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The effects of environment on *Arctica islandica* shell formation and architecture

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Abstract

Mollusks record valuable information in their hard parts that reflect ambient environmental conditions. For this reason, shells can serve as excellent archives to reconstruct past climate and environmental variability. However, animal physiology and biomineralization, which are often poorly understood, can make the decoding of environmental signals a challenging task. Many of the routinely used shell-based proxies are sensitive to multiple different environmental and physiological variables. Therefore, the identification and interpretation of individual environmental signals (e.g. water temperature) often is particularly difficult. Additional proxies not influenced by multiple environmental variables or animal physiology would be a great asset in the field of paleoclimatology. The aim of this study is to investigate the potential use of structural properties of *Arctica islandica* shells as an environmental proxy. A total of eleven specimens were analyzed to study if changes of the microstructural organization of this marine bivalve are related to environmental conditions. In order to limit the interference of multiple parameters, the samples were cultured under controlled conditions. Three shells presented here were grown at two different water temperatures (10 °C and 15 °C) for multiple weeks and exposed only to ambient food conditions. An additional eight specimens were reared under three different dietary regimes. Shell material was analyzed with two techniques: (1) Confocal Raman microscopy (CRM) was used to quantify changes of the orientation of microstructural units and pigment distribution and (2) Scanning electron microscopy (SEM) was used to detect changes in microstructural organization. Our results indicate that *A. islandica* microstructure is not sensitive to changes in the food source, and likely, shell pigment are not altered by diet. However, seawater temperature had a statistically significant effect on the orientation of the biomineral. Although additional work is required, the

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47 results presented here suggest that the crystallographic orientation of biomineral units of *A.*
48 *islandica* may serve as an alternative and independent proxy for seawater temperature.

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51 1. Introduction

52 Biomineralization is a process through which living organisms produce a protective, mineralized
53 hard tissue. The considerable diversity of biomineralizing species contributes to high variability in
54 terms of shape, organization and mineralogy of the structures produced (Lowenstam and Weiner,
55 1989; Carter et al., 2012). Different architectures at the micrometer and nanometer scale and
56 different biochemical compositions determine material properties that serve specific functions
57 (Weiner and Addadi, 1997; Currey, 1999; Merkel et al., 2007). Besides these differences, all
58 mineralized tissues are hybrid materials consisting in hierarchical arrangements of biomineral units
59 surrounded by organic matrix, also known as "microstructures" (Bøggild, 1930; Carter and Clark,
60 1985; Rodriguez-Navarro et al., 2006) or "ultrastructures" (Blackwell et al., 1977; Olson et al.,
61 2012) or overall "fabrics" (Schöne, 2013; Schöne et al., 2013). The carbonate and organic phases
62 represent the fundamental level of the organization of biomaterials (Aizenberg et al., 2005; Meyers
63 et al., 2006). The mechanisms of microstructure formation and shaping, especially in mollusks, has
64 attracted increasing attention during recent decades. At present, it is commonly accepted that the
65 organic compounds play an important role in the formation of the inorganic phases of biominerals
66 (Weiner and Addadi, 1991; Berman et al., 1993; Dauphin et al., 2003; Nudelman et al., 2006).
67 However, the identification of the exact mechanisms driving biomineralization is still an open
68 research question. Previous studies conducted on mollusks show that environmental parameters

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69 can influence microstructure formation (Lutz, 1984; Tan Tiu and Prezant, 1987; Tan Tiu, 1988;
70 Nishida et al., 2012). These results set the stage for a research interest toward the use of shell
71 microstructures as proxies for reconstructing environmental conditions (Tan Tiu, 1988; Tan Tiu
72 and Prezant, 1989; Olson et al., 2012; Milano et al., 2015).

73 Mollusks are routinely used as climate and environmental proxy archives because they can
74 record a large amount of environmental information in their shells (Richardson, 2001; Wanamaker
75 et al., 2011a; Schöne and Gillikin, 2013). Whereas structures at nanometric level are still
76 underexplored as potential environmental recorders, shell patterns at lower magnification, such as
77 individual growth increments, are commonly used for this purpose (Jones, 1983; Schöne et al.,
78 2005; Marali and Schöne, 2015; Mette et al., 2016). Mollusks deposit skeletal material on a
79 periodic basis and at different rates (Thompson et al., 1980; Deith, 1985). During periods of fast
80 growth, growth increments are formed whereas during periods of slower growth, growth lines are
81 formed (Schöne, 2008; Schöne and Gillikin, 2013). The periodicity of such structures ranges from
82 tidal to annual (Gordon and Carriker, 1978; Schöne and Surge, 2012). By crossdating time-series
83 with similar growth patterns it is possible to construct century and millennia-long master
84 chronologies (Marchitto et al., 2000; Black et al. 2008; Black et al., 2016; Butler et al., 2013). This
85 basic approach, in combination with geochemical methods, has great potential in reconstructing
86 past climatic conditions (Wanamaker et al., 2011b). At present, the most frequently used and well-
87 accepted geochemical proxy is oxygen isotopic composition of shell material ($\delta^{18}O_{shell}$) (Epstein,
88 1953; Grossman and Ku, 1986; Schöne et al., 2004; Wanamaker et al., 2007) which may serve as
89 a paleothermometer and/or paleosalinometer (Mook, 1971; Andrus, 2011); however, the $\delta^{18}O_{shell}$
90 is influenced by both seawater temperature and the isotopic composition of seawater ($\delta^{18}O_{water}$;
91 related to salinity). Thus, $\delta^{18}O_{shell}$-based temperature reconstructions are particularly challenging
92 in habitats with fluctuating $\delta^{18}O_{water}$ conditions such as estuaries or restricted basins (Gillikin et al.,

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2005). Because of the multiple impacts on $\delta^{18}O_{shell}$ values, there have been substantial efforts (including elemental ratios; cite Schone et al 2010 papers here) to develop alternative techniques to reconstruct environmental variables from mollusk shells.

This study investigates the possibility using shell microstructure properties to serve as a new environmental proxy. For this purpose, the effects of seawater temperature (grown at 10 °C and 15 °C) and dietary regime on the microstructural units of *Arctica islandica* cultured under controlled conditions were analyzed and quantified. *A. islandica* was chosen as model species because of its great potential in paleoclimatology and paleoceanography (see Schöne, 2013; Wanamaker et al., 2016). Its extreme longevity of up to more than 500 years makes this species a highly suitable archive for long-term paleoclimate and environmental reconstructions (Schöne et al., 2005; Wanamaker et al., 2008; Wanamaker et al., 2012; Butler et al., 2013).

