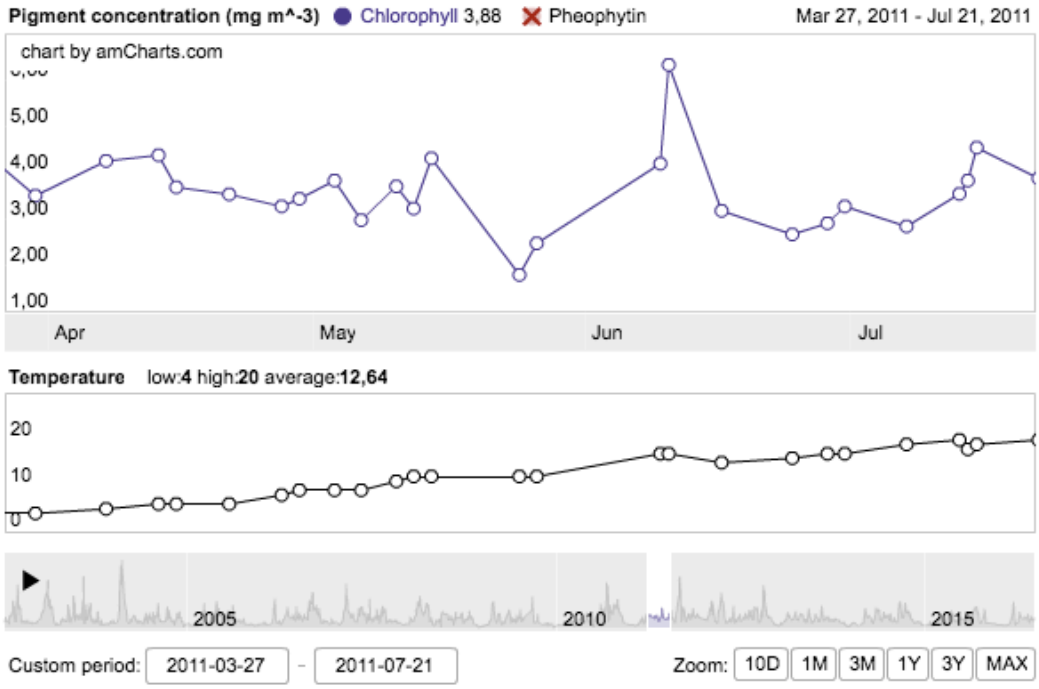
Dear Referee #1,

The authors thank you for your thoughtful comments and remarks.

- In regards to the food supply during the temperature experiment, the clams only received food from the outside ambient environment as seawater was pumped into the flow-through laboratory at the Darling Marine Center (Walpole Maine, USA). We were able to verify that Chlorophyll (pigment concentrations mg/m^3) remained fairly constant over the experiment (see screenshot below). We used data from the Perry phytoplankton & optics lab (http://perrylab.umeoce.maine.edu/docksampling.php), which is from the Darling Marine Center. From these data, we do not think it is likely that food availability was substantially different between the two culture periods.

- *A. islandica* optimal temperature range is between 6 and 10°C (Mann, 1982). The effect of water temperature on the shell growth in this species is described in section 4.3. Based on the studies cited, temperatures around 16 °C induce stress in the animals. In the current experiment, the temperatures selected are within the natural range for this region (4-20 °C; Beirne et al., 2012 and screen shot below). Therefore, they are representative for water conditions to which the species is regularly exposed and potentially influenced in the formation of its carbonate structure.



- The specific functions of the shell pigments have not been disclosed yet. However, Stemmer and Nehrke (2014) observed an enrichment in polyenes in the growth lines potentially suggesting their involvement in the biomineralization process. Furthermore, the high phenotypic

variation in pigmentation among and within mollusk species, indicates that these molecules may not have a primary function as adaptive tools (i.e. camouflage, warning signaling) as in other animals (Seilacher, 1972; Evans et al., 2009). This, in turn, can indicate a certain degree of influence of the environment on the pigments, in particular by diet (Hedegaard et al., 2006; Soldatov et al., 2013). In the current study, the effect of different dietary regimes was tested in order to explore the potential of polyenes as environmental proxy. This information was added to the section 2.4.

- The hypothetical explanation for a slightly thicker pigmented layer during the experimental phase can be found at the end of section 4.1. There, we discussed the possibility that pigments may be assimilated and therefore influenced by diet. However, the thickening that we observed was not large enough to anyhow be supported by this hypothesis.

- The methodology used for measuring the pigmented layer thickness was added to section 2.5: "*The images were analyzed using the software Panopea (© Schöne and Peinl). The thickness of the pigmented layer was calculated as distance between the outer shell margin and the point where the concentration of polyenes suddenly declined. The measurements were taken perpendicular to the shell outer margin."* The shells analyzed (food experiment) were all 1-year-old. Therefore, the effect of ontogeny could not be tested. For this purpose, we would suggest to select older specimen (> 20 years).

- The clams were collected in November 2009. They were then transported to the Darling Marine center where they were kept in flow-through conditions in sediment similar to their natural setting (fully described in Beirne et al., 2012). Again, these clams did not receive any "extra" food and their environmental conditions were not manipulated. They were exposed to the ambient conditions (temperature, salinity, and food) until this experiment began. Only then, were water temperatures manipulated. Again, salinity and food conditions were identical to those in the natural environment at the Darling Marine Center. We are confident that this transplantation in 2009 did not impact the findings noted in the manuscript.

- In regards to the growing season of *A. islandica,* there are discrepancies in the literature suggesting that the concept of a winter growth shutdown cannot be set in stone. Specimens from Iceland indicate a growth slowdown from October to January (Schöne et al., 2005; Marali and Schöne, 2015). However, *A. islandica* from Western Gulf of Maine display continuous growth throughout the year (Wanamaker et al., 2008). In good agreement with these results, Beirne et al. (2012) recorded growth during the winter months (January-March). Based on these observations, we can conclude that more studies are needed to fully understand the growing period of this species. However, we can already appreciate a certain degree of spatial variability. Likely, populations from different geographic locations are influenced by specific conditions which induce different growth responses. In addition, when talking about growing season, we must consider that ontogeny plays a very important role. It is well known that juvenile/young

shells grow more and for more extended periods of time compared to older specimens. In our work, we focus on juveniles from the Baltic Sea. In the light of the previous observations, this condition suggests an extended growing season throughout the year. As a consequence, it is likely that a stress line can be formed in January. In support to this hypothesis, shell length was measured at the beginning and at the end of the experiment. These data provided the exact amount of carbonate produced over the experimental phase. By using this measurements, we could locate the last shell portion formed before the experiment. In this spot all specimens presented a clearly visible line, likely related to the transposition stress. No other growth lines could be identified on the shells, supporting again the idea of a continuous growth.

- The shell portion mentioned as "deposited before the experiment,, in section 3.3 refers to the shell deposition during the acclimation phase. To make the text clearer, the sentence was rephrased. Section 2.2 describes the details of the food experiment. It is stated that the acclimation period lasted for 3 weeks before starting the experiment (therefore, 18 Jan-8 Feb. 2015). Generally, the acclimation phase in scientific experiments represents that period of time in which the animals are kept under the same conditions as during the experimental phase with the exception of the investigated variable(s), in this case food.

- The sentence starting with "As a consequence..." refer to the thickness of the calcitic layer. Not to the overall thickness of the shell. *Tridacna* and *Serripes* may not be the suitable species to take into consideration, given that their shells are fully aragonitic. As for *Spondylus*, the shell is composed by aragonite and calcite. The relative abundance of the single polymorphs support our statement and the cited work by Lowenstam (1954) and Taylor and Kennedy (1969). In fact, as a warm-water species, the aragonite layer is much thicker that the calcitic one (Maier and Titschack, 2010). Based on this data, we can conclude that the trend is indeed very well represented in numerous mollusk species.

- The polyenes peaks do not occur at the same wavelengths as the aragonite peaks. As explained in section 2.5, the two major polyene peaks are found at ~ 1130 (R_1) and 1520 cm^{-1} (R_4). On the other hand, the typical aragonite spectrum is characterized by peaks at 152, 206, 705 and 1085cm^{-1}.

