilid* mussels from Baltic Sea  
252 and North Sea, we interrogate the transcriptome databases of both *M. edulis* and *M. trossulus*  
253 against the shell proteomics dataset. The number of transcripts matched with at least two  
254 unique peptides for each site using *M. edulis* database are: 247 (Sylt), 436 (Usedom) and 187  
255 (Nynäshamn); transcripts matched using *M. trossulus* database are: 188 (Sylt), 224 (Usedom)  
256 and 257 (Nynäshamn). In total, 671 and 624 transcripts with at least 2 unique peptides could  
257 match using *M. Trossulus* (FDR peptide > 0.2%, protein =0.1%) and *M.edulis* (FDR peptide  
258 >0.4%, protein = 0%) databases, respectively (Supplementary Table 1). It should be noted  
259 that the matched transcripts include both proteins and proteoforms. The difference in the  
260 number of transcripts matched with respect to different sites and databases could be partially  
261 attributed to the taxonomy specificity of the mussels inhabiting the three sites: Sylt is popu-  
262 lated by *M. edulis*, Nynäshamn by *M. trossulus* and Usedom by *M. edulis* x *M. trossulus* hy-  
263 brid complex (Telesca et al., 2019). Carrying out BLAST using the matched sequences result-  
264 ed in the annotation of 327 and 462 sequences for *M. trossulus* and *M. edulis*, respectively  
265 (Supplementary Table 2). Among the annotated sequences, 144 SMPs are common to the  
266 shells from the three sites with *M. edulis* database and 131 SMPs in case of *M.trossulus*. Gene  
267 ontology count assigned top five molecular functions such as oxidoreductase activity, metal  
268 ion binding, chitin binding, peptidase activity and catalytic activity for transcripts matching  
269 *M. edulis* database (total sequence count = 58) and in case of *M.trossulus* database the molec-

270 ular functions were similar excepting that mono phenol monooxygenase activity replaced  
271 catalytic activity (total sequence count =74, Figure 3).

272 Label free semi-quantitative analysis of shell proteins from the three sites showed differential  
273 expression of many SMPs that could be attributed to different functional categories. 37 SMPs  
274 were found to be modulated when annotated against *M. trossulus* transcriptome database in-  
275 cluding SMPs comprising HHIP-like protein, shell protein-4, GA-rich protein, protease inhib-  
276 itor-like protein B1, matrix protein-1, metalloprotease inhibitor-3-like protein in addition to a  
277 WAP domain containing protein (Figure 4, Supplementary Table 3). 17 SMPs were modulat-  
278 ed when annotated using *M. edulis* transcriptome, and interestingly many are subset of the  
279 modulated SMPs identified in *M. trossulus* (Supplementary Figure 2, Supplementary Table  
280 1).

#### 281 **4. Discussion:**

282 The salinity gradient in the different regions of Baltic Sea is well established (Müller et al.,  
283 2016) and the adaptation of species to the brackish conditions in the Baltic Sea has caused  
284 both morphological and genetic differences compared to salt/water ancestors (Larsson et al.,  
285 2017). Under unfavorable environment, marine calcifiers reallocate the energy required for  
286 different cellular functions and generally this affects shell formation leading to shell pheno-  
287 types (Harper et al., 2012). Even though the SMPs employed in shell building by marine cal-  
288 cifiers are species specific, critical steps such as mineral deposition, crystal growth and orien-  
289 tation etc., could be effected by divergent sets of SMPs resulting in altered shell traits. The  
290 quantity of SMPs in the shell from natural populations may reflect the global phenotype of the  
291 organism during its entire life span and not a particular temporal scale.

292 Generally, *Mytilus* acclimation to varying temperatures and pH has shown limitation in pro-  
293 tein metabolism, thinner shells and CaCO<sub>3</sub> crystal disorientation, which may affect the mate-

294 rial properties of the shell (Fitzer et al., 2015, 2018; Fitzer et al., 2014a). Noticeably, our data  
295 revealed significant differences in shell morphology, microstructures and protein content in  
296 Baltic blue mussels from different population experiencing different salinity conditions. The  
297 production of a calcium carbonate exoskeleton depends not only on the seawater carbonate  
298 chemistry but also on the ability to produce bio-minerals (Fitzer et al., 2014b), and might be  
299 directly related to SMP content of individual shells.

300

#### 301 **4.1. Quantification of SMPs and shell phenotype**

302 Quantitative protein expression profiling is useful to gain insight on molecular mechanisms in  
303 marine shell forming organisms. For example, this approach was employed to investigate the  
304 impact of reduced seawater pH along with pathogen challenge in the immune response of  
305 *Crassostrea gigas* (Cao et al., 2018). In fact, the amount and nature of the SMPs in the shell  
306 alter its physical property affecting the resilience of mussels to rapidly changing environmen-  
307 tal conditions.

308 Shell formation involves the production of an extracellular organic matrix (the shell scaffold).  
309 This primarily contains  $\beta$ -chitin and SMPs and the crystals are induced to form inside these  
310 matrix voids (Falini et al., 1996). Chitinase-3 plays a key role in the metabolism of chitin in  
311 the shell scaffold, which provides the template for mineral nucleation. However, its expres-  
312 sion may vary in response to different abiotic factors as evidenced by the substantial increase  
313 in its expression in the outer mantle epithelial cells of *Pinctada fucata* during shell regenera-  
314 tion experiments (Li et al., 2017) and also during shell remodeling in the freshwater snail  
315 *Lymnaea stagnalis* (Yonezawa et al., 2016). In contrast, under pH stress the chitinase-3 gene  
316 expression was found to be down regulated in mussels (Hüning et al., 2013). In Baltic blue  
317 mussels, chitinase-3 was found in relatively higher amounts in the shell indicating altered  
318 scaffolding processes contributing to the shell phenotype in low salinity.

319 Label free quantification of the protein extracts from the shells collected from the North Sea  
320 (Sylt) and Baltic Sea (Usedom and Nynäshamn) indicated modulation in many SMPs (dis-  
321 cussed below). At this juncture, it is pertinent to examine if any disparity in aragonite or cal-  
322 cite content in the shells could affect the quantity of certain polymorph-specific SMPs. The  
323 FTIR data indicated that aragonite/calcite ratios did not vary significantly in the shells of in-  
324 dividuals from the three different locations and thus, it can be inferred that the relative modu-  
325 lation of SMPs could basically not be influenced by a specific calcium carbonate polymorph.  
326 Shell matrix proteins such as blue mussel shell protein (BMSP), sushi-like protein and chi-  
327 tinase-3 or several Von Willebrand factor-A (VWA) and chitin binding domain-2 (Chbt-2)  
328 containing proteins showed remarkable distinct quantity in the shells of individuals collected  
329 from the three sampling locations (Figure 4, Supplementary Figure 2 and Supplementary Ta-  
330 ble 3). In fact, proteins containing domains such as VWA and Chbt-2 contribute to the silk-  
331 like protein complexes (Weiner, 1979) and may act as glue assembling  $\beta$ -chitin sheets and  
332 minerals (Suzuki et al., 2011); their relatively higher content in Baltic blue mussels suggests  
333 that the scaffolding of  $\beta$ -chitin within the shell matrix space may be distinguishable too. Hy-  
334 drophobic glycine-alanine rich (GA-rich) proteins sandwich  $\beta$ -chitin and support the  $\beta$ -sheet  
335 conformation, whereas, serine-aspartic acid rich (SD-rich) proteins are believed to surround  
336 the above complex and interact with the mineral phase aiding in crystal formation (Falini et  
337 al., 1996; Nakahara et al., 1980; Weiner, 1979; Weiner & Traub, 1980). Intriguingly, these  
338 two chitin interacting proteins showed relatively lower contents in the Baltic blue mussels  
339 leading potentially to altered microstructural organization in the shell as observed on the  
340 scanning electron microscopy images (qualitative observations; Figure 2). Information about  
341 the orientation of  $\text{CaCO}_3$  crystals from these shells would better explain the altered micro-  
342 structures.

343 The thicker periostracum found in Baltic blue mussels are almost certainly associated with the  
344 increased protection compensating when the calcium carbonate saturation state is low (Thom-  
345 sen et al., 2010) and shell dissolution is more likely. Interestingly, tyrosinase and byssal pe-  
346 roxidase-like proteins levels were higher in Baltic mussel shells (Figure 4, Supplementary  
347 Table 3). This suggests that the processes important for periostracum maturation i.e. oxidation  
348 of tyrosine to L-Dopa and L-Dopa to O-quinone (Qin et al., 2016; Zhang et al., 2006) are also  
349 valuable for shell matrix formation, particularly under the low calcium carbonate saturation  
350 states encountered in the Baltic Sea. Tyrosinases have also been extracted from the shells of  
351 other *Mytilus* species (Gao et al., 2015) and acclimation of Baltic blue mussels to low pH  
352 (which produces a dramatic reduction in calcium carbonate saturation state) resulted in a very  
353 strong (>100 fold) increase in the mRNA expression of a mantle tissue-specific tyrosinase  
354 isoform (Hüning et al., 2013). Generally, a higher (shell) organic content, together with re-  
355 duced calcium and inorganic carbon availability lead to increased energy demands and this  
356 may explain the dwarfism in *Mytilus spp.* in the low saline Baltic Sea (Kautsky et al., 1990).  
357 Considering the projected decrease in salinity along with increased physiological osmotic  
358 stress, *Mytilus spp.* inhabiting the Baltic Sea are more likely to form a thicker periostracum  
359 (Telesca et al., 2019) pointing out that further adaptation by *Mytilus spp.* with regard to shell  
360 biomineralization should be anticipated.