2. Materials and Methods

The analyses were conducted on eleven *A. islandica* shells. Three juvenile *A. islandica* shells, sampled for the seawater temperature experiment, were collected alive on November 21, 2009 aboard the *F.V. Three of a Kind* off Jonesport, Maine USA (44° 26′ 9.829″N, 67° 26′ 18.045″W) in 82 m water depth. From 2009 to 2011, all animals were kept in a flowing seawater laboratory at the Darling Marine Center, Walpole, Maine, USA (see Beirne et al., 2012 for additional details). In 2011, clams were grown at two different temperature regimes for 16 weeks (Table 1). At the completion of the experiment, shells were estimated to be between 4 to 5 years old. Eight one-year

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115 old juveniles were collected in July 2014 from Kiel Bay, Baltic Sea (54° 32′ N, 10° 42′ E; Fig. 1)
116 and kept alive in tanks at 7 °C for six months at the Alfred Wegener Institute for Polar and Marine
117 Research (AWI), Bremerhaven, Germany. During this time interval, the animals were fed with an
118 algal mix of *Nannochloropsis* sp., *Isochrysis galbana* and *Pavlova lutheri*. Then, they were
119 transferred to the Royal Netherlands Institute for Sea Research (NIOZ), Texel, The Netherlands,
120 and cultured in tanks at three different dietary conditions for 11 weeks (Table 1).

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123 ## 2.1 Seawater temperature experiment

124 The seawater temperature experiment started on 27 March 2011 and ended on 21 July 2011. Prior
125 to the start of the experiment the animals were marked with calcein. The staining leaves a clear
126 fluorescent marker in the shells that can be used to identify which shell material has formed prior
127 to and during the experiment. Initially, the animals were kept at 10.3 ± 0.2 °C for 48 days. Then,
128 they were briefly removed from the tanks and marked again. Subsequently, the clams were cultured
129 for 69 more days at 15.0 ± 0.3 °C. Ambient seawater was pumped from the adjacent Damariscotta
130 River estuary and adjusted to desired temperature. The salinity was measured with a Hydrolab®
131 MiniSonde. It ranged between 30.2 ± 0.7 and 30.7 ± 0.7, in the two experimental phases,
132 respectively. During the entire culture period, all clams were exposed to ambient food conditions.
133 At the end of the experiment the soft tissues were removed.

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136 ## 2.2 Food experiment

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137 The food experiment was carried out from 9 February 2015 to 29 April 2015. The animals were
138 placed in aquaria inside a climate room at 9 °C. Water temperature in the tanks ranged between 8
139 and 10 °C. Water salinity was measured by using an ENDECO 102 refractometer and ranged
140 between 29.6 and 29.9 ± 0.1 in each aquarium. The 15-liter tanks were constantly supplied with
141 aerated water from the Wadden Sea. The clams were acclimated for three weeks before the start of
142 the experiment. Three dietary regimes were chosen. One treatment consisted of feeding the animals
143 with Microalgae Mix (food type 1), a ready-made solution of four marine microalgae (25 %
144 *Isochrysis*, 25 % *Tetraselmis*, 25 % *Thalassiosira*, 25 % *Nannochloropsis*) with a particle size
145 range of 2 - 30 µm. A second treatment was based on PhytoMaxx (food type 2), a solution of living
146 *Nannochloropsis* algae with 2 - 5 µm size range. A third treatment served as control, i.e., the
147 animals were not fed with any additional food. In treatments with food type 1 and 2, microalgae
148 were provided at the constant optimum concentration of 20×10^6 cells/liter (Winter, 1969). A
149 dispenser equipped with a timer was used to distribute the food five times per day. At the end of
150 the experiment the soft tissues were removed. A distinct dark line in the shells indicated the
151 transposition to the NIOZ aquaria and the associated stress. This line marks the beginning of the
152 tank experiment.

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155 ## 2.3 Sample preparation

156 The right valve of each specimen was cut into two 1.5 millimeter-thick sections along the axis of
157 maximum growth. For this purpose, a low speed precision saw (Buehler Isomet 1000) was used.
158 Given the small size and fragility of the juvenile shells used in the food experiment, the valves
159 were fully embedded in a block of Struers EpoFix (epoxy) and air-dried overnight prior the

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160 sectioning. Sections of the clams used in the temperature experiment were embedded in epoxy after
161 the cutting. All samples were ground using a Buehler Metaserv 2000 machine equipped with
162 Buehler silicon carbide papers of different grit sizes (P320, P600, P1200, P2500). In addition, the
163 samples were manually ground with Buehler P4000 grit paper and polished with a Buehler diamond
164 polycrystalline suspension (3 µm). Sample surfaces were rinsed in demineralized water and air-
165 dried. In the samples of the temperature experiment, the calcein marks were located under a
166 fluorescence light microscope (Zeiss Axio Imager.A1m microscope equipped with a Zeiss
167 HBO100 mercury lamp and filter set 38: excitation wavelength, ca. 450 - 500 nm; emission
168 wavelength, ca. 500 - 550 nm).

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171 ## 2.4 *A. islandica* shell organization

172 The shell of *A. islandica* consists or pure aragonite and it is divided in two major layers, an outer
173 (OSL) and the inner shell layer (ISL). The OSL is further subdivided in outer (oOSL) and inner
174 portion (iOSL) (Schöne, 2013). These layers are characterized by specific microstructures (Ropes
175 et al., 1984). The oOSL largely consists of homogenous microstructure with a bulky and granular
176 aspect (Schöne et al., 2013). The iOSL and ISL are largely composed of crossed-lamellar to cross-
177 acicular microstructures (Dunca et al., 2009). The present study focuses on ventral margin of the
178 shells. Analyses were carried out exclusively in the OSL.

179 Similar to other mollusks, the shell of *A. islandica* contains pigment polyenes which are
180 obviously visible when using CRM (Hedegaard et al., 2006). Polyenes are organic compounds
181 containing single (C-C) and double (C=C) carbon-carbon bonds forming a polyenic chain. Their

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182 distribution across the shell is not homogenous. The pigments are abundant in the oOSL whereas
183 they become scarce in the iOSL (Stemmer and Nehrke, 2014).

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186 ## 2.5 Confocal Raman microscopy and image processing

187 Shells were mapped with a WITec alpha 300 R (WITec GmbH, Germany) confocal Raman
188 microscope. Scans of 50 × 50 µm, 100 × 50 µm and 150 × 50 µm were performed using a
189 piezoelectric scanner table. All Raman measurements were carried out using a 488 nm diode laser.
190 A spectrometer (UHTS 300, WITec, Germany) was used with a 600 mm^{-1} grating, a 500 nm blaze
191 and an integration time of 0.03 s. On each sample two to six scans were made, depending of the
192 thickness of the shell. For instance, in juvenile shells (food experiment), two scans of each sample
193 were made. On larger shells used in the temperature experiment, six maps were completed, i.e.,
194 two maps in the oOSL, two in the middle of the iOSL and two in the inner portion of the iOSL.
195 Each scan contained between 40,000 and 120,000 data points, depending on the map size. The
196 spatial resolution equaled 250 nm. Half of the maps were performed on the shell portion formed
197 before the experiments. The other half were made on the shell portion formed under experimental
198 conditions. In order to avoid areas affected by transplantation or marking stress, the scans were
199 located far off the calcein and stress lines. Raman maps on food experiment shells were performed
200 300 µm away from the stress line. In the shells from the temperature experiment, the scans were
201 made 1 mm away from the calcein mark.