Technical corrections

- Line 38: "Shells,, was changed into "specimens,, as suggested.
- Although the absence of strict rules, the space between values and units is commonly used and accepted.
- Line 89: The word "value,, was added to "$\delta^{18}O_{shell}$,,.
- The missing references at line 94 were added.
- Line 202: in the literature cited the method was referred as polarized Raman microscopy.
- Line 314: the typo was corrected.
- Line 352: the typo was corrected.

- Line 359: the typo was corrected.
- Line 389: “and many other bivalves,, was deleted.
- Line 434: the typo was corrected.
- Line 750: the distinction between adult/juvenile is not strictly fixed. Witbaaard et al. (1997) define “juveniles,, shells with a height between 10 and 23 mm. The shells of the food experiment were in the same range (10-14 mm) so they were defined as juveniles. The shells from the temperature experiment measured between 38 and 44 mm. Based on the size classes of the specimen analyzed in this work, they were categorized as juveniles and adults, respectively. This distinction was also made to reinforce clarity in the text.
- Line 751: the typo was corrected.

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Dear Referee #2,

We appreciate your positive review and the constructive comments.

In regards to the first remark: to our knowledge a detailed description of the orientation of the microstructural units in the different shell layers of *Arctica islandica* currently not available. We are only aware of one single paper that has been published on a similar subject (Karney et al., 2012). However, in that case, the EBSD analyses were exclusively conducted in the hinge plate and not in the ventral margin. The study characterized the microstructural orientation in the growth increments and in the growth lines, respectively. However, no data was shown on the three shell layers mentioned in the current manuscript. Given the frequent use of *A. islandica* in sclerochronological studies, the community will benefit from a map of crystal orientation in the whole shell, not just in very small shell portions. The used of CRM is therefore clearly advantageous.

As suggested, a more detailed description of the microstructures will be added to a revised version of the manuscript (paragraph 2.4: *A. islandica* shell organization) and a sketch (new Fig. 2) will be provided to better locate each type of microstructure described in the article. However, a superimposition of SEM images and CRM spectral maps would be imprecise since the two analyses were conducted on different machines without common coordinates as reference. We therefore prefer to show the two outputs separately.

The paragraph 4.2 will be edited as suggested. The main difference between the two techniques is the output of absolute (EBSD) and relative (CRM) data. This can be considered the main advantage of EBSD over the CRM. The information has been added to the paragraph. However, it should be realized that the "absolute orientation" is determined for the actual cross section. Therefore we regard a relative change in orientation as sufficient to for the questions asked in this study.

As for the third point highlighted by the referee, we agree that an experimental setup in which more parameters had been varied would be very interesting. However, this was outside the scope of the present study, which focused on the general feasibility of the applied methods. The positive results obtained by this first study set the basis for further studies in which now a more complex matrix of parameters can be investigated.

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

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Keywords: Confocal Raman microscopy; Scanning electron microscopy; Shell microstructure; Water temperature; Diet; Bivalve shell

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

1. Introduction

Biomineralization is a process through which living organisms produce a protective, mineralized hard tissue. The considerable diversity of biomineralizing species contributes to high variability in terms of shape, organization and mineralogy of the structures produced (Lowenstam and Weiner, 1989; Carter et al., 2012). Different architectures at the micrometer and nanometer scale and different biochemical compositions determine material properties that serve specific functions (Weiner and Addadi, 1997; Currey, 1999; Merkel et al., 2007). Besides these differences, all mineralized tissues are hybrid materials consisting in hierarchical arrangements of biomineral units surrounded by organic matrix, also known as "microstructures" (Bøggild, 1930; Carter and Clark, 1985; Rodriguez-Navarro et al., 2006) or "ultrastructures" (Blackwell et al., 1977; Olson et al., 2012) or overall "fabrics" (Schöne, 2013; Schöne et al., 2013). The carbonate and organic phases represent the fundamental level of the organization of biomaterials (Aizenberg et al., 2005; Meyers et al., 2006). The mechanisms of microstructure formation and shaping, especially in mollusks, has attracted increasing attention during recent decades. At present, it is commonly accepted that the organic compounds play an important role in the formation of the inorganic phases of biominerals

252 (Weiner and Addadi, 1991; Berman et al., 1993; Dauphin et al., 2003; Nudelman et al., 2006).
253 However, the identification of the exact mechanisms driving biomineralization is still an open
254 research question. Previous studies conducted on mollusks show that environmental parameters
255 can influence microstructure formation (Lutz, 1984; Tan Tiu and Prezant, 1987; Tan Tiu, 1988;
256 Nishida et al., 2012). These results set the stage for a research interest toward the use of shell
257 microstructures as proxies for reconstructing environmental conditions (Tan Tiu, 1988; Tan Tiu
258 and Prezant, 1989; Olson et al., 2012; Milano et al., 2015).

259 Mollusks are routinely used as climate and environmental proxy archives because they can
260 record a large amount of environmental information in their shells (Richardson, 2001; Wanamaker
261 et al., 2011a; Schöne and Gillikin, 2013). Whereas structures at nanometric level are still
262 underexplored as potential environmental recorders, shell patterns at lower magnification, such as
263 individual growth increments, are commonly used for this purpose (Jones, 1983; Schöne et al.,
264 2005; Marali and Schöne, 2015; Mette et al., 2016). Mollusks deposit skeletal material on a
265 periodic basis and at different rates (Thompson et al., 1980; Deith, 1985). During periods of fast
266 growth, growth increments are formed whereas during periods of slower growth, growth lines are
267 formed (Schöne, 2008; Schöne and Gillikin, 2013). The periodicity of such structures ranges from
268 tidal to annual (Gordon and Carriker, 1978; Schöne and Surge, 2012). By crossdating time-series
269 with similar growth patterns it is possible to construct century and millennia-long master
270 chronologies (Marchitto et al., 2000; Black et al. 2008; Black et al., 2016; Butler et al., 2013). This
271 basic approach, in combination with geochemical methods, has great potential in reconstructing
272 past climatic conditions (Wanamaker et al., 2011b). At present, the most frequently used and well-
273 accepted geochemical proxy is oxygen isotopic composition of shell material ($\delta^{18}O_{shell}$) (Epstein,
274 1953; Grossman and Ku, 1986; Schöne et al., 2004; Wanamaker et al., 2007) which may serve as
275 a paleothermometer and/or paleosalinometer (Mook, 1971; Andrus, 2011); however, $\delta^{18}O_{shell}$ value

276 is influenced by both seawater temperature and the isotopic composition of seawater ($\delta^{18}O_{water}$;
277 related to salinity). Thus, $\delta^{18}O_{shell}$-based temperature reconstructions are particularly challenging
278 in habitats with fluctuating $\delta^{18}O_{water}$ conditions such as estuaries or restricted basins (Gillikin et al.,
279 2005). Because of the multiple impacts on $\delta^{18}O_{shell}$ values, there have been substantial efforts to
280 develop alternative techniques to reconstruct environmental variables from mollusk shells (Schöne
281 et al., 2010; Milano et al., 2017).

282 This study investigates the possibility using shell microstructure properties to serve as a new
283 environmental proxy. For this purpose, the effects of seawater temperature (grown at 10 °C and 15
284 °C) and dietary regime on the microstructural units of *Arctica islandica* cultured under controlled
285 conditions were analyzed and quantified. *A. islandica* was chosen as model species because of its
286 great potential in paleoclimatology and paleoceanography (see Schöne, 2013; Wanamaker et al.,
287 2016). Its extreme longevity of up to more than 500 years makes this species a highly suitable
288 archive for long-term paleoclimate and environmental reconstructions (Schöne et al., 2005;
289 Wanamaker et al., 2008; Wanamaker et al., 2012; Butler et al., 2013).