361 Surprisingly, several other key-domain shell proteins were not observed to present quantifica-  
362 tion difference between the different location populations, including carbonic anhydrase  
363 (CA), epidermal growth factor (EGF) and fibronectin-3 (FN3). While CA converts carbon  
364 dioxide to bicarbonate ions (Miyamoto et al., 1996), EGF may interact with calcium ions, and  
365 fibronectin brings anions and cations into close proximity to enhance nucleation as proposed  
366 for calcium phosphate biomineralization (Ba et al., 2010). Due to this apparently ubiquity of  
367 the observed quantity in shell of CA, it may be presumed that bicarbonate ion nucleation

368 might not be a decisive factor for processes underlying shell phenotypic plasticity under low  
369 salinity conditions. Alternatively, it is also possible that the shell protein composition does not  
370 reflect the actual quantity of different isoforms of CA in the mantle tissue. Even though the  
371 *M. edulis* shell is composed of both calcite and aragonite layers, calcite-specific EGF contain-  
372 ing protein was not differentially quantified within the shell of the sampled populations.

373 Although to date, the complete set of proteins within the mollusc shell biomineralization tool  
374 kit remains to be described in-depth, recent studies have demonstrated the presence of an evo-  
375 lutionarily conserved toolkit for shell formation that is crystal-specific and species-specific  
376 from four highly divergent bivalves including *M. edulis* (Arivalagan et al., 2017). In addition,  
377 Feng *et al.*, observed that proteins containing domains such as carbonic anhydrase, sushi,  
378 VWA and chitin binding domains are conserved in the shell matrix of many bivalve species  
379 (Feng et al., 2017). In this study, proteins such as BMSP, sushi-like protein, chitinase-3 and  
380 tyrosinase, which contain domains that are potentially part of the basic tool kit present quanti-  
381 ty variations within the shell of the three different Baltic populations sampled, suggesting that  
382 variation in the overall biomineralization processes between those populations, supporting the  
383 shell structure and morphology differences observed in parallel.

#### 384 **4.2. Shell fracture resistance**

385 The mechanical properties of bivalve shells are also attributed to the minor organic compo-  
386 nents present in the CaCO<sub>3</sub> inorganic structure that is characterized by highly ordered crystal  
387 state. Deviations from this state are considered to indicate impaired or irregular biominerali-  
388 zation resulting in weaker shells (Fitzer et al., 2016). Common structural proteins such as  
389 elastin, collagen, silk and keratins are characterized by a long range ordered molecular sec-  
390 ondary structures (e.g.  $\beta$ -pleated sheets,  $\beta$ -spirals, coiled coils, triple helices etc.) that accom-  
391 modate a broader spectrum of functional requirements such as elasticity to support diverse  
392 tissues (Hu *et al.*, 2012). The sequence/motif repeats are responsible for the conformation of

393 their secondary structures. For example, a recent study on teeth-like structural proteins present  
394 inside squid suckers using proteomics and molecular biology clearly highlighted the relation-  
395 ship between the molecular features of the low-complexity and repeated proteinaceous se-  
396 quences and mechanical properties of the resulting biomineral (Jung et al., 2016). The tandem  
397 repeats of proteins that are embedded within the squid ring teeth are responsible for the ex-  
398 traordinary toughness and flexibility of the teeth biomaterial. Based on the observed differ-  
399 ences in the mechanical properties of the *Mytilus* shells of blue mussels originating from the  
400 compared Baltic area (Figure 1b), we hypothesized that the shells, especially those presenting  
401 higher mechanical properties might harbor certain enrichment in such structural proteins with  
402 a similar set of repetitive sequences and secondary structures. The primary sequences of two  
403 proteins (TR78416\_c1\_g1\_i2 and TR71052\_c0\_g1\_i1, Figure 6a and b) that are highly ex-  
404 pressed in North Sea mussel shells (homologous to MSI-60 of *P. fucata* and insoluble pro-  
405 teins of *Mytilus spp*) contain several repeat sequences of this kind. The poly-Ala regions in  
406 these proteins are similar to that of silk protein, which forms  $\beta$ -sheet structures by placing  
407 successive alanine residues on alternate sides of a backbone (Hayashi et al., 1999) with each  
408 alanine chain interlocking with an adjacent chain *via* hydrophobic interactions. Similarly,  
409 GGXGG repeats, as those found in mussel shell proteins, were previously reported in the in-  
410 sect glycine rich cuticle protein family (CPG) including resilin (a rubber-like protein in insect  
411 cuticle), which provides higher elasticity and acts as an efficient elastic-energy storage com-  
412 ponent in insect wings (Adams, 2000; Andersen and Weis-Fogh, 1964). 3-D model simula-  
413 tions of the protein sequences (TR78416\_c1\_g1\_i2 and TR71052\_c0\_g1\_i1) predicted  $\beta$ -  
414 strands in both the cases (Figure 5a and b) and the tertiary structure of TR78416 resembled a  
415  $\beta$ -solenoid, in particular the  $\beta$ -roll structure of spider silk protein (Figure 5c), which confers  
416 unique mechanical properties to silk. Disordered regions rich in Asp, Ser and Gly that have  
417 the potential to bind  $\text{CaCO}_3$  minerals, or to allow calcium ion super-saturation conditions

418 were also found in different amount in the shell of the mussel populations. The capacity of  
419 these proteins to bind CaCO<sub>3</sub> minerals may play a critical role in making shell more resistant  
420 to fracture as observed for Sylt shells. The observation that these proteins were present in  
421 relatively lower amounts along with thinner shell in Baltic blue mussels may explain the fra-  
422 gility of those biomineral structures (Figure1b). Variations in shell thickness indicate altera-  
423 tion in biomineralisation control by the SMPs and may alter the fracture force.

#### 424 **4.3. Alteration in biochemical defense**

425 The identification in the mussel matrix of many proteins that could be rely to immune classes  
426 raises the question of “what other functions could bear certain SMPs that would not basically  
427 be involved in shell building process?” The presence of proteins with immunity-related func-  
428 tions argues for the occurrence of an efficient biochemical defense system operating in the  
429 shell, in addition to the primary shell edification processes. Recent studies on gastropods sug-  
430 gest that the shell has been co-opted as a defense system to encase and kill parasitic nema-  
431 todes which validates the data here (Rae, 2017).

432 An innate immune cascade involving phenoloxidase (PO) leads to several responses such as  
433 pigmentation (melanization) and repair of damaged insect exoskeletons including hardening  
434 (sclerotization), pathogen elimination by phagocytes (opsonization) etc., (Cerenius &  
435 Söderhäll, 2004; Jiravanichpaisal et al., 2006). POs are a copper containing class of enzymes  
436 including tyrosinase, catecholase and laccase (Luna-Acosta et al., 2011). In mussels, tyrosi-  
437 nase is involved in periostracum formation (Zhang et al., 2006) and the presence of laccase-4  
438 like protein (pro-PO) and several other components of the PO cascade imply the participation  
439 of certain POs in the mussel immune response. Generally, PO activation starts with the ag-  
440 gregation of several proteins and enzymes, which convert pro-PO to PO (Figure 6), thanks to  
441 the action of proteinases. Here, no shell proteinase present observable differences in quantity  
442 within either North Sea or Baltic blue mussels. However, serine protease inhibitors containing



443 domains such as kunitz, serpin and TIMP (tissue inhibitor of metalloproteinase), that are neg-  
444 ative regulators of the PO pathway (Jiravanichpaisal et al., 2006), were present in high  
445 amount in Baltic mussel shells even though pro-PO (Laccase-4 like protein) did not present  
446 different quantification within the three populations. It is worth to note that cellular cost of  
447 investing energy in systems such as PO is expensive. Generally, organisms opt for constitu-  
448 tive defenses and therefore activate the PO cascade only when there is a constant pathogen  
449 threat in their environment (Moret, 2003). Baltic waters may contain relatively fewer mussel  
450 pathogens and shell boring organisms than the North Sea, which might explain the observed  
451 differential regulation of the PO cascade.

## 452 **5. Conclusion:**

453 To refine predictions of the effects of environmental change on marine calcifiers, it is essen-  
454 tial to decipher the intricate relationship between shell phenotype, biological control of bio-  
455 mineralization and the environment. This study is the first to show that modulation in SMPs  
456 correlates to the shell phenotype and might support environmental adaptability of the mussels.  
457 Clearly, our findings show that alteration in the mechanical properties and along with poten-  
458 tial biochemical defense offered by the shell are correlated with specific SMP features of the  
459 populations. Although Baltic blue mussels have in first order successfully experiment life  
460 under low-salinity conditions, they may also have encountered a lower predator environment  
461 pressure over several generations, that also may have influenced the composition of the shell  
462 calcifying matrices, through selective or adaptative mechanisms. Further declines in salinity  
463 over short time-spans in the Baltic Sea (Gräwe et al., 2013) might irreversibly alter the calci-  
464 fication processes because of increased energy costs, altered protein metabolism, decreased  
465 shell thickness etc. Also, a recent study showed that low salinity induced strong immunosup-  
466 pressive effects on the functional and immune molecular traits of *M. edulis* (Wu et al., 2020)  
467 endangering further the survival of populations.