202 Polarized Raman microscopy is known to provide comprehensive information about the
203 crystallographic properties of the materials (Hopkins and Farrow, 1985). The aragonite spectrum

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204 is characterized by two lattice modes (translation mode T_a, 152cm^{-1} and librational mode L_a,
205 206cm^{-1}) and the two internal modes (in-plane band ν_4, 705cm^{-1} and symmetric stretch ν_1, 1085cm^{-1}
206). The ratio ($R_{\nu1/Ta}$) between peak intensities belonging to ν_1 and T_a is caused by different
207 crystallographic orientations of the aragonitic units (Hopkins and Farrow, 1985; Nehrke and Nouet,
208 2011). Within each scan, $R_{\nu1/Ta}$ was calculated for each data point. New spectral images were
209 generated using WITecProject software (version 4.1, WITec GmbH, Germany). These images were
210 then binarized by replacing all values above 2.5 with 1 and the others with 0. The orientation was
211 quantified by calculating the area formed by pixels of value 1 over the total scan area. The imaging
212 software Gwyddion (http://gwyddion.net/last checked: June 2016) was used for this purpose. The
213 results were expressed in percentage.

214 The Raman scans of the food experiment shells were analyzed to investigate the pigment
215 composition. Polyene peaks have definite positions in the spectrum according to the number of the
216 C-C and C=C bonds of the chain, which are specific for certain types of pigments. The two major
217 polyene peaks at ~ 1130 (R_1) and 1520 cm^{-1} (R_4) were identified by using the "multipeak fitting 2"
218 routine of IGOR Pro (version 7.00, WaveMetrics, USA). Their exact position was determined
219 adopting a Gaussian fitting function (Fig. 2). The number of single (N_1) and double carbon bonds
220 (N_4) was calculated by applying the equations by Schaffer et al. (1991):

221 (1) $N_1 = 476 (R_1 - 1{,}082)$

222 (2) $N_4 = 830 (R_4 - 1{,}438)$

223 Spectral images of the R_4 band were used to locate the polyenes in the shell and measure the
224 thickness of the pigmented layer. This analysis was conducted only on the shells of the food
225 experiment. Given the larger size of the shells used in the temperature experiment, the spectral

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maps were not sufficient for a correct localization of the pigmented layer boundaries and estimation of its thickness.

To quantify changes of the orientation of individual biomineral units of the juvenile shells (food experiment), the spectral maps were subdivided into two portions. The outermost shell portion (oOSL) was enriched in pigments whereas the iOSL showed a decrease in polyene content.

2.6 Scanning electron microscopy

After performing Raman measurements, the samples were prepared for SEM analysis. Each shell slab was ground with a Buehler Metaserv 2000 machine and Buehler silicon F2500 grit carbide paper. To reduce the impact of grinding on the sample surface of juvenile shells, extra grinding was done by hand. Then, the slabs were polished with a Buehler diamond polycrystalline suspension (3 µm). Afterward, shell surfaces were etched in 0.12 N HCl solution for 10 (food experiment samples) to 90 s (temperature experiment samples) and subsequently placed in 6 vol % NaClO solution for 30 min. After being rinsed in demineralized water, air-dried samples were sputter-coated with a 2 nm-thick platinum film by using a Low Vacuum Coater Leica EM ACE200.

A scanning electron microscope (LOT Quantum Design 2nd generation Phenom Pro desktop SEM) with backscattered electron detector and 10 kV accelerating voltage was used to analyze the shells. Images were taken at the same distances from the calcein and stress lines as in the case of the Raman measurements to assure comparability of the data.

In addition, stitched SEM images of the ventral margins were used to accurately determine the shell growth advance during the culturing experiments. Growth increment widths were

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248 measured with the software Panopea (© Schöne and Peinl). Given the difference in duration of the
249 two phases of the temperature experiment, the measurements were expressed as total growth and
250 instantaneous growth rate (Fig. 3a + b). The latter was calculated using the following equation
251 (Brey et al., 1990; Witbaard et al., 1997):

252 (3) Instantaneous growth rate = $(\ln (y_t / y_0) / a)$

253 where y_0 represents the initial shell height, y_t is the final shell height and a is the duration of the
254 experiment. In the case of the food experiment, only the total growth was calculated (Fig. 3c).

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256

257

258 # 3. Results

259 ## 3.1 Effect of seawater temperature and diet on *A. islandica* shell growth

260 When exposed to a water temperature of 10 °C, the shells grew between 11.67 and 14.17 mm
261 during a period of 48 days. During a period of 69 days at 15 °C, the growth ranged between 2.32
262 and 5.77 mm (Fig. 3a). Instantaneous growth rate showed a decrease between the two experimental
263 phases. At 10 and 15 °C, the average instantaneous growth per day was 0.0091 and 0.0013,
264 respectively (Fig. 3b). The decrease in total growth and growth rate between the two temperatures
265 was statistically significant (*t*-test, $p < 0.01$).

266 During the food experiment, shells grew between 0.37 and 3.71 mm with large differences
267 due to the different food types. Growth of specimens exposed to food type 1 ranged between 1.87
268 to 3.71 mm, whereas those cultured with food type 2 grew between 0.55 to 0.96 mm. Both control

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269 specimens added 0.37 mm of shell during the experimental phase (Fig. 3c). ANOVA and Tukey´s
270 HSD post hoc tests showed significant differences between specimens cultured with food type 1
271 and 2 ($p < 0.05$) and between food type 1 and control shells ($p < 0.05$).

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274 ## 3.2 Effect of seawater temperature on *A. islandica* microstructure

275 At a water temperature of 10 °C, the area occupied by microstructural units oriented with $R_{v1/Ta}$
276 higher than 2.5 a.u. (= arbitrary units) ranged between 31.3 and 50.6 % in the oOSL and between
277 21.3 and 33.5 % in the iOSL. When exposed to 15 °C, values ranged between 25.6 and 48.7 % and
278 between 45.7 and 55.9 % in the oOSL and iOSL, respectively (Fig. 4). Whereas the slight difference
279 of area with $R_{v1/Ta} > 2.5$ in the oOSL was not significant between the two water temperatures (*t*-
280 test, $p = 0.62$), the area with $R_{v1/Ta} > 2.5$ in the iOSL significantly increased at 15 °C (*t*-test, $p =$
281 0.02). Under the SEM, no difference was visible between units formed at 10 °C and 15 °C (Fig. 5).

282

283

284 ## 3.3 Effect of food on *A. islandica* microstructure and pigments

285 In the shells cultured with food type 1, the area occupied by biomineral units oriented with $R_{v1/Ta}$
286 higher than 2.5 a.u. during the experiment ranged between 24.8 % (oOSL) and 43.0 % (iOSL). In
287 the shell portion deposited before the experiment, the ratio varied between 19.4 % (oOSL) and 36.2
288 % (iOSL). Although a trend was recognized, these variations were not statistically different (*t*-tests.
289 OSL: $p = 0.43$; ISL: $p = 0.57$; Fig. 6a). On the contrary, in the clams exposed to food type 2, the

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area occupied by units oriented with $R_{v1/Ta}$ > 2.5 ranged between 11.7 % (oOSL) and 20.4 % (iOSL). Before the experiment, the proportions were higher, i.e., 18.1% (oOSL) and 26.3% (iOSL) (Fig. 6b). As for the other treatment, the difference was not significant (*t*-tests. oOSL: $p = 0.34$; iOSL: $p = 0.28$). In the control shells grown with no extra food supply, the area with $R_{v1/Ta}$ > 2.5 ranged between 24.6 % (oOSL) and 44.8 % (iOSL) during the experiment and 21.2 % (oOSL) and 44.5 % (iOSL) before the experiment (Fig. 6c). Hence, no trend was visible and the two portions did not show significant differences (*t*-tests. oOSL: $p = 0.59$; iOSL: $p = 0.99$). As for the temperature experiment, under the SEM, the microstructure of the shells from the food experiment did not show any change (Fig. 7).