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292

293 2. Materials and Methods

294 The analyses were conducted on eleven *A. islandica* shells. Three juvenile *A. islandica* shells,
295 sampled for the seawater temperature experiment, were collected alive on November 21, 2009
296 aboard the *F.V. Three of a Kind* off Jonesport, Maine USA (44° 26′ 9.829″N, 67° 26′ 18.045″W)
297 in 82 m water depth. From 2009 to 2011, all animals were kept in a flowing seawater laboratory at

298 the Darling Marine Center, Walpole, Maine, USA (see Beirne et al., 2012 for additional details).
299 In 2011, clams were grown at two different temperature regimes for 16 weeks (Table 1). At the
300 completion of the experiment, shells were estimated to be between 4 to 5 years old. Eight one-year
301 old juveniles were collected in July 2014 from Kiel Bay, Baltic Sea (54° 32′ N, 10° 42′ E; Fig. 1)
302 and kept alive in tanks at 7 °C for six months at the Alfred Wegener Institute for Polar and Marine
303 Research (AWI), Bremerhaven, Germany. During this time interval, the animals were fed with an
304 algal mix of *Nannochloropsis* sp., *Isochrysis galbana* and *Pavlova lutheri.* Then, they were
305 transferred to the Royal Netherlands Institute for Sea Research (NIOZ), Texel, The Netherlands,
306 and cultured in tanks at three different dietary conditions for 11 weeks (Table 1).

307

308

309 ## 2.1 Seawater temperature experiment

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

320

2.2 Food experiment

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

2.3 Sample preparation

342 The right valve of each specimen was cut into two 1.5 millimeter-thick sections along the axis of
343 maximum growth. For this purpose, a low speed precision saw (Buehler Isomet 1000) was used.
344 Given the small size and fragility of the juvenile shells used in the food experiment, the valves
345 were fully embedded in a block of Struers EpoFix (epoxy) and air-dried overnight prior the
346 sectioning. Sections of the clams used in the temperature experiment were embedded in epoxy after
347 the cutting. All samples were ground using a Buehler Metaserv 2000 machine equipped with
348 Buehler silicon carbide papers of different grit sizes (P320, P600, P1200, P2500). In addition, the
349 samples were manually ground with Buehler P4000 grit paper and polished with a Buehler diamond
350 polycrystalline suspension (3 µm). Sample surfaces were rinsed in demineralized water and air-
351 dried. In the samples of the temperature experiment, the calcein marks were located under a
352 fluorescence light microscope (Zeiss Axio Imager.A1m microscope equipped with a Zeiss
353 HBO100 mercury lamp and filter set 38: excitation wavelength, ca. 450 - 500 nm; emission
354 wavelength, ca. 500 - 550 nm).

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357 ## 2.4 *A. islandica* shell organization

358 The shell of *A. islandica* consists of pure aragonite and it is divided in two major layers, an outer
359 (OSL) and the inner shell layer (ISL). The OSL is further subdivided in outer (oOSL) and inner
360 portion (iOSL) (Schöne, 2013). These layers are characterized by specific microstructures (Ropes
361 et al., 1984; Fig. 2). The oOSL largely consists of homogenous microstructure with granular aspect
362 (Schöne et al., 2013). This type of architecture is characterized by approximately equidimensional
363 units of about 5 µm in width. The unit shape tends to be irregular with a bulky aspect. The
364 organization lacks of specific structural arrangement typical of other type of microstructures such

365 as the crossed-lamellar and cross-acicular microstructures. The latter are the main component
366 characterizing the iOSL and ISL (Dunca et al., 2009). Here, elongated units are arranged with two
367 main dip directions, resulting in a relative oblique alignment. As shown in Fig. 2, the elongation of
368 the structures becomes more evident in the ISL.

369 The present study focuses on ventral margin of the shells. Analyses were carried out exclusively in
370 the OSL.

371 Similar to other mollusks, the shell of *A. islandica* contains pigment polyenes which are
372 obviously visible when using CRM (Hedegaard et al., 2006). Polyenes are organic compounds
373 containing single (C-C) and double (C=C) carbon-carbon bonds forming a polyenic chain. Their
374 distribution across the shell is not homogenous. The pigments are abundant in the oOSL whereas
375 they become scarce in the iOSL. Furthermore, an enrichment in polyenes has been observed in the
376 growth lines, potentially indicating their involvement in the biomineralization process (Stemmer
377 and Nehrke, 2014). However, the specific functions of these organic compounds have not been
378 disclosed yet (Hedegaard et al., 2006; Karampelas et al., 2009). Given the high phenotypic
379 variation in pigmentation among and within mollusk species, it has been proposed that coloration
380 does not have a primary function as adaptive tool (i.e. camouflage, warning signaling) as in other
381 animals (Seilacher, 1972; Evans et al., 2009). This, in turn, can indicate a certain degree of
382 influence of the environment on the pigments, in particular by diet (Hedegaard et al., 2006;
383 Soldatov et al., 2013). In the current study, the effect of different dietary regimes was tested in
384 order to explore the potential of polyenes as environmental proxy.

385

386

2.5 Confocal Raman microscopy and image processing

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

Polarized Raman microscopy is known to provide comprehensive information about the crystallographic properties of the materials (Hopkins and Farrow, 1985). The aragonite spectrum is characterized by two lattice modes (translation mode T_a, 152cm^{-1} and librational mode L_a, 206cm^{-1}) and the two internal modes (in-plane band ν_4, 705cm^{-1} and symmetric stretch ν_1, 1085cm^{-1}). The ratio ($R_{\nu1/Ta}$) between peak intensities belonging to ν_1 and T_a is caused by different crystallographic orientations of the aragonitic units (Hopkins and Farrow, 1985; Nehrke and Nouet, 2011). Within each scan, $R_{\nu1/Ta}$ was calculated for each data point. New spectral images were

generated using WITecProject software (version 4.1, WITec GmbH, Germany). These images were then binarized by replacing all values above 2.5 with 1 and the others with 0. The orientation was quantified by calculating the area formed by pixels of value 1 over the total scan area. The imaging software Gwyddion (http://gwyddion.net/last checked: June 2016) was used for this purpose. The results were expressed in percentage.

The Raman scans of the food experiment shells were analyzed to investigate the pigment composition. Polyene peaks have definite positions in the spectrum according to the number of the C-C and C=C bonds of the chain, which are specific for certain types of pigments. The two major polyene peaks at ~ 1130 (R_1) and 1520 cm^{-1} (R_4) were identified by using the "multipeak fitting 2" routine of IGOR Pro (version 7.00, WaveMetrics, USA). Their exact position was determined adopting a Gaussian fitting function (Fig. 3). The number of single (N_1) and double carbon bonds (N_4) was calculated by applying the equations by Schaffer et al. (1991):

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

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

Spectral images of the R_4 band were used to locate the polyenes in the shell and measure the thickness of the pigmented layer. The images were analyzed using the software Panopea (© Schöne and Peinl). The thickness of the pigmented layer was calculated as distance between the outer shell margin and the point where the concentration of polyenes suddenly declined. The measurements were taken perpendicular to the shell outer margin. This analysis was conducted only on the shells of the food experiment. Given the larger size of the shells used in the temperature experiment, the spectral 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 measured with the software Panopea. Given the difference in duration of the two phases of the temperature experiment, the measurements were expressed as total growth and instantaneous

454 growth rate (Fig. 4a + b). The latter was calculated using the following equation (Brey et al., 1990;
455 Witbaard et al., 1997):

456 (3) Instantaneous growth rate = (ln (y_t / y_0) / a)

457 where y_0 represents the initial shell height, y_t is the final shell height and a is the duration of the
458 experiment. In the case of the food experiment, only the total growth was calculated (Fig. 4c).

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461

462 # 3. Results

463 ## 3.1 Effect of seawater temperature and diet on *A. islandica* shell growth

464 When exposed to a water temperature of 10 °C, the shells grew between 11.67 and 14.17 mm
465 during a period of 48 days. During a period of 69 days at 15 °C, the growth ranged between 2.32
466 and 5.77 mm (Fig. 4a). Instantaneous growth rate showed a decrease between the two experimental
467 phases. At 10 and 15 °C, the average instantaneous growth per day was 0.0091 and 0.0013,
468 respectively (Fig. 4b). The decrease in total growth and growth rate between the two temperatures
469 was statistically significant (*t*-test, $p < 0.01$).