468 To conclude, SMPs seem to constitute valuable indicators of altered molecular mechanisms of  
469 shell edification for Baltic blue mussels encountering contrasted marine conditions. Shell  
470 traits such as periostracum thickness, organic content, microstructure and fracture resistance  
471 qualitatively correlates with the modulation of SMPs. Large scale studies in this context and  
472 assessing genetic background will help to discern alteration in biomineralization control by  
473 environmental and genetic factors and further enable to identify molecular signatures of resil-  
474 ience or sensitivity in marine calcifiers.

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675

676 **Data availability**

677 The mass spectrometry proteomics data have been deposited to the ProteomeXchange Con-  
678 sortium *via* the PRIDE partner repository with the dataset identifiers PXD013091 and  
679 10.6019/PXD013091; PXD013148 and 10.6019/PXD013148.

680

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690 60611, USA

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692 Author contributions: A.M. and S.B. designed the experiments; J.A. prepared the samples for  
693 proteomic experiments; G.C. and J.V. performed mass spectrometry analysis; J.A., B.M. and  
694 A.M. analyzed the mass spectrometry data and validated the results; J.A. and S.B. generated  
695 scanning electron microscopy images. S.M.Z. carried out the compression tests. P.D. is a Re-  
696 search Director of the National Fund for Scientific Research (Belgium), designed the com-  
697 pression tests and analyzed the results. M.L. ran FTIR analysis and S.B. performed statistical  
698 analysis. J.A., S.B. and A.M. wrote the paper and all authors discussed and approved the  
699 manuscript.

700 The authors declare no conflict of interest

701 **Legends for Figures and Tables:**

702 **Figure 1:**

703 (a) Mussel sampling locations. (b) Dot plot indicates the size range of the blue mussel shells are ho-  
704 mogenous and the histogram shows the shell fracture resistance ( $F_{max}$ ) of shells from different sites.  
705  $F_{max}$  of shells from North Sea (Sylt) varies considerably compared to shells from Baltic Sea  
706 (Nynäshamn and Usedom; Kruskal-wallis chi-squared = 8.43,  $p$ -value = 0.01). (c) FT-IR of aragonite  
707 and calcite reference materials. (d) FT-IR spectra of North Sea (Sylt) and mussel shell samples show  
708 highly superimposable spectra. Panels (e) and (f) represent scatter and box plot of IR peak absorp-  
709 tion ratio 875/1785 (kruskal-wallis chi-squared = 0.78,  $p$ -value = 0.67) and 1793/1785 (kruskal-wallis  
710 chi-squared = 0.86,  $p$ -value = 0.65) respectively indicating that shells from Sylt, Usedom and  
711 Nynäshamn consist of rather similar mixture of aragonite and calcite. Red line in the panel e repre-  
712 sents 100% calcite

713 **Figure 2:**

714 Scanning electron microscopy images show altered microstructure of Baltic mytilid shells. The region  
715 of the shell used for acquiring the images is shown at the top. Rows a, b and c show the interface of  
716 the nacreous layer, myostracum and prismatic layer of the shells from Sylt, Usedom and Nynäshamn  
717 respectively. Each row followed by magnified images of nacreous and prismatic layers. In Baltic myti-  
718 lid mussels (rows b and c) the nacreous layer is formed by relatively thin aragonite tablets compared to  
719 North Sea mussels (row a). Ladders in the nacre layer are shown for visualizing tablet thickness. Pris-  
720 matic layers are formed by irregular calcite prisms in Baltic mytilid mussels and are indicated by ar-  
721 rows

722 **Figure 3:**

723 Gene ontology (GO) count of molecular functions represented using the matched transcripts from *M.*  
724 *edulis* and *M.trossulus* databases

725 **Figure 4:**

726 Heat map generated using Label free LC-MS/MS data indicating modulated SMPs identified with *M.*  
727 *trossulus* transcriptome database

728 **Figure 5:**

729 Simulated structure of proteins TR78416 and TR71052 predicted using the I-Tasser tool (a and b re-  
730 spectively). TR78416 proteins contain motifs that are formed by  $\beta$ -strand-turn- $\beta$ -strand and are similar

731 to that of spider silk. (c)  $\beta$ -roll structure formed by GAGAGAGX repeats of *B. Mori* silk protein. Pri-  
732 mary protein sequences are given for both the proteins and the GGXGG repeating motifs (see text) are  
733 shaded in yellow.

734 **Figure 6:**

735 The phenoloxidase (PO) pathway in arthropods and crustaceans indicating the different proteins that  
736 are modulated in Baltic mussels under the influence of low salinity and biotic factors that characterize  
737 their habitat. Down-regulated proteins are represented in green font and up- regulated proteins are  
738 denoted in red font.

739

740 **Supplementary Figure 1:**

741 (a) Graph shows periostracum/mineral layer thickness ratio (expressed as %) of shells from three loca-  
742 tions and clusters into separate groups Baltic mussels (Nynäshman and Usedom) and North Sea mus-  
743 sels (Sylt). Boxplots constructed with periostracum thickness/mineral layer thickness ratio (as %) and  
744 mineral layer thickness illustrates the separation of the Baltic and North Sea shells. (b) Box plot show-  
745 ing the organic content of the shells excluding periostracum. (c) Principal component analysis per-  
746 formed by combining mineral layer thickness (Mn), periostracum thickness (P) and organic content  
747 (OC) separates the Baltic mussels from the North Sea mussels.

748 **Supplementary figure 2:**

749 Heat map generated using Label free LC-MS/MS data showing modulated SMPs that are identified  
750 using *M.edulis* transcriptome database.

751 **Supplementary Table 1:**

752 List of matched transcripts from *M.trossulus* and *M.edulis* database using Mascot and grouped using  
753 Scaffold software. The list indicates proteins and proteoforms from all the three sites matched to each  
754 database.