All treatments showed a slightly thicker pigmented layer formed during the experiment than during the acclimation phase (Fig. 8a). During the experiment, clams cultured with food type 1 showed, on average, a thickening by 6.4 %. In the food type 2 specimens, the layer thickness increased by 9.9 %. Control shells showed an increase of 10.4 % (Fig. 8b). However, none of these differences was statistically significant (*t*-test. Food type 1: $p = 0.43$; Food type 2: $p = 0.39$; Control: $p = 0.10$). According to the position of the polyene peaks, the number of single carbon bonds in the pigment chain did not change between the acclimation and experimental phase ($N_1 = 10.1 \pm 1.3$ and $N_1 = 10.0 \pm 0.9$, respectively). Likely, no significant variation was observed in the number of double carbon bonds ($N_4 = 10.5 \pm 0.2$ and $N_4 = 10.4 \pm 0.3$, respectively; Table 2).

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4. Discussion

According to the results, variations of both food type and water temperature can influence the shell production rate of *A. islandica.* However, the shell microstructure and pigmentationreact differently to these two environmental variables. Whereas changes of the dietary conditions do not affect the shell architecture and pigment composition, the crystallographic orientation of the biomineral units responds to seawater temperature fluctuations.

4.1 Environmental influence on shell microstructure

The environmental conditions experienced by mollusks during the process of biomineralization appear to influence shell organization (Carter, 1980). Among the different environmental variables, water temperature is the most studied driving force of structural changes of the shell. For instance, shell mineralogy can vary depending on water temperature (Carter, 1980). According to the thermal potentiation hypothesis, nucleation and growth of calcitic structural units is favored at low temperatures by kinetic factors (Carter et al., 1998). As a consequence, bivalve species living in cold water environments exhibit additional or thicker calcitic layers compared to the corresponding species from warm waters (Lowenstam, 1954; Taylor and Kennedy, 1969). Changes in the calcium carbonate polymorph also affect the type of microstructures (Milano et al., 2016). However, architectural variations often occur without mineralogical impact (Carter, 1980).

The present results indicate that temperature induces a change in the crystallographic orientation of the biomineral units of *A. islandica*. Although water temperature was previously shown to have an impact on microstructure formation, the attention has been mainly addressed to

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334 the effects on the morphometric characteristics (e.g. size and shape) or on the type of
335 microstructure. Milano et al. (2015) demonstrated that size and elongation of prismatic structural
336 units of *Cerastoderma edule* were positively correlated to seawater temperature variation
337 throughout the growing season. Likely, low temperatures induced the formation of small nacre
338 tablets in *Geukensia demissa* (Lutz, 1984). Seasonal changes of the microstructural type were
339 reported in the freshwater bivalve *Corbicula fluminea* (Prezant and Tan Tiu, 1986; Tan Tiu and
340 Prezant, 1989). During the warm months, crossed acicular structure was produced, whereas simple
341 crossed-lamellae were formed during the winter period. So far, variations of the crystallographic
342 properties of bivalve biominerals have been exclusively investigated as a response to hypercapnic
343 (acidified) conditions. *Mytilus galloprovincialis* and *Mytilus edulis* showed a significant change in
344 the orientation of the prisms forming shell calcitic layer when subjected to hypercapnia (Hahn et
345 al., 2012; Fitzer et al., 2014). Altered crystallographic organization may derive from the animal
346 exposure to suboptimal conditions. These findings together with the present results suggest that
347 thermal- and hypercapnic-induced stress are likely to affect the ability of the bivalves to preserve
348 the orientation of their microstructural units (Fitzer et al., 2015).

349 Different food sources do not significantly influence the orientation of the biomineral units
350 or the composition and distribution of pigments in shells of *A. islandica*. In previous studies, the
351 relationship between microstructure and diet was virtually overlooked resulting in a lack of data in
352 the literature. As suggested by Hedegaard et al., (2006), however, the type of polyenes is influenced
353 by food. The ingestion of pigment-enriched microalgae potentially leads to an accumulation of
354 pigments in mollusk tissues and the shell (Soldatov et al., 2013). On the other hand, it has been
355 argued that polyenes do not generate from food sources like other pigments (i.e., carotenoids), but
356 they are locally synthesized (Karampelas et al., 2009). In accordance to Stemmer and Nehrke
357 (2014), the results presented here support the view that the specific diets on which the animals rely

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358 on do not influence shell pigment composition. The chemical characteristics of the polyenes are
359 likely to be specie-specific and independent from the habitats.

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362 ## 4.2 Confocal Raman microscopy as tool for microstructural analysis

363 From a methodological perspective, the present study represents an innovative approach in
364 the investigation of shell microstructural organization. Electron backscatter diffraction (EBSD) has
365 been previously used to determine the crystallographic orientation of gastropod (Fryda et al., 2009;
366 Pérez-Huerta et al., 2011) and bivalve microstructural units (Checa et al., 2006; Frenzel et al., 2012;
367 Karney et al., 2012). Whereas, CRM on mollusk shells is generally applied within studies on
368 taphonomic mineralogical alteration and pigment identification (Stemmer and Nehrke, 2014;
369 Beierlein et al., 2015). Both techniques provide considerably high spatially resolved analysis up to
370 250 nm, allowing the identification of individual structural units at µm- and nm-scale (Cusack et
371 al., 2008; Karney et al., 2012). CRM offers important advantages supporting a broader application
372 of this methodology in the biomineralization research field. For instance, samples do not require
373 any pre-treatment. Unlike EBSD, there is no need of preparing thin-sections (~ 150 µm thick) or
374 etching the shell surface (Griesshaber et al., 2010; Hahn et al., 2012). Therefore, further structural
375 and geochemical analyses can be easily performed on the same sections (Nehrke et al., 2012). In
376 addition, the size of CRM scans can be remarkably large (~ 7-8 mm^2) without compromising the
377 achievable resolution. By overlapping adjacent scans, it is possible to produce stitched scans
378 allowing to further increase the region of interest on the shell surface.

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379 SEM has previously been demonstrated to provide a convenient approach for the
380 identification of individual structural units and the quantification of potential changes occurring
381 within them (Milano et al., 2015, 2016b). However, SEM exclusively provides information about
382 the morphometric characteristics of the microstructural units. As highlighted by the present study,
383 to achieve an exhaustive examination, it is suggested to combine SEM with techniques assessing
384 crystallographic properties of the biomaterials. For instance, our results show that the effect of
385 water temperature is detectable in crystallographic orientation but not in morphometric features of
386 the biomineral units.