470 During the food experiment, shells grew between 0.37 and 3.71 mm with large differences
471 due to the different food types. Growth of specimens exposed to food type 1 ranged between 1.87
472 to 3.71 mm, whereas those cultured with food type 2 grew between 0.55 to 0.96 mm. Both control
473 specimens added 0.37 mm of shell during the experimental phase (Fig. 4c). ANOVA and Tukey´s

HSD post hoc tests showed significant differences between specimens cultured with food type 1 and 2 ($p < 0.05$) and between food type 1 and control shells ($p < 0.05$).

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

At a water temperature of 10 °C, the area occupied by microstructural units oriented with $R_{v1/Ta}$ higher than 2.5 a.u. (= arbitrary units) ranged between 31.3 and 50.6 % in the oOSL and between 21.3 and 33.5 % in the iOSL. When exposed to 15 °C, values ranged between 25.6 and 48.7 % and between 45.7 and 55.9 % in the oOSL and iOSL, respectively (Fig. 5). Whereas the slight difference of area with $R_{v1/Ta} > 2.5$ in the oOSL was not significant between the two water temperatures (*t*-test, $p = 0.62$), the area with $R_{v1/Ta} > 2.5$ in the iOSL significantly increased at 15 °C (*t*-test, $p = 0.02$). Under the SEM, no difference was visible between units formed at 10 °C and 15 °C (Fig. 6).

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

In the shells cultured with food type 1, the area occupied by biomineral units oriented with $R_{v1/Ta}$ higher than 2.5 a.u. during the experiment ranged between 24.8 % (oOSL) and 43.0 % (iOSL). In the shell portion deposited during the acclimation phase, the ratio varied between 19.4 % (oOSL) and 36.2 % (iOSL). Although a trend was recognized, these variations were not statistically different (*t*-tests. OSL: $p = 0.43$; ISL: $p = 0.57$; Fig. 7a). On the contrary, in the clams exposed to food type 2, the 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. 7b). 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. 7c). 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. 9).

All treatments showed a slightly thicker pigmented layer formed during the experiment than during the acclimation phase (Fig. 9a). 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. 9b). 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).

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 pigmentation react 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 the effects on the morphometric characteristics (e.g. size and shape) or on the type of

539 microstructure. Milano et al. (2017) demonstrated that size and elongation of prismatic structural
540 units of *Cerastoderma edule* were positively correlated to seawater temperature variation
541 throughout the growing season. Likely, low temperatures induced the formation of small nacre
542 tablets in *Geukensia demissa* (Lutz, 1984). Seasonal changes of the microstructural type were
543 reported in the freshwater bivalve *Corbicula fluminea* (Prezant and Tan Tiu, 1986; Tan Tiu and
544 Prezant, 1989). During the warm months, crossed acicular structure was produced, whereas simple
545 crossed-lamellae were formed during the winter period. So far, variations of the crystallographic
546 properties of bivalve biominerals have been exclusively investigated as a response to hypercapnic
547 (acidified) conditions. *Mytilus galloprovincialis* and *Mytilus edulis* showed a significant change in
548 the orientation of the prisms forming shell calcitic layer when subjected to hypercapnia (Hahn et
549 al., 2012; Fitzer et al., 2014). Altered crystallographic organization may derive from the animal
550 exposure to suboptimal conditions. These findings together with the present results suggest that
551 thermal- and hypercapnic-induced stress are likely to affect the ability of the bivalves to preserve
552 the orientation of their microstructural units (Fitzer et al., 2015).

553 Different food sources do not significantly influence the orientation of the biomineral units
554 or the composition and distribution of pigments in shells of *A. islandica*. In previous studies, the
555 relationship between microstructure and diet was virtually overlooked resulting in a lack of data in
556 the literature. As suggested by Hedegaard et al. (2006), however, the type of polyenes is influenced
557 by food. The ingestion of pigment-enriched microalgae potentially leads to an accumulation of
558 pigments in mollusk tissues and the shell (Soldatov et al., 2013). On the other hand, it has been
559 argued that polyenes do not generate from food sources like other pigments (i.e., carotenoids), but
560 they are locally synthesized (Karampelas et al., 2009). In accordance to Stemmer and Nehrke
561 (2014), the results presented here support the view that the specific diets on which the animals rely

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

564

565

566 ## 4.2 Confocal Raman microscopy as tool for microstructural analysis

567 From a methodological perspective, the present study represents an innovative approach in
568 the investigation of shell microstructural organization. Electron backscatter diffraction (EBSD) has
569 been previously used to determine the crystallographic orientation of gastropod (Fryda et al., 2009;
570 Pérez-Huerta et al., 2011) and bivalve microstructural units (Checa et al., 2006; Frenzel et al., 2012;
571 Karney et al., 2012). Whereas, CRM on mollusk shells is generally applied within studies on
572 taphonomic mineralogical alteration and pigment identification (Stemmer and Nehrke, 2014;
573 Beierlein et al., 2015). Both techniques provide considerably high spatially resolved analysis up to
574 250 nm, allowing the identification of individual structural units at µm- and nm-scale (Cusack et
575 al., 2008; Karney et al., 2012). CRM offers important advantages supporting a broader application
576 of this methodology in the biomineralization research field. For instance, samples do not require
577 any pre-treatment. Unlike EBSD, there is no need of preparing thin-sections (~ 150 µm thick) or
578 etching the shell surface (Griesshaber et al., 2010; Hahn et al., 2012). Therefore, further structural
579 and geochemical analyses can be easily performed on the same sections (Nehrke et al., 2012). In
580 addition, the size of CRM scans can be remarkably large (~ 7-8 mm^2) without compromising the
581 achievable resolution. By overlapping adjacent scans, it is possible to produce stitched scans
582 allowing to further increase the region of interest on the shell surface. On the other side, EBSD
583 provides a relevant advantage to take into consideration. It allows absolute measures of the

584 crystallographic orientation of the carbonate structures. The CRM, instead, determines the relative
585 change in the orientation between the single units without providing absolute values.

586 SEM has previously been demonstrated to provide a convenient approach for the
587 identification of individual structural units and the quantification of potential changes occurring
588 within them (Milano et al., 2017, 2016b). However, SEM exclusively provides information about
589 the morphometric characteristics of the microstructural units. As highlighted by the present study,
590 to achieve an exhaustive examination, it is suggested to combine SEM with techniques assessing
591 crystallographic properties of the biomaterials. For instance, our results show that the effect of
592 water temperature is detectable in crystallographic orientation but not in morphometric features of
593 the biomineral units.

594

595 ## 4.3 Environmental influence on shell growth

596 Numerous previous studies demonstrated that growth rate of *A. islandica* is linked to environmental
597 variables (e.g., Witbaard et al., 1997, 1999; Schöne et al., 2004; Butler et al., 2010; Mette et al.,
598 2016). However, the relative importance of the main factors, temperature and food supply/quality
599 driving shell formation are still not well understood. Positive correlations between shell growth
600 and water temperature have been identified (i.e., Schöne et al., 2005; Wanamaker et al., 2009;
601 Marali et al., 2015), but the relationship between shell growth and environment is more complex
602 (Marchitto et al., 2010; Stott et al., 2010; Schöne et al., 2013) and likely dependent on the synergic
603 effect of food availability and water temperature (Butler et al., 2013; Lohmann and Schöne, 2013;
604 Mette et al., 2016). Tank experiments were run in order to precisely identify the role of these two
605 parameters of shell growth of *A. islandica* (Witbaard et al., 1997; Hiebenthal et al., 2012). A tenfold
606 increase in instantaneous growth rate was observed between 1 and 12 °C, with the greatest variation

607 occurring below 6 °C (Witbaard et al., 1997). On the contrary, a temperature increase between 4
608 and 16 °C was shown to produce a slowdown of shell production (Hiebenthal et al., 2012). Our
609 results are in agreement with the latter study and show a decrease in the instantaneous growth rate
610 between 10 and 15 °C. High temperatures are often associated with an increase of free radical
611 production (Abele et al., 2002). A large amount of energy then has to be allocated to limit oxidative
612 cellular damage (Abele and Puntarulo, 2004). This translates into a higher accumulation of
613 lipofuscin and slower shell production rate (Hiebenthal et al., 2013). The contrasting results of
614 previous studies may be explained by individual differences in the tolerance toward temperature
615 change (Marchitto et al., 2000).

