

RESEARCH ARTICLE

Understanding Irregular Shell Formation of *Nautilus* in Aquaria: Chemical Composition and Structural Analysis

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Irregular shell formation and black lines on the outside of live chambered nautilus shells have been observed in all adult specimens at aquariums and zoos soon after the organisms enter aquaria. Black lines have also been observed in wild animals at sites of broken shell, but continued growth from that point returns to a normal, smooth structure. In contrast, rough irregular deposition of shell continues throughout residence in aquaria. The composition and reasons for deposition of the black material and mitigation of this irregular shell formation is the subject of the current study. A variety of analytical techniques were used, including stable isotope mass spectrometry (SI-MS), inductively coupled plasma mass spectrometry (ICP-MS), micro x-ray fluorescence (μ XRF), X-ray diffraction (XRD), and scanning electron microscopy (SEM) based X-ray microanalysis. Results indicate that the black material contains excess amounts of copper, zinc, and bromine which are unrelated to the *Nautilus* diet. The combination of these elements and proteins plays an important role in shell formation, growth, and strengthening. Further study will be needed to compare the proteomics of the shell under aquaria versus natural wild environments. The question remains as to whether the occurrence of the black lines indicates normal healing followed by growth irregularities that are caused by stress from chemical or environmental conditions. In this paper we begin to address this question by examining elemental and isotopic differences of *Nautilus* diet and salt water. The atomic composition and light stable isotopic ratios of the *Nautilus* shell formed in aquaria versus wild conditions are presented. Zoo Biol. 33:285–294, 2014. © 2014 Wiley Periodicals, Inc.

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INTRODUCTION

Nautilus is the sole surviving genus of the ectocochliate (external shelled) cephalopods. The depth range at which *Nautilus* live in their wild native environment makes human observation of their general life habits difficult except with remote cameras. Despite an increase in the number of studies, the details of their natural environment and the extent to which environmental changes impact development and survival of *Nautilus* are not well known. Exploring the degree to which shell formation in aquaria diverge from shell formed in natural conditions will begin to uncover these details.

Nautiloids date to the Late Cambrian (~520–540 Ma). Descriptions of chambered nautilus appear in literature and scholarly writings since Aristotle's time. Unfortunately, determining the accuracy of these references is complicated by inconsistent descriptions and by large gaps in the fossil record. Fossils most similar to modern *Nautilus* are in the order Nautilida and are abundant in Jurassic sediments

[Teichert and Matsumoto, 1987]. *Nautilus* research has significantly increased with the availability of living specimens in aquariums and zoos [Davis, 1987]. The first public display of *Nautilus* was in New Caledonia's Noumea Aquarium in 1958 [Catala, 1996]. Hawaii's Waikiki Aquarium was the first United States aquarium to successfully keep *Nautilus* in 1976 [Carlson, 1977]. Twenty-five aquariums and zoos report to have maintained *Nautilus* in

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their collection according to the International Species Information System (ISIS), questionnaires [Carlson, 1987], and the Association of Zoos and Aquariums (AZA) institutional registrars (personal communication).

Nautilus inhabit the waters of the Indo-Pacific seas, with a range from southern Japan to northern Australia [House, 2009]. Chronic overfishing and recognition of shell value prompted the establishment of an inter-agency group to determine if *Nautilus* should be added to Appendix II of the Convention on International Trade in Endangered Species of Wild Flora and Fauna [Saunders and Landman, 2009]. Ongoing census studies suggest the situation is worse than anticipated, with one species possibly extinct [Broad, 2011]. Even though no official declaration of the status of *Nautilus* has been made, wild populations are thought to be threatened by habitat shrinkage. The United Nation's International Panel on Climate Change (IPCC) predicts that continued dissolution of atmospheric carbon dioxide into oceans will promote shoaling of the calcite compensation depth [Kleypas et al., 1999], decreasing the depth at which calcareous shells are stable and so eliminating deeper habitats [Sarma et al., 2002]. Portions of the 0–600 m functional depth range of *Nautilus* was brought into view with remote camera traps at 73–538 m [Saunders, 1984]. More recently many hours of remote video filming was made at 450 m in 2013 as reported by Gregory Barord (personal communication). Aquariums and zoos have become crucial both as a potential species refuge and for facilitating behavioral and developmental studies of *Nautilus*. However, abnormal shell growth in aquaria specimens has been reported since the 1970s [Keupp and Riedel, 1995] and, to date, only four aquariums have successfully bred and reared live *Nautilus* with two captive offspring living 376 days [Carlson et al., 1992].