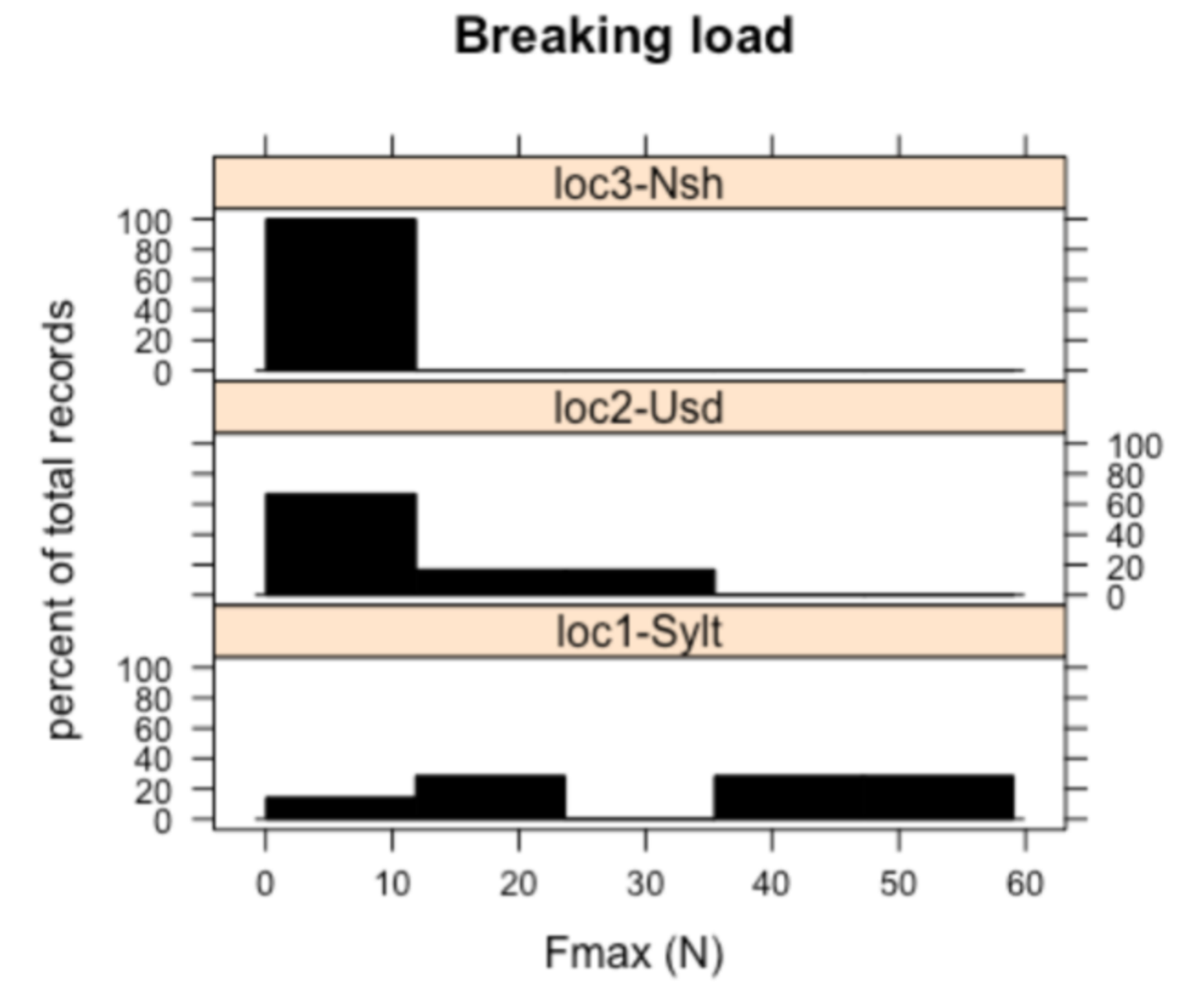
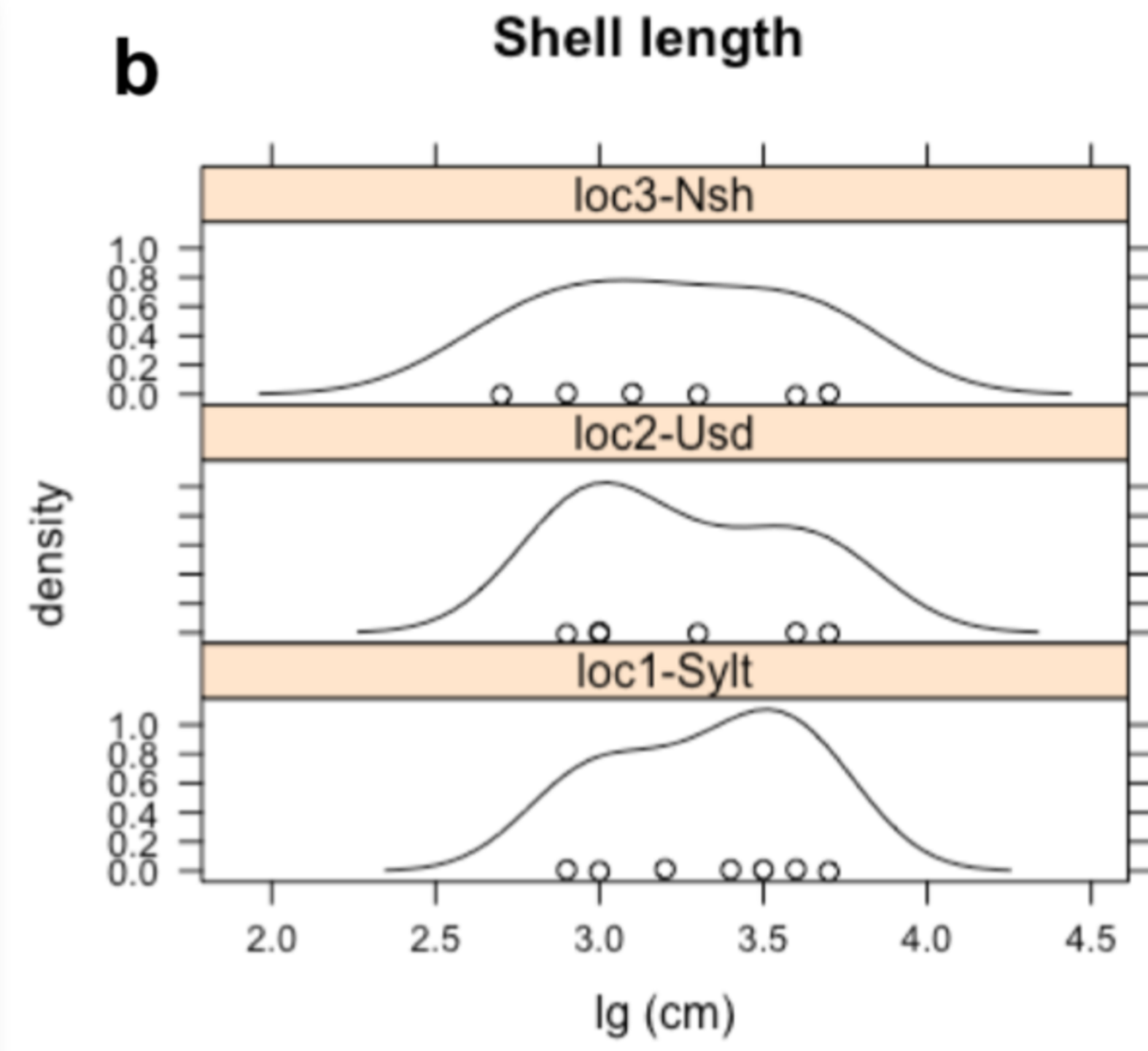
755 **Supplementary Table 2:**