616 Along with water temperature, food availability was also shown to influence *A. islandica*
617 shell growth (Witbaard et al., 1997). At high algal cell densities, the siphon activity increased. This,
618 in turn, was positively correlated to shell growth. Previous experiments used different combinations
619 of algae such as *Isochrysis galbana* and *Dunaliella marina* (Witbaard et al., 1997), or
620 *Nannochloropsis oculata, Phaeodactylum tricornutum* and *Chlorella* sp. (Hiebenthal et al., 2012)
621 to grow the clams. However, there are still uncertainties about the composition of the primary food
622 source for this species (Butler et al., 2010). Even though it is challenging to determine the preferred
623 algal species, our results show that the use of a mixture of different algal species results in
624 significantly faster shell growth than the used of just one algal species. In the natural environment,
625 suspension feeders such as *A. islandica* preferentially ingest certain particle sizes (Rubenstein and
626 Koehl, 1977; Jorgensen, 1996; Baker et al., 1998). The exposure to a limited algal size range, as in
627 the case of food type 2, may affect shell growth. Furthermore, multispecific solutions contain a
628 higher variability of biochemical components that better meet the nutritional requirements of the
629 animal (Widdows, 1991). Our results are in good agreement with previous findings. For instance,
630 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 composed by *Isochrysis galbana* and *Thalassiosira pseudonana* and diets consisting of the single species. Faster growth was measured in the mixed diet treatment, indicating a synergic effect of the relative food composition (Epifanio, 1979). Likely, *Mytilus edulis* grew faster when reared with different types of mixed diets as opposed to monospecific diets (Galley et al., 2010).

5. Conclusions

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

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

654

655

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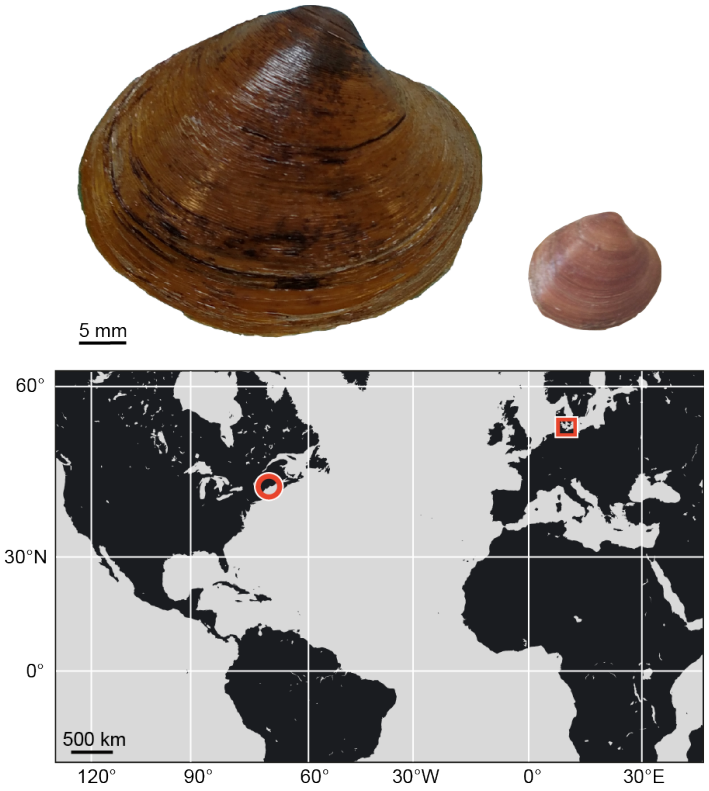
964 # Figures and tables



965

966 **Fig. 1.** Shell of adult *Arctica islandica* used in the temperature experiment (left) and juvenile from the Baltic

967 Sea used in the food experiment (right). The map indicates the localities where the two sets of shells were

968 collected: Jonesport, Maine (circle) and Kiel Bay (square).

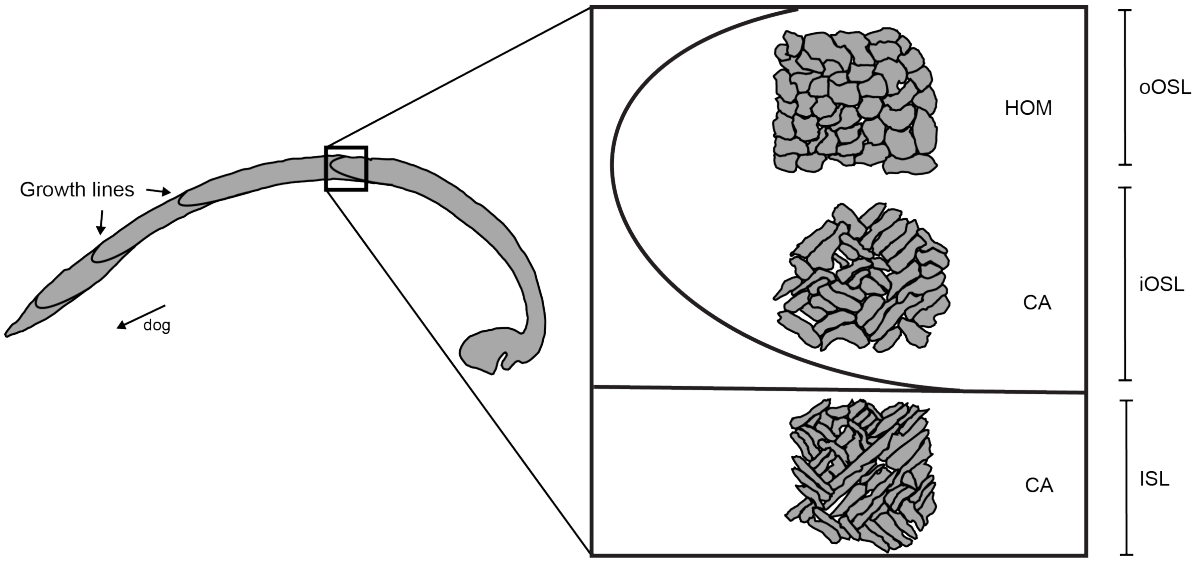


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Fig. 2. Sketch showing the microstructures characterizing the different shell layers of *Arctica islandica*. The oOSL is formed by homogenous microstructure (HOM), whereas the oOSL and ISL are composed of crossed acicular structure (CA). dog = direction of growth.

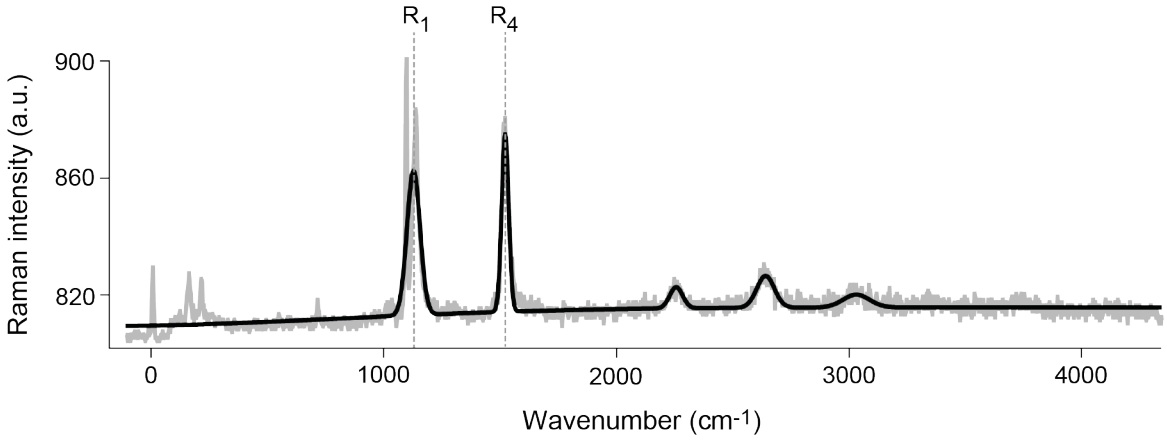

Fig. 3. Raman spectrum of *Arctica islandica* showing the typical aragonite peaks (grey line). The exact position of the polyene peaks R_1 and R_4 was determined by using a peak fitting routine based on a Gaussian function (black line).