387

388 ## 4.3 Environmental influence on shell growth

389 Numerous previous studies demonstrated that growth rate of *A. islandica* - and many other bivalves
390 - is linked to environmental variables (e.g., Witbaard et al., 1997, 1999; Schöne et al., 2004; Butler
391 et al., 2010; Mette et al., 2016). However, the relative importance of the main factors, temperature
392 and food supply/quality driving shell formation are still not well understood. Positive correlations
393 between shell growth and water temperature have been identified (i.e., Schöne et al., 2005;
394 Wanamaker et al., 2009; Marali et al., 2015), but the relationship between shell growth and
395 environment is more complex (Marchitto et al., 2010; Stott et al., 2010; Schöne et al., 2013) and
396 likely dependent on the synergic effect of food availability and water temperature (Butler et al.,
397 2013; Lohmann and Schöne, 2013; Mette et al., 2016). Tank experiments were run in order to
398 precisely identify the role of these two parameters of shell growth of *A. islandica* (Witbaard et al.,
399 1997; Hiebenthal et al., 2012). A tenfold increase in instantaneous growth rate was observed
400 between 1 and 12 °C, with the greatest variation occurring below 6 °C (Witbaard et al., 1997). On
401 the contrary, a temperature increase between 4 and 16 °C was shown to produce a slowdown of

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shell production (Hiebenthal et al., 2012). Our results are in agreement with the latter study and show a decrease in the instantaneous growth rate between 10 and 15 °C. High temperatures are often associated with an increase of free radical production (Abele et al., 2002). A large amount of energy then has to be allocated to limit oxidative cellular damage (Abele and Puntarulo, 2004). This translates into a higher accumulation of lipofuscin and slower shell production rate (Hiebenthal et al., 2013). The contrasting results of previous studies may be explained by individual differences in the tolerance toward temperature change (Marchitto et al., 2000).

Along with water temperature, food availability was also shown to influence *A. islandica* shell growth (Witbaard et al., 1997). At high algal cell densities, the siphon activity increased. This, in turn, was positively correlated to shell growth. Previous experiments used different combinations of algae such as *Isochrysis galbana* and *Dunaliella marina* (Witbaard et al., 1997), or *Nannochloropsis oculata, Phaeodactylum tricornutum* and *Chlorella* sp. (Hiebenthal et al., 2012) to grow the clams. However, there are still uncertainties about the composition of the primary food source for this species (Butler et al., 2010). Even though it is challenging to determine the preferred algal species, our results show that the use of a mixture of different algal species results in significantly faster shell growth than the used of just one algal species. In the natural environment, suspension feeders such as *A. islandica* preferentially ingest certain particle sizes (Rubenstein and Koehl, 1977; Jorgensen, 1996; Baker et al., 1998). The exposure to a limited algal size range, as in the case of food type 2, may affect shell growth. Furthermore, multispecific solutions contain a higher variability of biochemical components that better meet the nutritional requirements of the animal (Widdows, 1991). Our results are in good agreement with previous findings. For instance, it has been shown by Strömgren and Cary (1984) that *Mytilus edulis* shell growth increased as a result of a diet based on three different algal species. Furthermore, Epifanio (1979) tested the differences on the growth of *Crasssostrea virginica* and *Mercenaria mercenaria* of a mixed diet

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426 composed by *Isochrysis galbana* and *Thalassiosira pseudonana* and diets consisting of the single
427 species. Faster growth was measured in the mixed diet treatment, indicating a synergic effect of
428 the relative food composition (Epifanio, 1979). Likely, *Mytilus edulis* grew faster when reared with
429 different types of mixed diets as opposed to monospecific diets (Galley et al., 2010).

430

431

432

433 5. Conclusions

434 *Arctica islandica* shell growth and biomineral orientation vary with changes in sewater
435 temperature. However, exposure to different food sources affect shell deposition rate but do not
436 influence the organization of the biomineral units. Given the exclusive sensitivity to one
437 environmental variable, the orientation of biomineral units may represent a promising new
438 temperature proxy for paleoenvironmental reconstructions. However, additional studies are needed
439 to further explore the subject. In particular, intra-individual variability influence on the results
440 needs to be assessed. In the present study, a variation in the orientation between individuals was
441 well visible and the risks associated have to be taken in account when considering further
442 application of the possible proxy. Furthermore, the effect of other environmental variables such as
443 salinity needs to be tested.

444 The innovative application of CRM for microstructural orientation and proxy development
445 proved that the technique has large potential in this research direction. More studies are needed to
446 validate its suitability in paleoclimatology experimental works.

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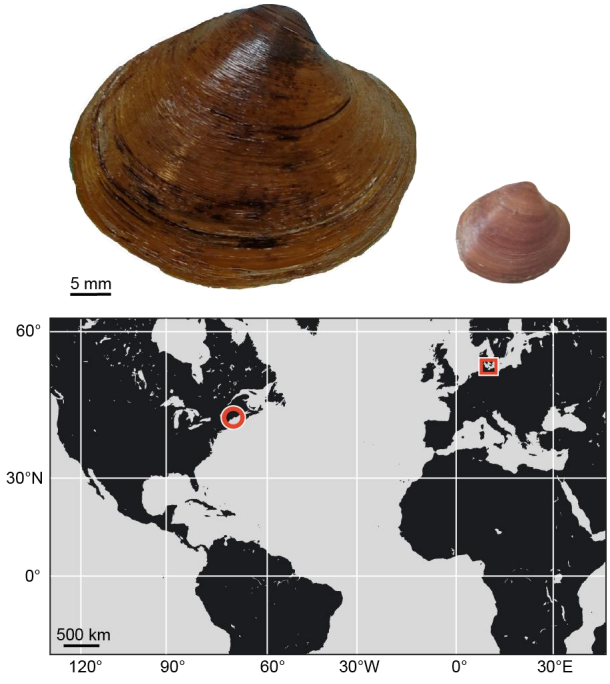
748 Figures and tables



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750 **Fig. 1.** Shell of adult *Arctica islandica* used in the temperature experiment (left) and juvenile from the Baltic
751 Sea used in the food experiment (right). The map indicates the localities where the two sets of shells where
752 collected: Jonesport, Maine (circle) and Kiel Bay (square).