756 List of matched proteins from Supplementary Table 1 and annotated using BLAST.

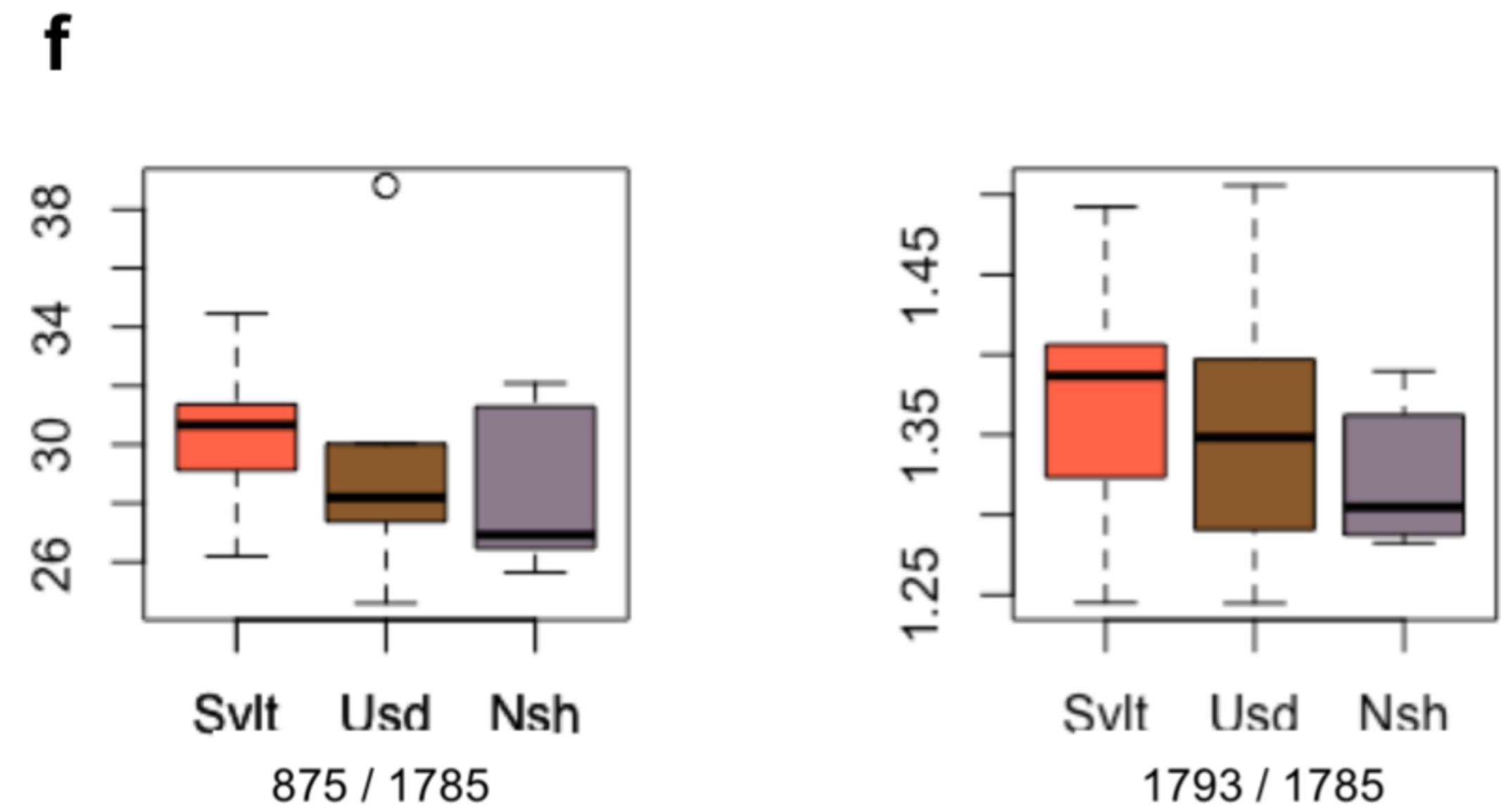
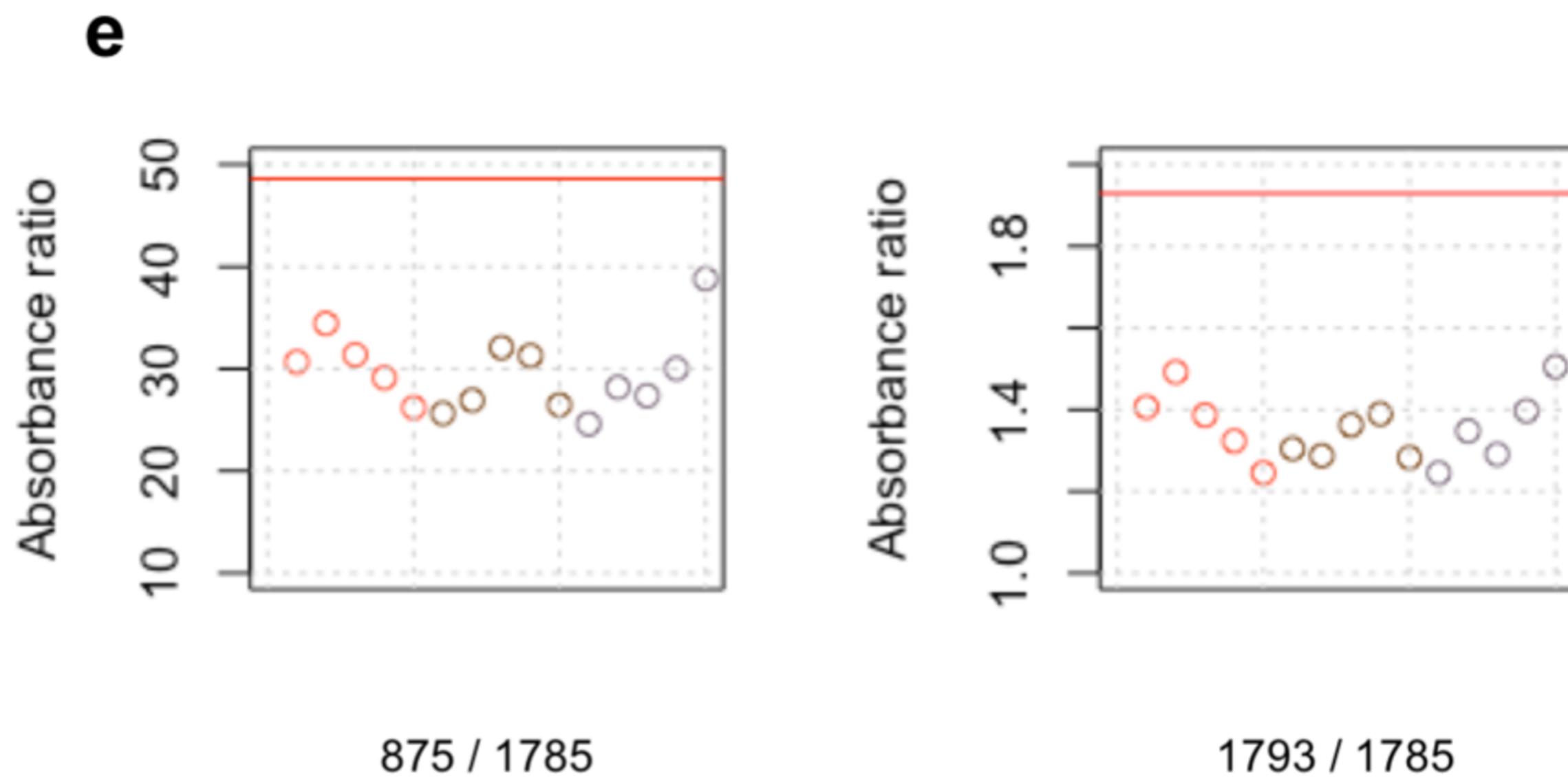
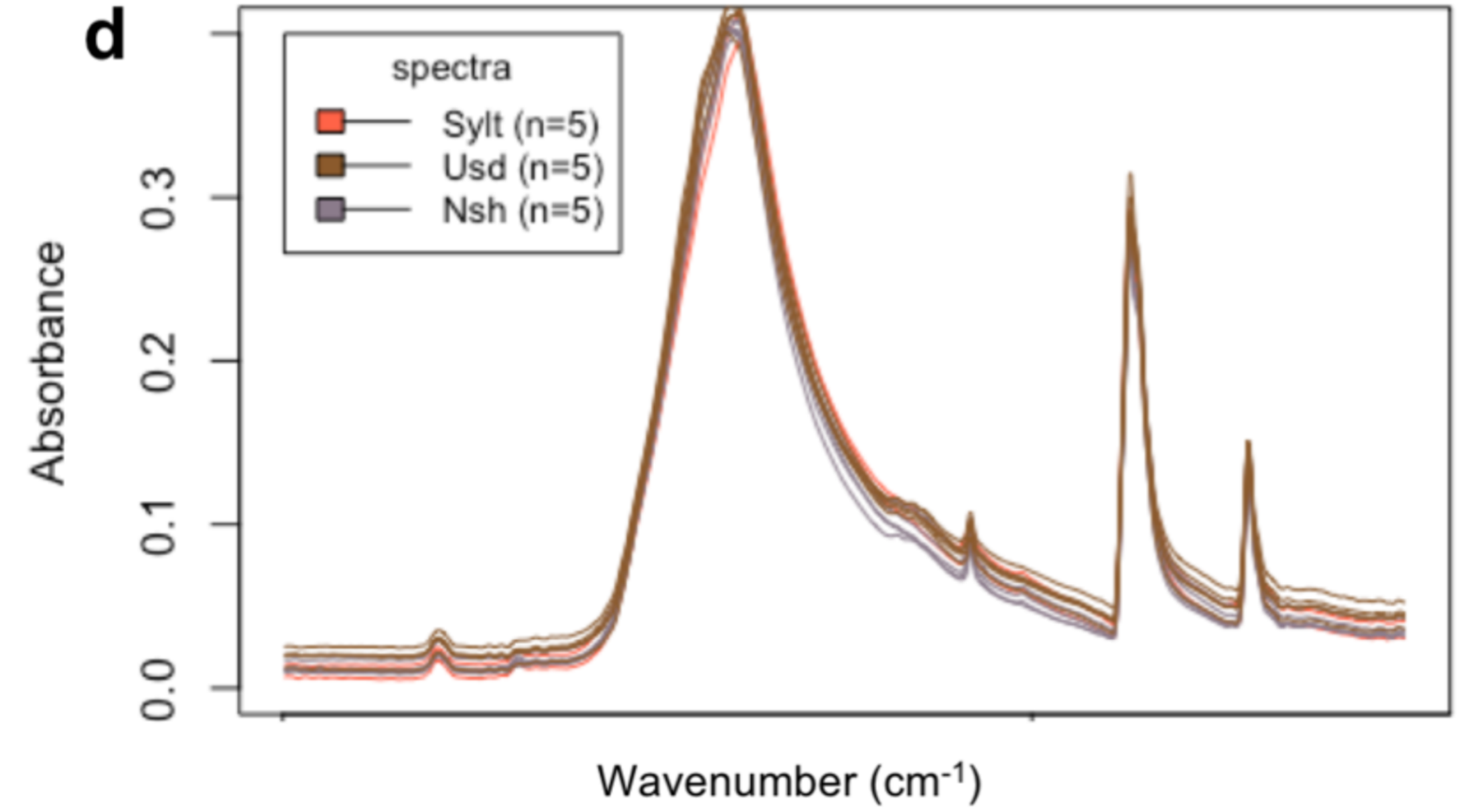
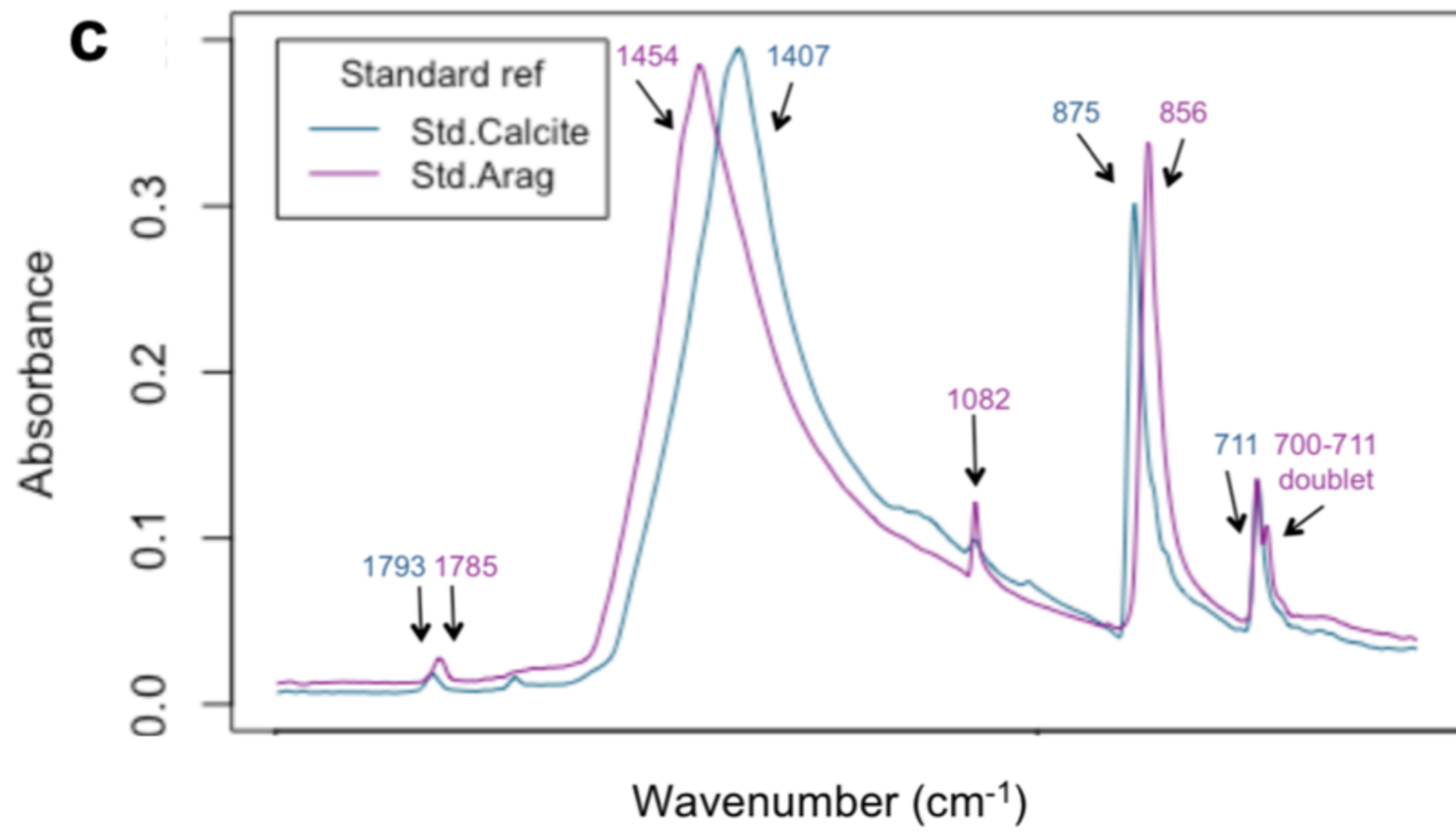
757 **Supplementary Table 3:**