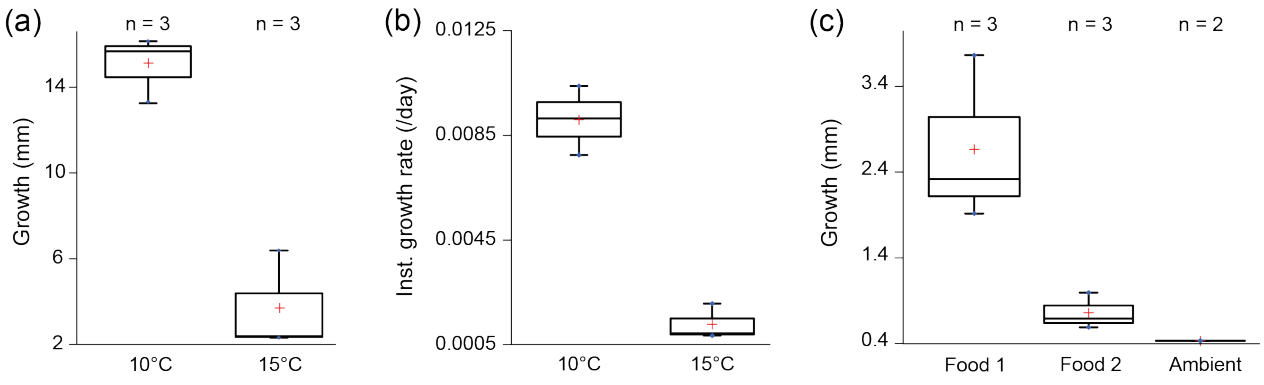

Fig. 4. *Arctica islandica* shell growth under controlled conditions. (a) Total growth and (b) instantaneous growth rate during the temperature experiment. (c) Total growth during the food experiment.

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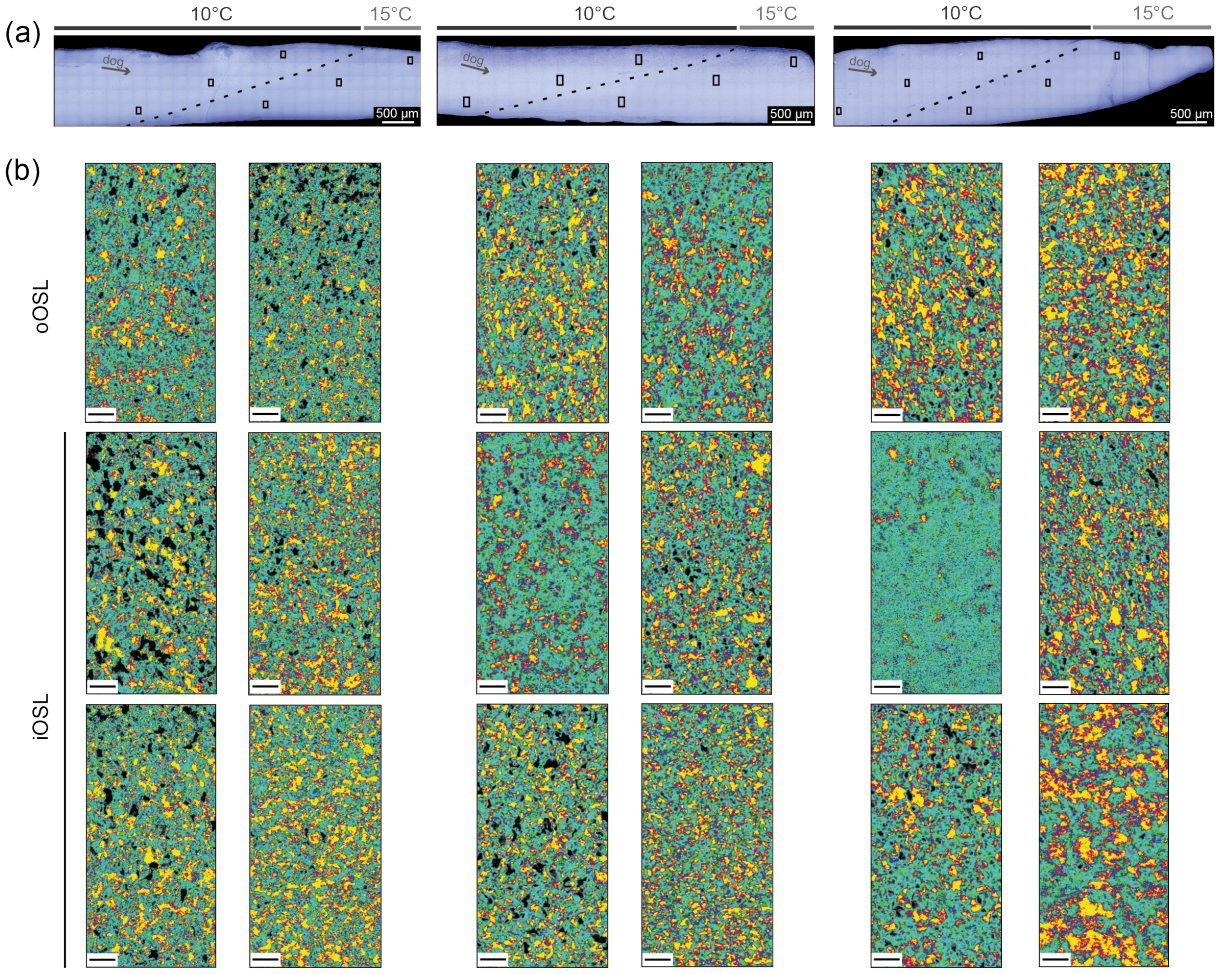
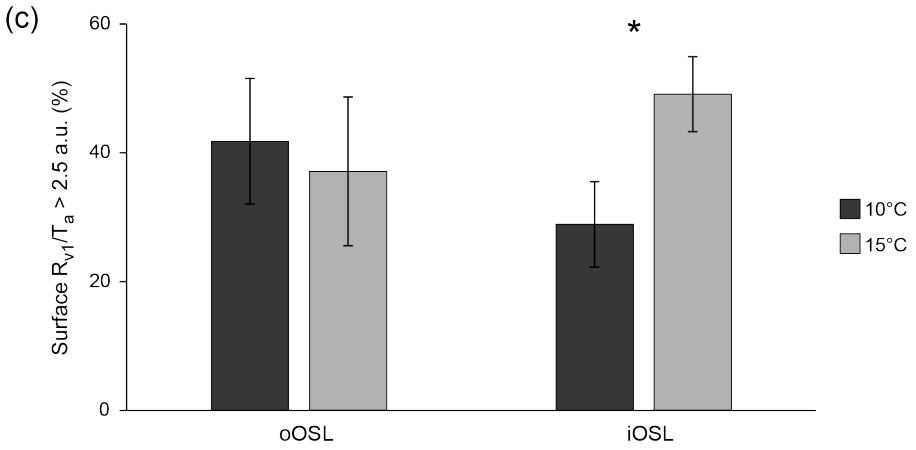

(a)
10°C
15°C
10°C
15°C
10°C
15°C
dog
dog
dog
500 µm
500 µm
500 µm
(b)
oOSL
iOSL


(c)
Surface R_{v1}/T_a > 2.5 a.u. (%)
60
40
20
0
*
oOSL
iOSL
10°C
15°C

985

Fig. 5. Effect of temperature increase on biomineral orientation. (a) Position of the Raman maps of the three specimens reared at 10 °C and 15 °C. Dotted lines indicate the location of the calcein marks. dog = direction of growth. (b) Raman spectral maps of $R_{v1/Ta}$. Left images of each column represents shell portion formed at 10 °C, right images represent shell portions formed at 15 °C. First row of pairs refers to oOSL, the other two represent the iOSL. Scale bars = 10 µm. (c) Proportions of biominerals with $R_{v1/Ta} > 2.5$ a.u. with respect to the total map area. Asterisks indicate significant difference between the orientation of iOSL microstructures formed at 10 and 15°C ($p < 0.05$).