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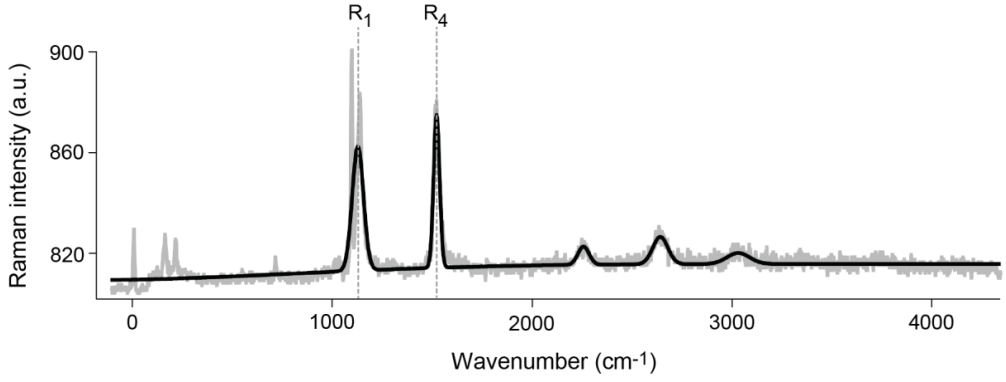

753

754 **Fig. 2.** Raman spectrum of *Arctica islandica* showing the typical aragonite peaks (grey line). The exact
755 position of the polyene peaks R_1 and R_4 was determined by using a peak fitting routine based on a Gaussian
756 function (black line).

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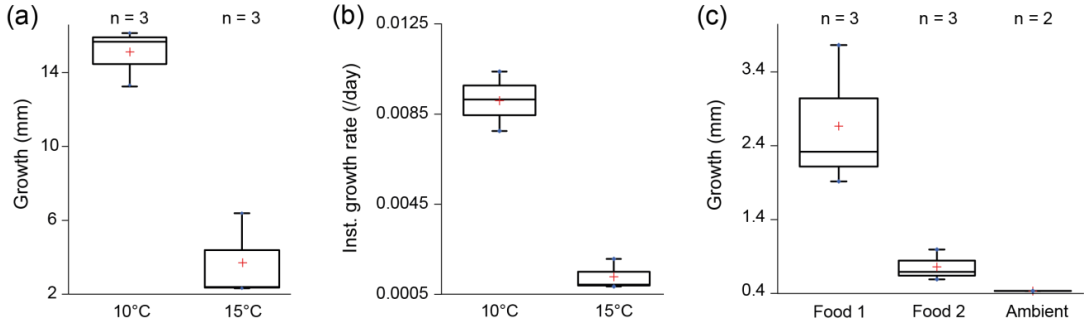

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760 **Fig. 3.** *Arctica islandica* shell growth under controlled conditions. (a) Total growth and (b) instantaneous
761 growth rate during the temperature experiment. (c) Total growth during the food experiment.

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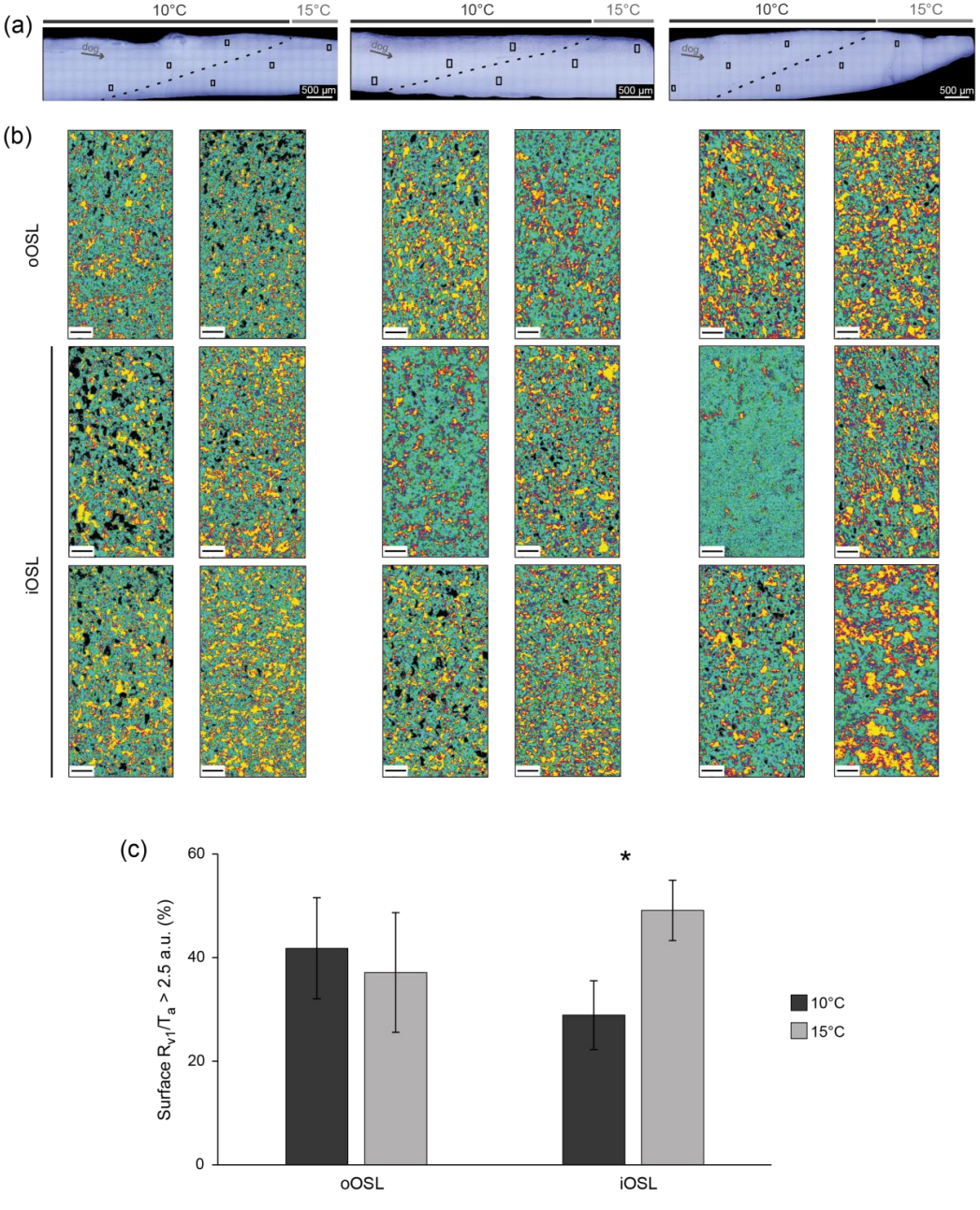

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766 **Fig. 4.** Effect of temperature increase on biomineral orientation. (a) Position of the Raman maps of the three
767 specimens reared at 10 °C and 15 °C. Dotted lines indicate the location of the calcein marks. dog = direction
768 of growth. (b) Raman spectral maps of $R_{v1/Ta}$. Left images of each column represents shell portion formed

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769 at 10 °C, right images represent shell portions formed at 15 °C. First row of pairs refers to oOSL, the other
770 two represent the iOSL. Scale bars = 10 µm. (c) Proportions of biominerals with $R_{v1/Ta}$ > 2.5 a.u. with respect
771 to the total map area. Asterisks indicate significant difference between the orientation of iOSL
772 microstructures formed at 10 and 15°C ($p < 0.05$).