758 Combined list of modulated SMPs and corresponding SMART functional domains from *M. trossulus*  
759 (Figure 4) and the *M. edulis* (Supplementary Figure 2) databases.

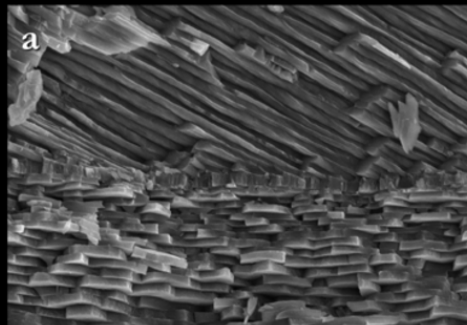
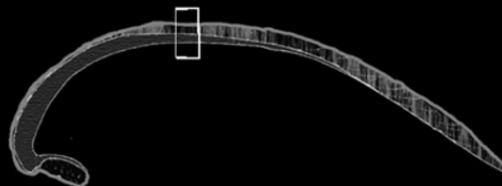
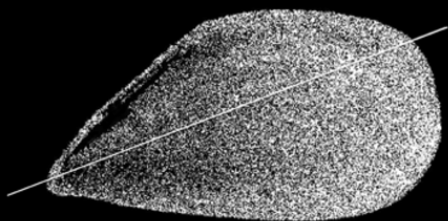




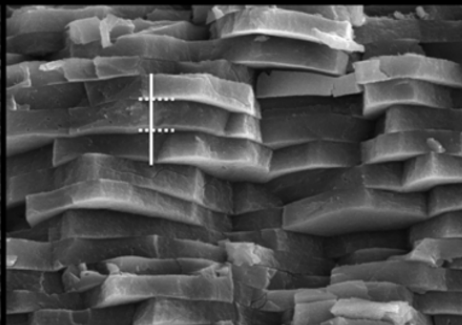
FTIR - ATR



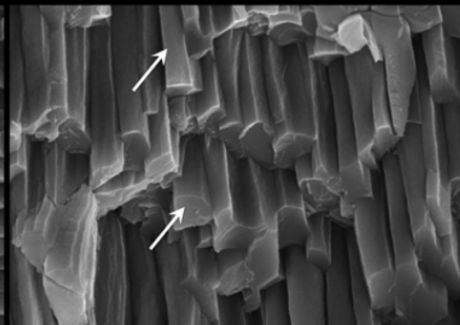




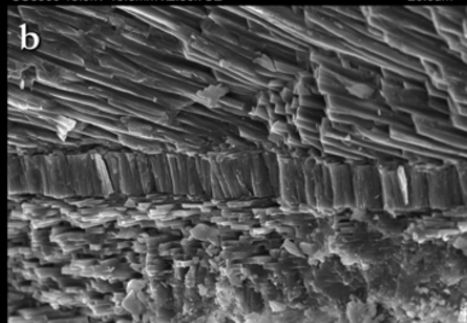
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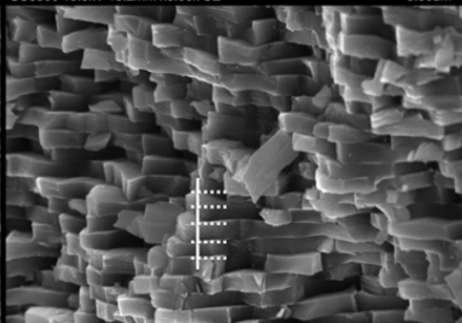
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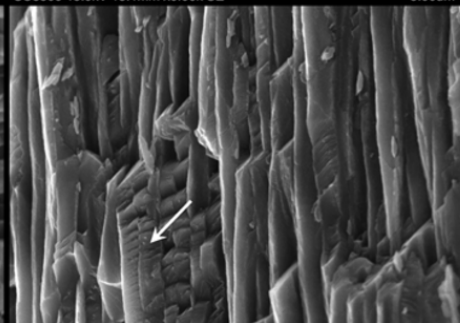
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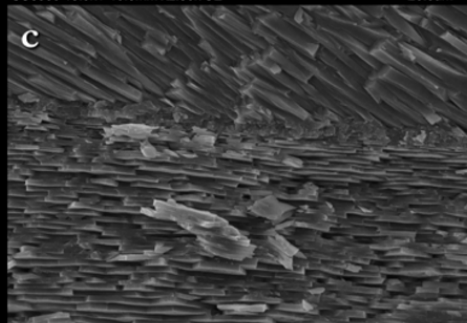
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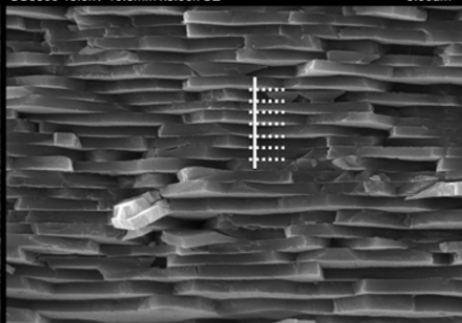
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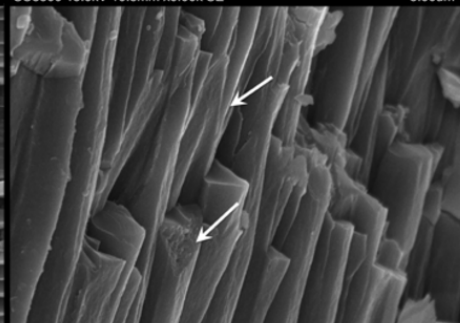
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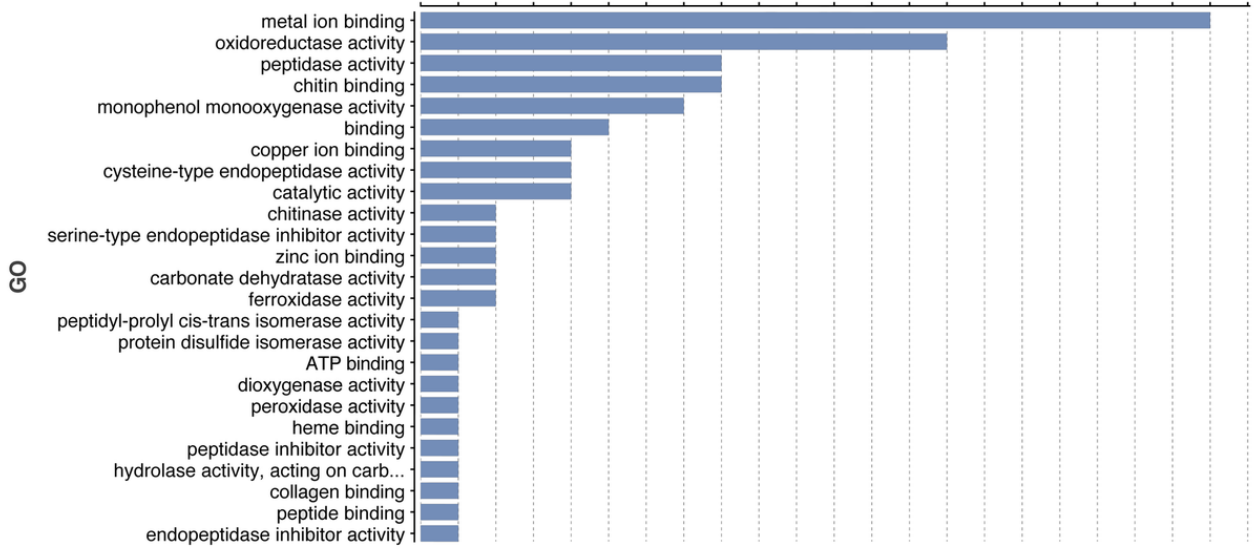