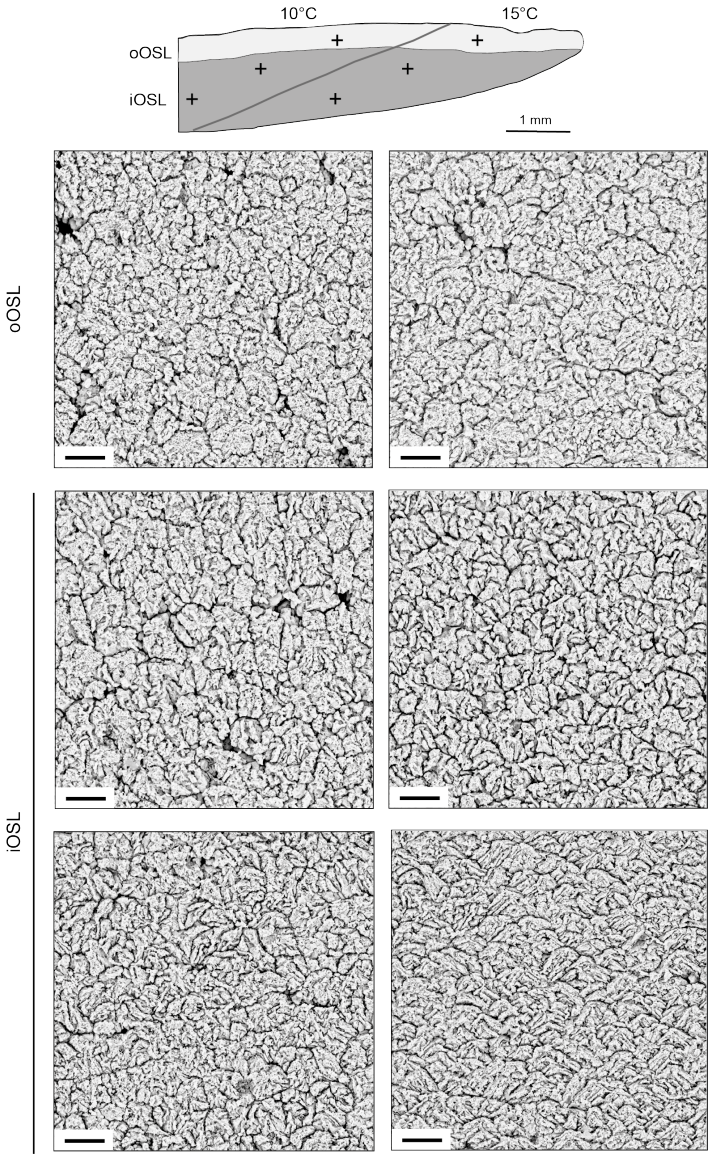


Fig. 6. SEM images of *Arctica islandica* shell microstructures formed at 10 °C (left column) and at 15 °C (right column). The sketch indicates the position of the images 1 mm away from the calcein mark (grey line). The first row of images refers to the oOSL, the other two row refers to the iOSL. Scale bars if not otherwise indicated = 5 µm.

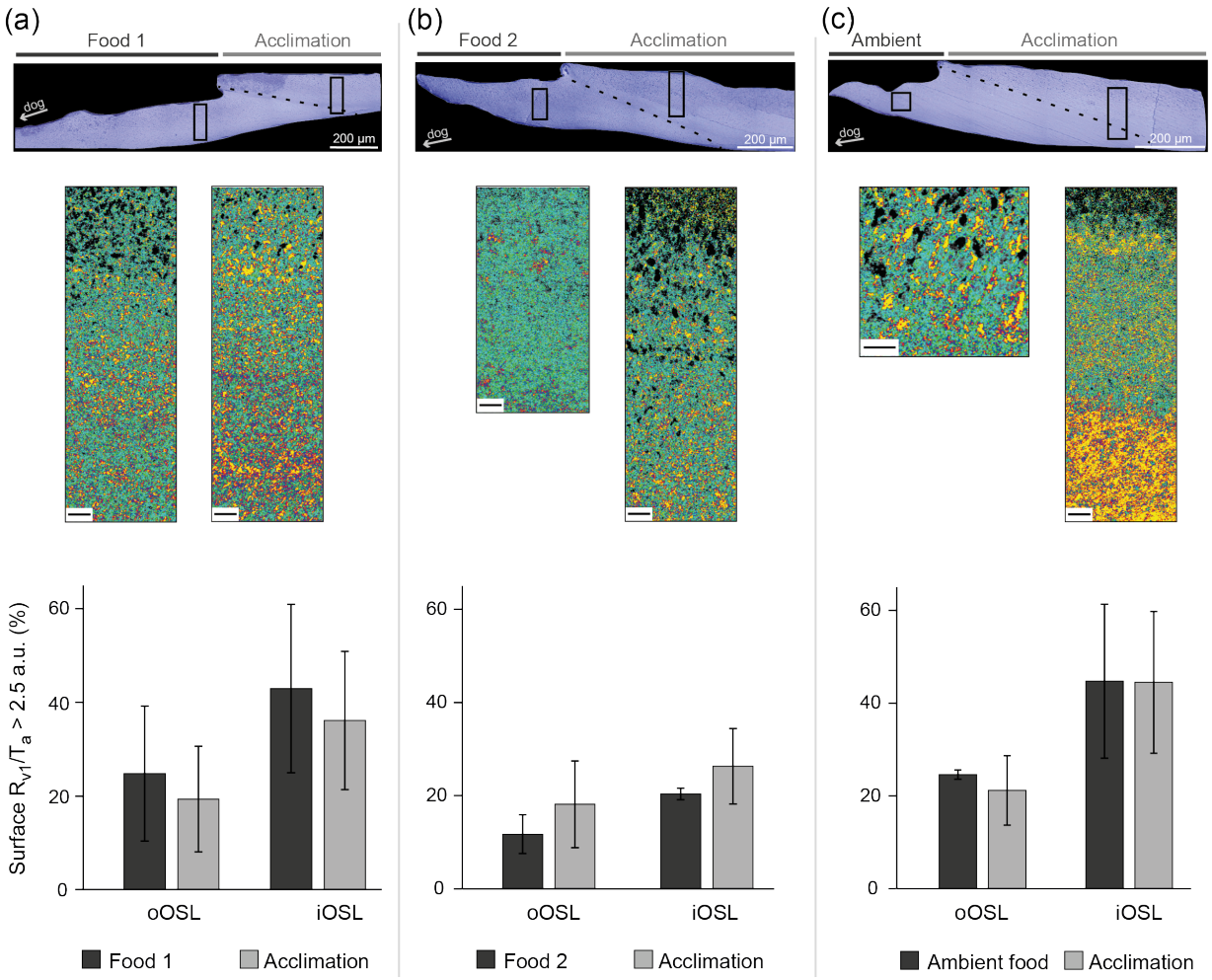


Fig. 7. Effect of different diets based on (a) food type 1, (b) food type 2 and (c) ambient food on biomineral orientation. The optical microscope images indicate the position of the Raman scans. Dotted line marks the start of the experiment. The portion of shell prior the line was formed during the acclimation phase. dog = direction of growth. The Raman spectral maps indicate the ratio $R_{v1/Ta}$ for each data point of the scan. For each shell, maps on the left represent shell portions during the experiment, maps on the right represent shell portions formed during the acclimation phase. In the acclimation portion of the sample reared with ambient food, a significant change in the microstructure orientation is visible. The respective area of the Raman map was not considered in further calculations because it was influenced by the emersion and transportation stress at the start of the experiment. Scale bars = 10 µm. The graphs show the proportions of biominerals of oOSL and iOSL with $R_{v1/Ta}$ > 2.5 a.u. with respect to the total map area.