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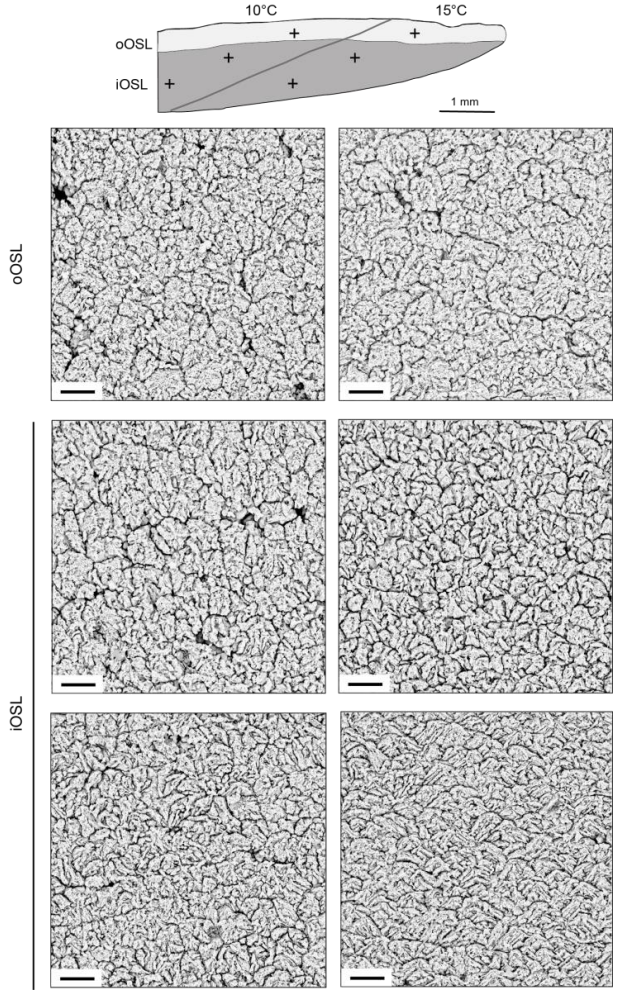

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777 **Fig. 5.** SEM images of *Arctica islandica* shell microstructures formed at 10 °C (left column) and at 15 °C
778 (right column). The sketch indicates the position of the images 1 mm away from the calcein mark (grey
779 line). The first row of images refers to the oOSL, the other two row refers to the iOSL. Scale bars if not
780 otherwise indicated = 5 µm.

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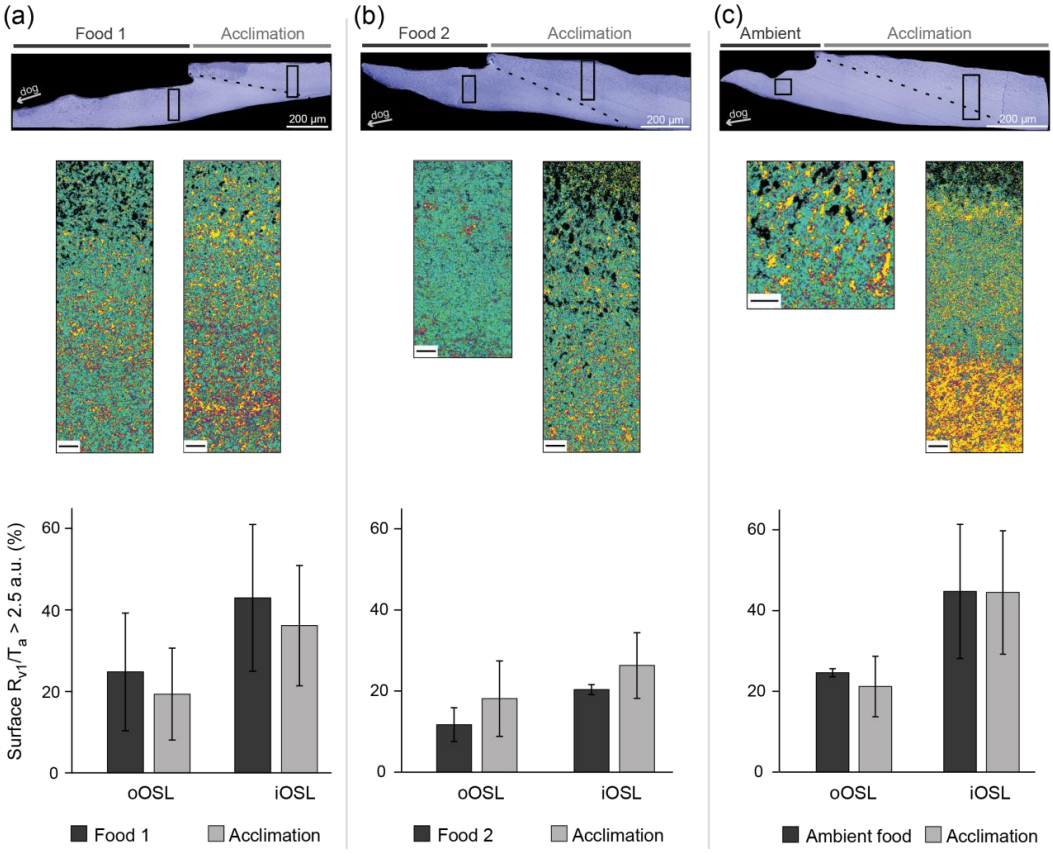

783

784 **Fig. 6.** Effect of different diets based on (a) food type 1, (b) food type 2 and (c) ambient food on biomineral
785 orientation. The optical microscope images indicate the position of the Raman scans. Dotted line marks the
786 start of the experiment. The portion of shell prior the line was formed during the acclimation phase. dog =
787 direction of growth. The Raman spectral maps indicate the ratio $R_{v1/Ta}$ for each data point of the scan. For

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788 each shell, maps on the left represent shell portions during the experiment, maps on the right represent shell
789 portions formed during the acclimation phase. In the acclimation portion of the sample reared with ambient
790 food, a significant change in the microstructure orientation is visible. The respective area of the Raman map
791 was not considered in further calculations because it was influenced by the emersion and transportation
792 stress at the start of the experiment. Scale bars = 10 µm. The graphs show the proportions of biominerals of
793 oOSL and iOSL with $R_{v1/Ta}$ > 2.5 a.u. with respect to the total map area.