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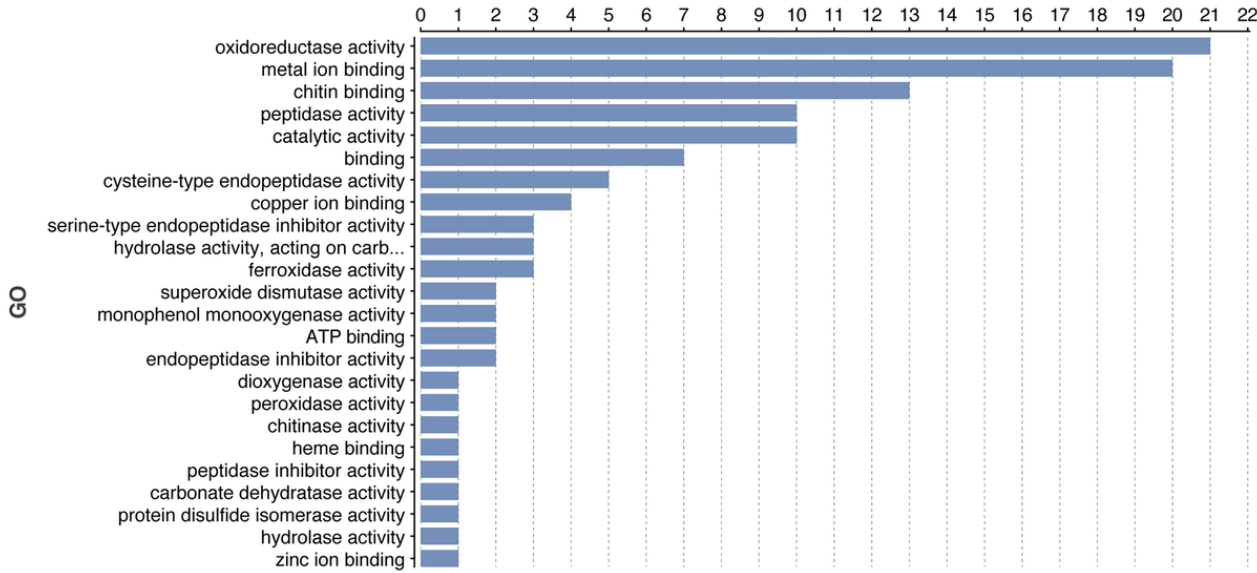


**GO count**  
**Molecular function**

**#Seqs *M. edulis***

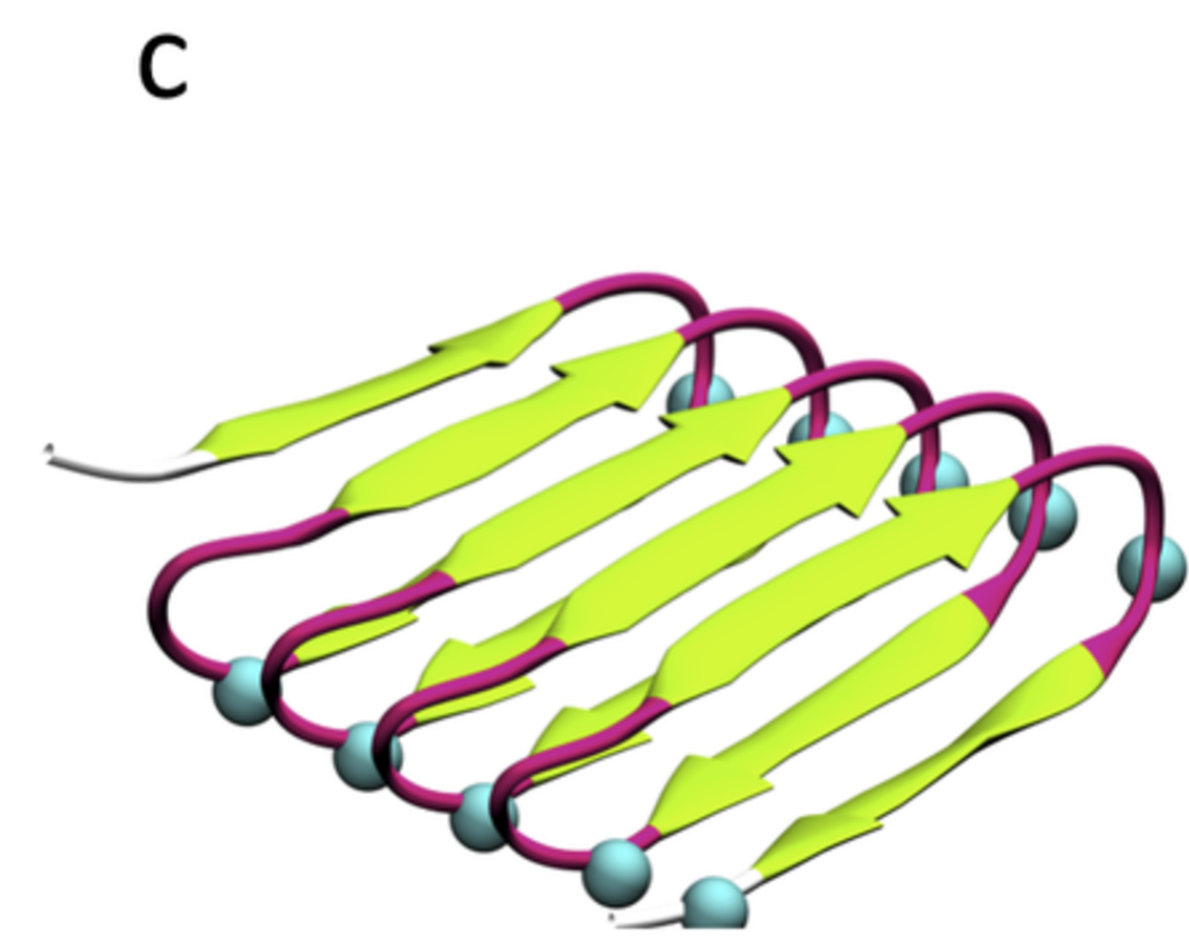
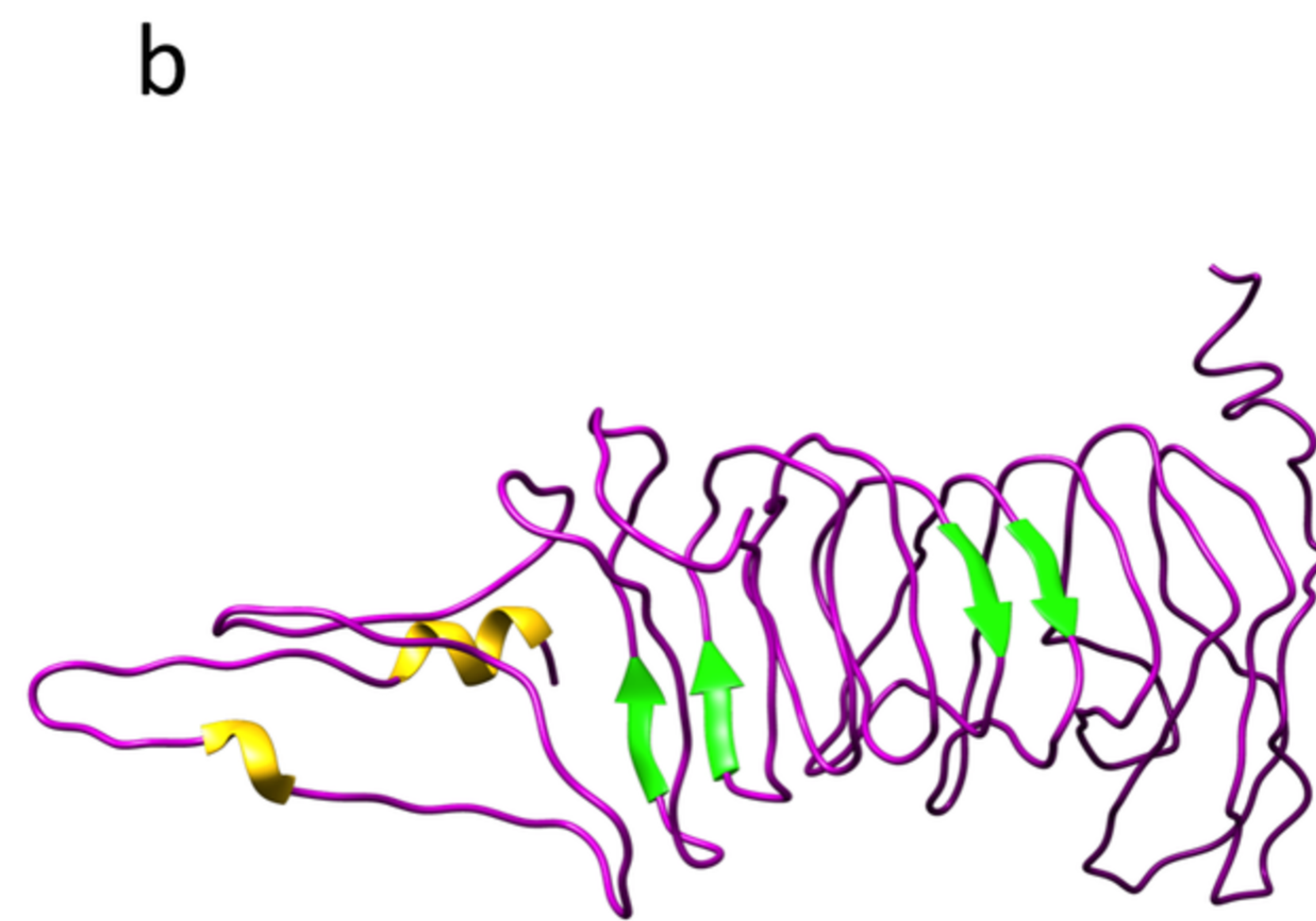
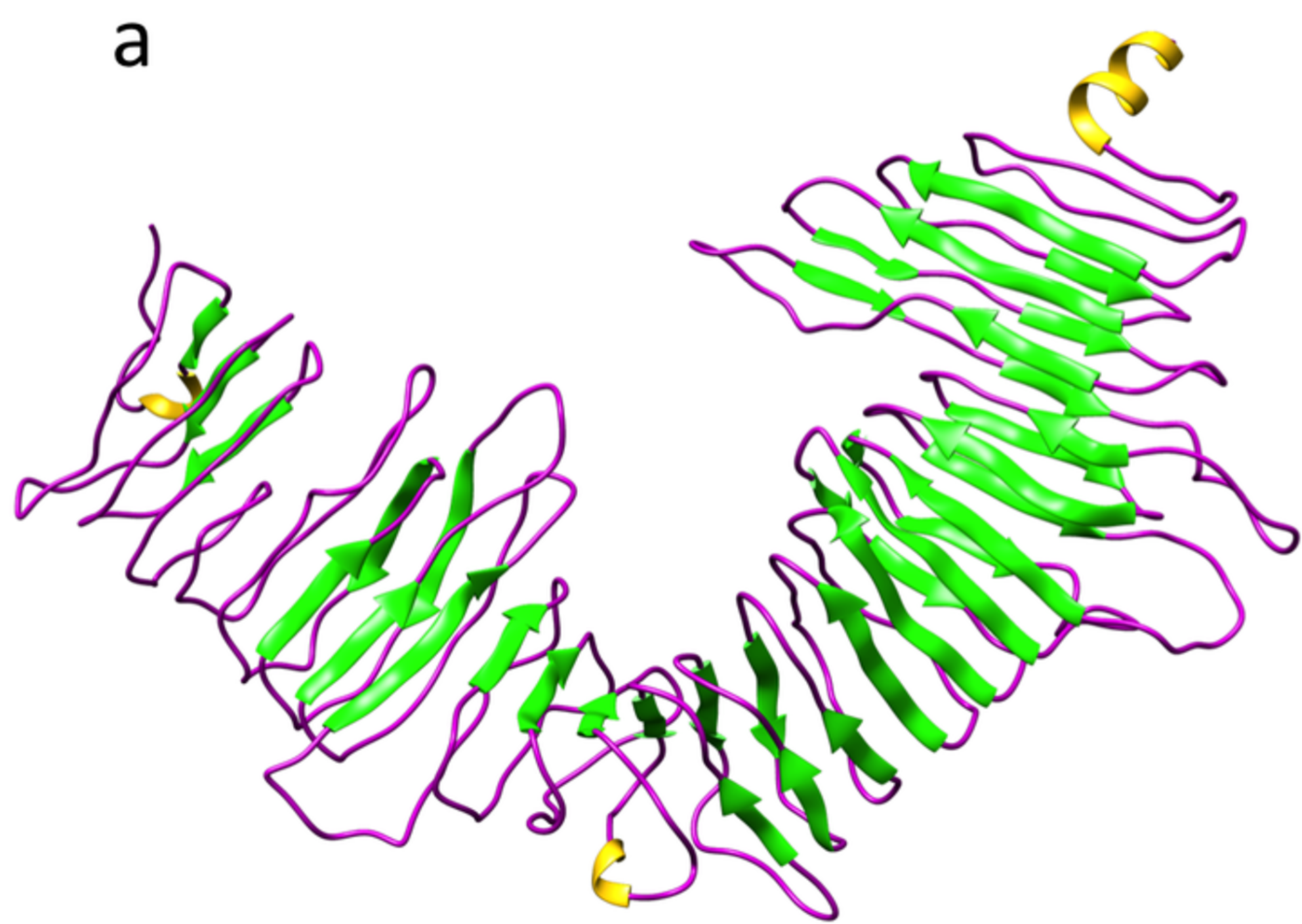