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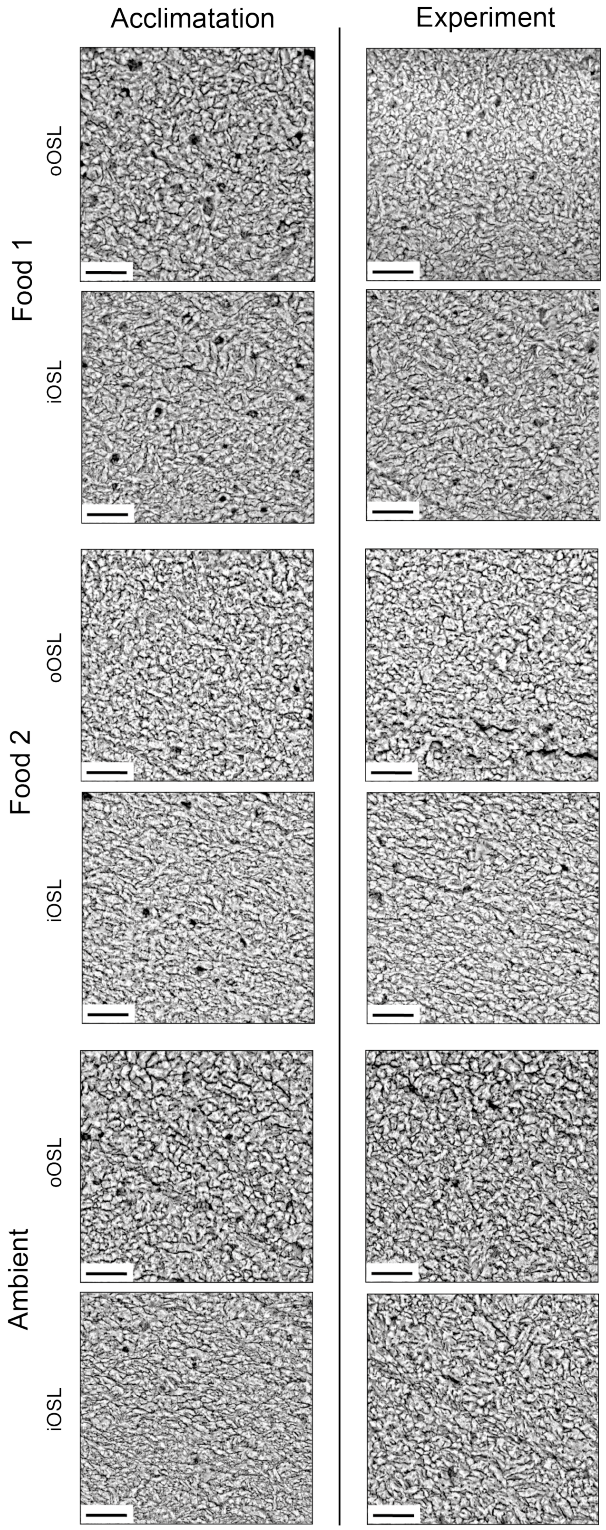
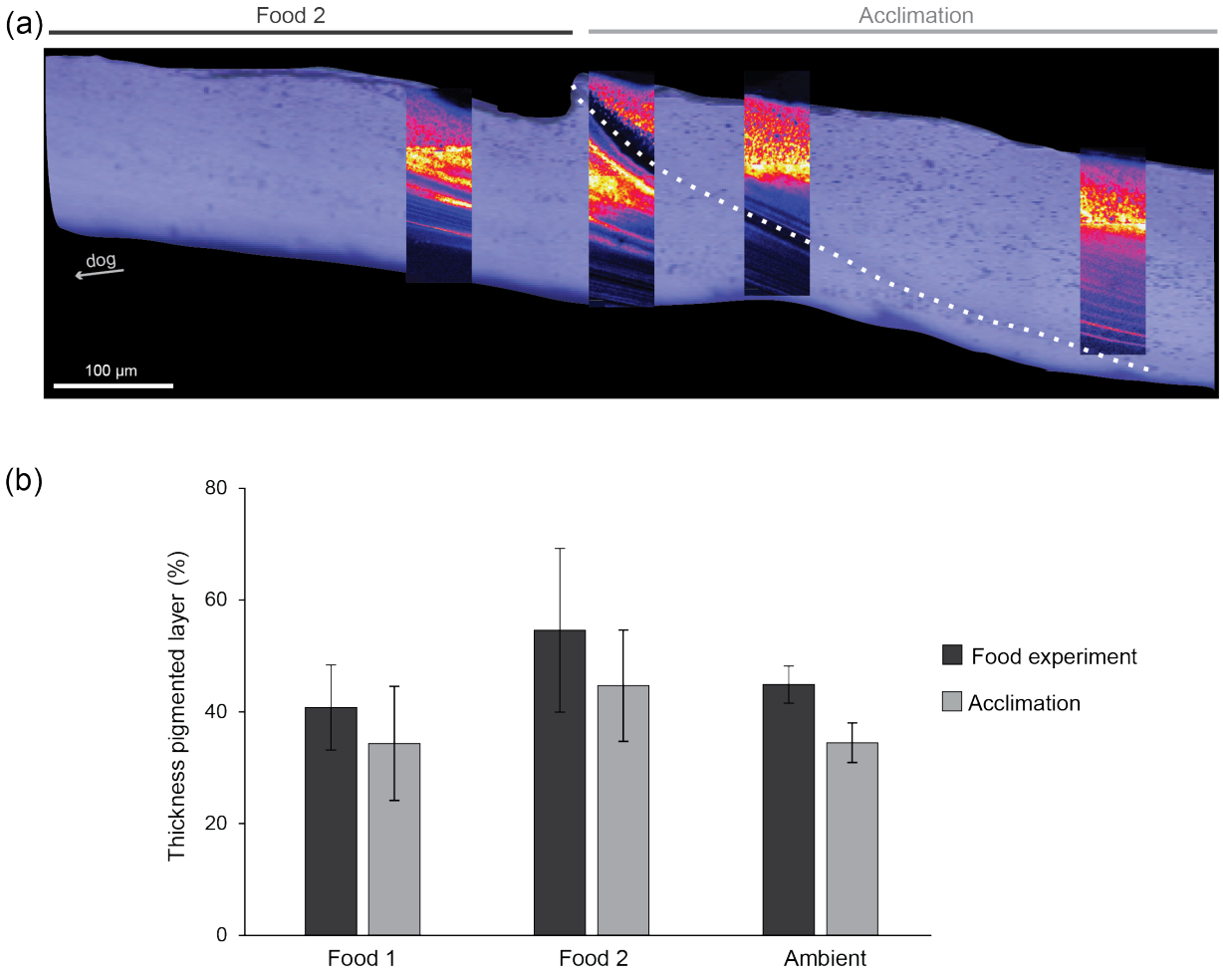


1016

1017 **Fig. 8.** SEM images of *Arctica islandica* shell microstructures formed during the acclimation phase at AWI

1018 (left column) and during the food experiment (right column). Scale bars = 4 µm.



1019

1020 **Fig. 9.** Effects of diet on shell pigment distribution. (a) Raman spectral maps of the 1524 cm^{-1} band

1021 representing the distribution of the polyenes in the shell cultured with food type 2. Dotted line marks the

1022 start of the experiment. dog = direction of growth. (b) The graph shows the thickness of the pigmented layer

1023 over the whole shell thickness before and during the food experiments.

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1026 **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

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1028 **Table 2.** Details of the pigment composition of the *Arctica islandica* shells used in the food experiment.
1029 The position of the major polyene peaks R_1 and R_4 in the Raman spectrum is indicated together with the
1030 number of single and double carbon bonds of the pigment molecular chain (N_1 and N_4). Each shell was
1031 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