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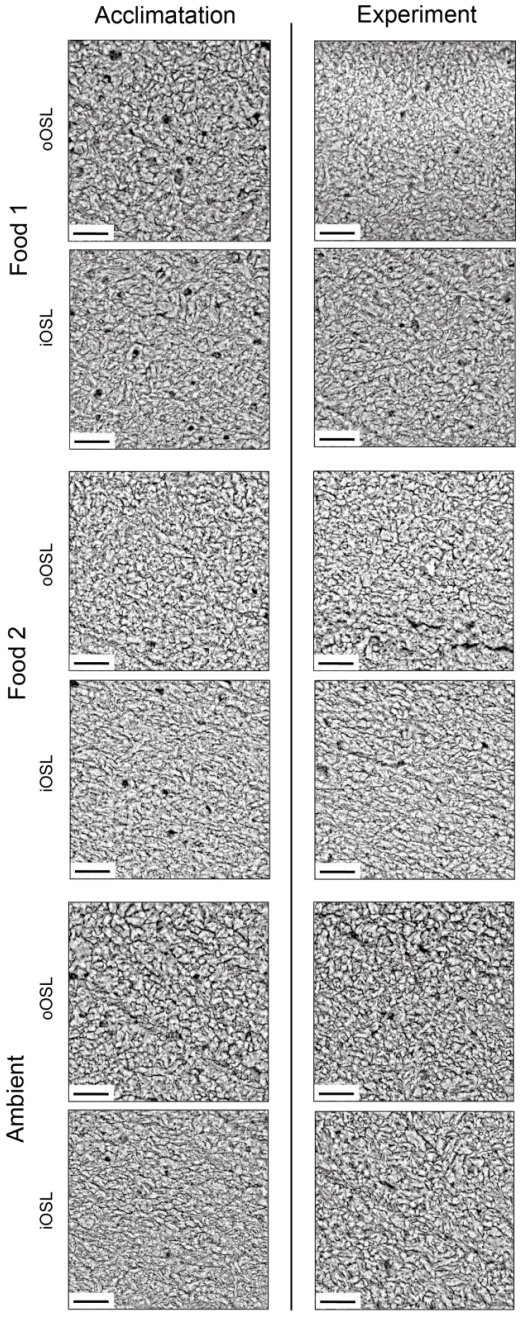

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797 **Fig. 7.** SEM images of *Arctica islandica* shell microstructures formed during the acclimation phase at AWI

798 (left column) and during the food experiment (right column). Scale bars = 4 µm.

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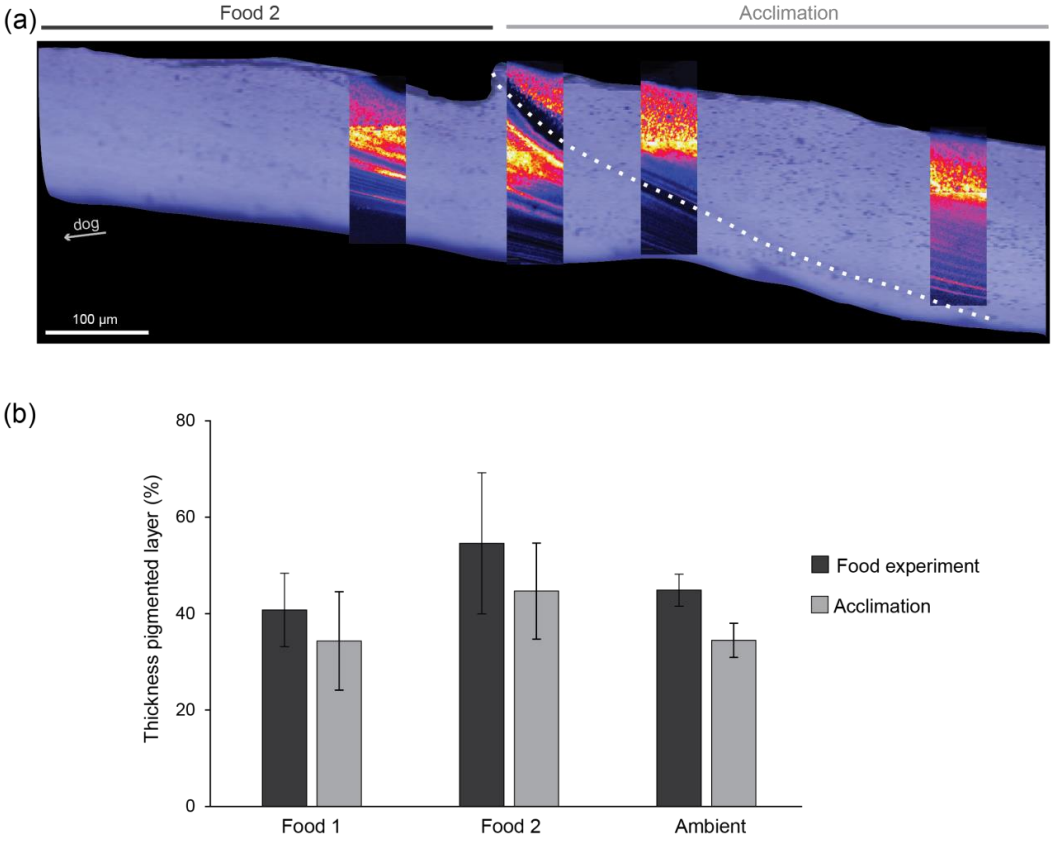

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800 **Fig. 8.** Effects of diet on shell pigment distribution. (a) Raman spectral maps of the 1524 cm^{-1} band
801 representing the distribution of the polyenes in the shell cultured with food type 2. Dotted line marks the
802 start of the experiment. dog = direction of growth. (b) The graph shows the thickness of the pigmented layer
803 over the whole shell thickness before and during the food experiments.

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806 **Table 1.** List of the studied specimens of *Arctica islandica* and experimental conditions.

Sample ID	Locality	Age	Experiment	Treatment
A2	Maine	5	Temperature	10 °C + 15 °C
A4	Maine	4	Temperature	10 °C + 15 °C
A5	Maine	4	Temperature	10 °C + 15 °C
S12	Kiel Bay	1	Diet	Food 1
S14	Kiel Bay	1	Diet	Food 1
S15	Kiel Bay	1	Diet	Food 1
G11	Kiel Bay	1	Diet	Food 2
G12	Kiel Bay	1	Diet	Food 2
G15	Kiel Bay	1	Diet	Food 2
N13	Kiel Bay	1	Diet	No additional food
N15	Kiel Bay	1	Diet	No additional food

807 808 809 810 811 812 813

814 **Table 2.** Details of the pigment composition of the *Arctica islandica* shells used in the food experiment.
815 The position of the major polyene peaks R_1 and R_4 in the Raman spectrum is indicated together with the
816 number of single and double carbon bonds of the pigment molecular chain (N_1 and N_4). Each shell was
817 analyzed in the portions formed before and during the experimental phase.

Sample ID	Shell portion	R_1 (cm^{-1})	R_4 (cm^{-1})	N_1	N_4
S12	Acclimation	1130.9	1515.2	9.7	10.8
	Food 1	1121.4	1515.3	12.1	10.7
S14	Acclimation	1133.2	1519.4	9.3	10.2
	Food 1	1132.2	1518.6	9.5	10.3
S15	Acclimation	1129.5	1516.5	10.0	10.6
	Food 1	1132.1	1519.8	9.5	10.1
G11	Acclimation	1132.6	1518.4	9.4	10.3
	Food 2	1129.5	1517.0	10.0	10.5
G12	Acclimation	1131.7	1518.7	9.6	10.3
	Food 2	1132.1	1518.2	9.5	10.4
G15	Acclimation	1132.4	1519.5	9.4	10.2
	Food 2	1128.0	1520.9	10.3	10.0
N13	Acclimation	1130.2	1515.6	9.9	10.7
	Ambient food	1131.4	1514.1	9.6	10.9
N15	Acclimation	1117.9	1516.0	13.3	10.6
	Ambient food	1130.7	1517.0	9.8	10.5
Average		1129.7 ± 4.2	1517.5 ± 2.0	10.1 ± 1.1	10.4 ± 0.3

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