One of six extant species, *Nautilus pompilius*, has been kept at numerous institutions including the Smithsonian's National Zoo, Washington, DC. Most specimens kept at the National Zoo survive 2–5 years in residence (not a direct reference to age). All inquiries indicate that all *Nautilus* entering aquaria exhibit abnormal shell growth. This study examines the atomic composition and structural differences of shells within individuals as they have formed under both natural and aquaria conditions. *Nautilus pompilius* shell and soft tissue samples collected from necropsies at the National Zoo (post-2005) were compared to soft tissue samples from wild *Nautilus macromphalus*. Additionally, chemical variables such as food and water were compared. The long-range goal of this research is to determine preventative measures, effective treatment for the irregular shell formation of *Nautilus*, potential impacts of climate change on shell formation in the ocean, and to gather data that will help preserve this animal before it becomes endangered.

MATERIALS AND METHODS

Chemical comparisons of shell and soft tissue, food, and water used a variety of analytical instrumentation. Stable

isotope mass spectrometry (SI-MS) was used to examine differences in temperature, food sources, and trophic structure between aquaria and wild environments. Inductively coupled plasma mass spectrometry (ICP-MS) and micro X-ray fluorescence (μ XRF) were used to identify the differences in elemental compositions of tissue, shell, water, and food sources between aquaria and wild conditions. Energy dispersive spectrometry in the scanning electron microscope (SEM-EDS) was used to examine the physical structure of aquaria and wild shell formation, and to provide information for a wide range of elements present in the shell. Finally, morphological imaging with SEM was used to compare the crystalline structure of the shell between the aquaria and wild timeframes.

Materials

National Zoo *N. pompilius* shell and soft tissue samples recovered during necropsies were selected for analysis (Table 1) and *N. macromphalus* soft tissue only from animals that never lived in the aquaria environment (Table 2). Shells were bisected along the vertical plane. One half was used for current analyses and the other preserved for future studies. Soft tissue samples varied between individuals but included at least one sample of mantle, tentacle, and/or hood. All soft tissue samples were frozen at -80°C prior to analyses. Instant Ocean[®] salt was freshly mixed with deionized (DI) water to artificially simulate sea water in the aquaria. Aquaria tank waters were frozen within 12 hr of sampling and were thawed before analyses.

Sample Preparation

Prior to analyses, shells were hand-washed with DI water to remove any clinging salts. Shells were then either broken apart into small chunks with pliers for coarse sampling or powdered with a rotary tool for high-resolution, small-quantity sampling. Shell pieces were powdered by hand in an agate mortar and pestle. It was observed that the aquaria formed shell is thicker and harder than wild formed shell. Black material from the aquaria formed shell was retrieved via scraping with forceps. All soft tissue samples were washed, cut into small pieces, and lyophilized before analyses. Remnants were re-frozen at -80°C . For carbon and oxygen isotope analyses, samples were taken from three wild and three aquaria formed shell areas, including both the exterior and interior shell surfaces. Interior samples consisted of the septa, while ten exterior samples were taken from each shell. Samples were powdered and soaked in 2.5% sodium hypochlorite for 24 hr to remove organic material. Samples were then rinsed five times in ultra-pure water and dried at 60°C for 48 hr. Samples were weighed, reacted with 102% H_3PO_4 in pure helium at 25°C for 18 hr, and analyzed on a Thermo Delta V Advantage mass spectrometer coupled to a Thermo Gas Bench II in continuous flow mode. Analyses for dietary carbon and nitrogen isotopes were carried out on all aquaria and wild specimen soft tissue samples, scraped black

TABLE 1. Accession numbers were given sequentially to individual *Nautilus* upon arrival at National Zoo

Pathology number	Accession number	Time in aquaria (months)	Sampled shell sections	Soft tissue parts
05-0355	601135	28	Left hemisphere	N/A
06-0054	601187	17	Left hemisphere	N/A
08-0052	601214	30	Both hemispheres	N/A
08-0060	601215	30	Right hemisphere	N/A
09-0214	601243	31	N/A	Mantle, Tentacle
09-0216	601240	36	Right hemisphere	N/A
09-0250	601244	37	Left hemisphere	Mantle
09-0283	601212	38	Left hemisphere	Mantle, Tentacle
09-0327	601239	39	Left hemisphere	Mantle, Hood
10-0053	601237	43	Left hemisphere	Mantle
10-0082	601238	44	Left hemisphere	Mantle, Tentacle
10-0109	601241	46	Right hemisphere	Mantle, Tentacle

Pathology numbers were assigned upon death with the first two digits indicating the year of death.