**#Seqs *M. trossulus***









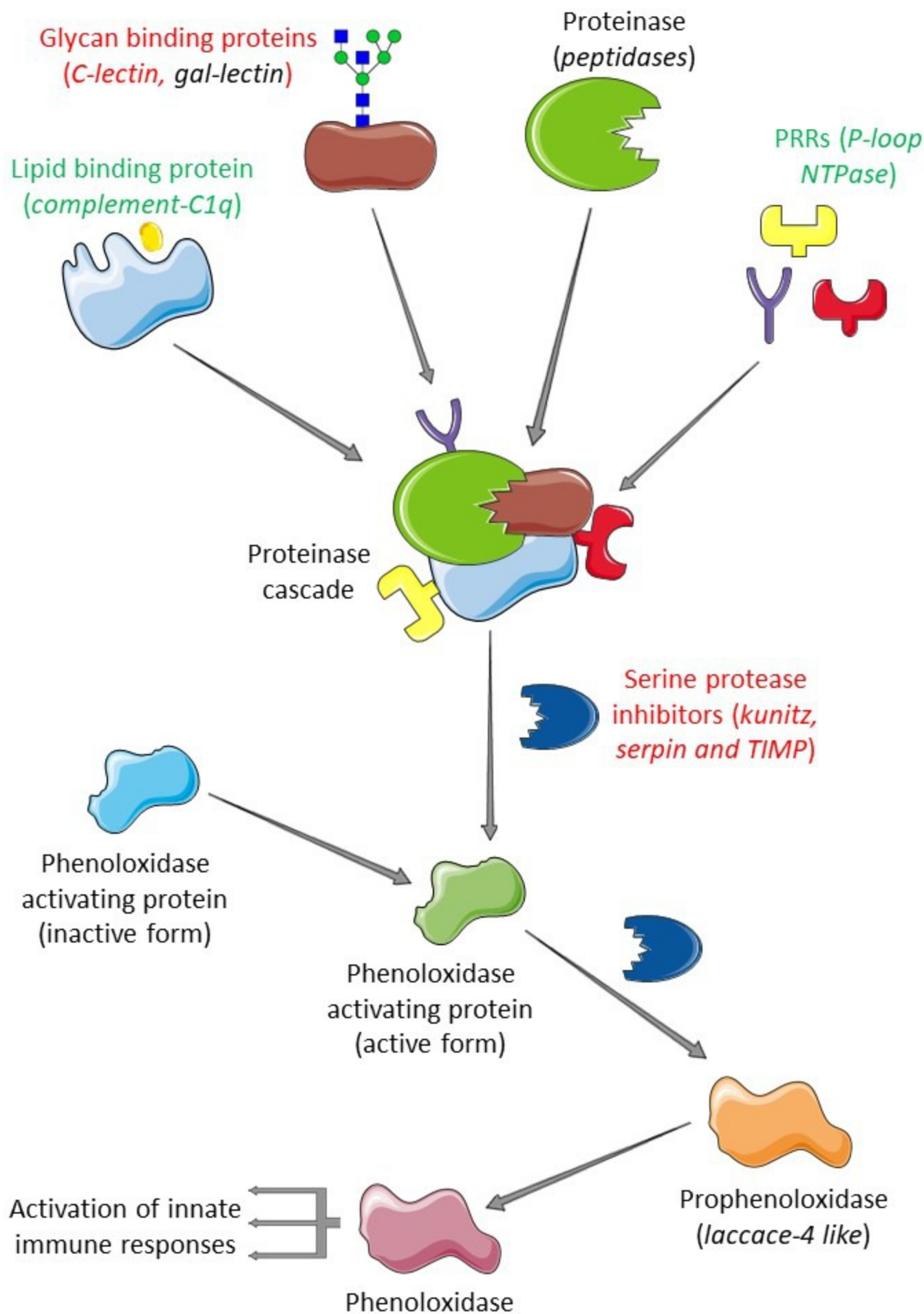
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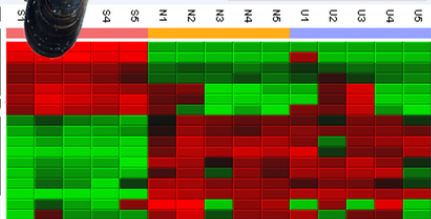
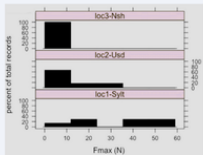
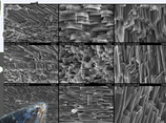




## changing conditions

- ↑ riverine input
- salinity
- ↓ buffering capacity
- pH

## altered shell characteristics



## altered shell proteome