material formed while in aquaria, food samples, and on the shell's internal organic framework. Tissue samples and food material were analyzed directly after lyophilization. Scraped black material formed while in aquaria was treated with 0.6 M HCl at 4°C to remove any carbonate shell accidentally incorporated during scraping. Internal organic framework of the shell was isolated by soaking at least 30 mg of shell material in 0.6 M HCl at 4°C for 24 hr increments until reaction ceased with daily acid replacement. All samples were rinsed in ultra-pure water and dried at 60°C after treatments. Samples were weighed into tin cups and analyzed on a Thermo Delta V Advantage mass spectrometer coupled to a Costech 4010 Element Analyzer via a ConFlo IV interface.

All stable isotope data are reported in standard delta notation: $\delta = [(R_{\text{sample}} - R_{\text{standard}})/R_{\text{standard}}] \times 1,000$, where δ represents the isotope system of interest (i.e., $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, or $\delta^{18}\text{O}$) and R represents the ratio of the heavy to light isotope (i.e., $^{13}\text{C}:^{12}\text{C}$, $^{15}\text{N}:^{14}\text{N}$, $^{18}\text{O}:^{16}\text{O}$). Units are in permil (‰). The standards are Vienna Pee-dee Belemnite (V-PDB) for carbon and oxygen, and atmospheric N_2 for nitrogen. All values are linearly corrected against internal standards and reported with an error of $\pm 0.2\text{‰}$ (1σ) [Coplen, 1994].

The metal content of shells and organic materials was measured with both ICP-MS and μXRF . Soft tissue, food, black material, and shell samples were weighed and digested in Optima grade HNO_3 , and then diluted with ultra-pure DI

water for ICP-MS analysis. Elemental abundances of Br, Cr, Cu, Hg, Mg, Pb, Zn, As, and Ba were measured using a GBC Optimass time of flight ICP-MS. These elements have become of interest either because of their roles in bone production (Zn, Cu, Br) or are frequently named as problematic from tap water supplies (Hg, As, Pb) [Seo et al., 2010, Mai et al., 2003, Opsahl et al., 1982]. Water samples from various tanks and the Instant Ocean[®] powder were diluted with ultra-pure DI water and analyzed. All ICP-MS results are plotted against Ba as it has a low abundance and even distribution because it forms an insoluble precipitate in sea water and is not bioavailable in large quantities [Neff, 2008]. The μXRF analyses of shell, food, aquaria *Nautilus* soft tissue, and Instant Ocean[®] were conducted using a Bruker AXS ARTAX 800 spectrometer to measure Ca, Fe, Cu, Zn, Sr, S, Cl, and Br. The instrument is equipped with a rhodium target poly-capillary lens X-ray tube that has ca. 80 μm X-Y spatial resolution. A Silicon drift x-ray detector (SDD) was used with a 10 mm² active area and energy resolution of ca. 142 eV for the Mn K α peak at 100 kcps. All spectra were collected at 50 kV and 600 μA with a live-time count of 200 sec.

The physical structure of the shell and its elemental composition were examined using an SEM equipped with EDS. The SEM analyses were conducted using a conventional electron source Hitachi S-3700N variable pressure SEM and FEI Quanta 200F field emission scanning electron microscope, and both instruments are equipped with Bruker SDD EDS systems (XFlash 4010 and 5030, respectively). Samples were mounted to aluminum stubs using carbon tape and placed directly into the SEM chamber without any further modification for morphological examination, while particles were dispersed on double-sided carbon tape adhered to a brass plate for microanalytical studies. The SEM was operated using a primary electron beam energy of 15 kV. Photons were collected using a pulse processing time constant associated with a maximum rate of 130,000 counts/sec. SDD data were collected and processed using Bruker ESPRIT v1.9 software and spectra were quantified using the Phi-Ro-Z matrix

TABLE 2. Identified wild *Nautilus* soft tissue samples were provided courtesy of Claire Goiran of University of New Caledonia and originated from Lifou Island

Sample name	Sample abbreviation
Lifou Nautilus 1	LN1
Lifou Nautilus 2	LN2
Lifou Nautilus 3	LN3
Lifou Nautilus 4	LN4
LN4_Gut Contents	LN4_GC
Lifou Nautilus 5	LN5
Lifou Nautilus 6	LN6
Lifou Nautilus 7	LN7

correction method. Brass was selected over the typical aluminum SEM mounting pins given the severe X-ray line overlap between Al, K, and Br (an element of interest for the study) L-line X-rays.

RESULTS

Stable Isotope Analyses—Latitudinal Changes

Figure 1 shows carbon ($\delta^{13}\text{C}$) and oxygen ($\delta^{18}\text{O}$) values from six National Zoo individual *Nautilus* that lived in the wild and then in aquaria. For each individual, shell formed in the wild includes more enriched values than shell formed while living in aquaria, which included depleted values. The depletion upon entering aquaria was $\sim 4\text{--}6\text{‰}$ for both carbon and oxygen values which could be due to changes in latitude with the move to a new environment.

Stable Isotope Analyses—Temperature Changes

Figure 1 also shows the mean $\delta^{18}\text{O}$ in the aquaria-formed shell ($-3.8 \pm 0.7\text{‰}$, 1σ) was approximately $\sim 5.6\text{‰}$ depleted compared to its wild shell components ($+1.8 \pm 0.6\text{‰}$, 1σ) (Fig. 1). Given that *Nautilus* and other Mollusca shell deposition show an oxygen isotope depletion of $\sim 0.25\text{‰}/^\circ\text{C}$ between water and carbonate shells [Epstein et al., 1953; Killingley and Newman, 1982; Grossman and Ku, 1986; Landman et al., 1994; Lecuyer et al., 2004], *Nautilus* would have to experience a temperature increase of $\sim 22^\circ\text{C}$ upon entering the aquaria to produce the observed isotope depletion. In addition, the variability in $\delta^{18}\text{O}$ values in wild shell was noticeably more cyclic than the observed pattern in aquaria-formed shell (Fig. 1).

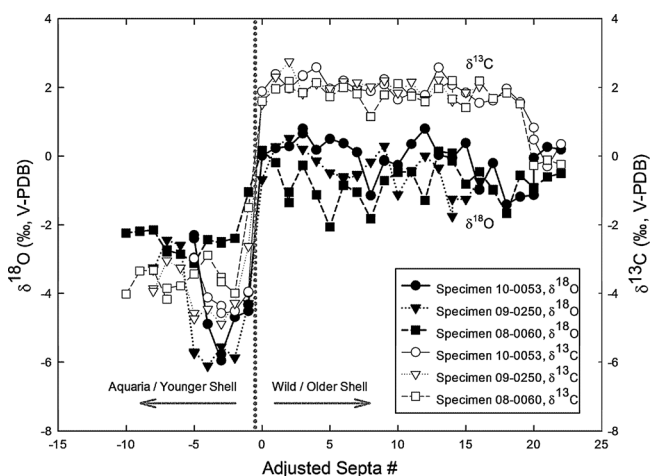


Fig. 1. Carbon ($\delta^{13}\text{C}$) and oxygen ($\delta^{18}\text{O}$) values from the aquaria and wild environments, in which wild formed shell shows more enriched values and aquaria formed shell shows depleted values of carbon and oxygen. Septa numbers are adjusted by placing 0 as the point at which the individual animal entered the aquaria with positive numbers indicating wild shell formation and negative numbers indicating shell formation in the aquaria of the same animal. Negative numbers indicate later adult animal shell formation.

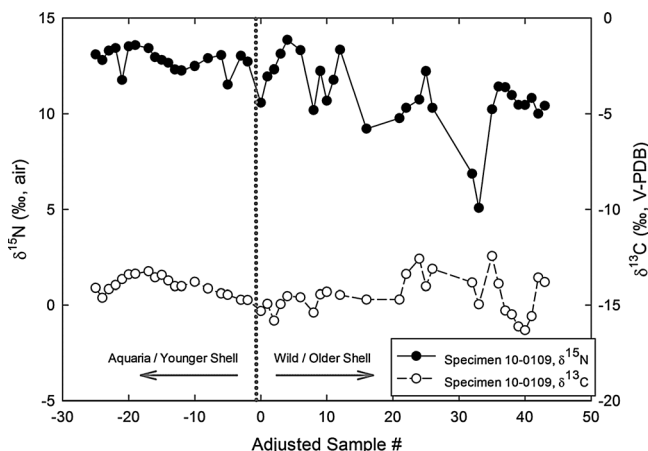


Fig. 2. Carbon and nitrogen isotope values from aquaria versus wild formed shells are plotted against the adjusted sample numbers on the x-axis with 0 being the point when the animal entered the aquaria environment just as Figure 1.

Stable Isotope Analyses—Contribution of Food

The organic components of shell 10-0109 show very small to no change in organic nitrogen ($\delta^{15}\text{N}$) and carbon ($\delta^{13}\text{C}$) composition over the lifetime of the animal (Fig. 2). However, it should be noted that the offset of isotope values between food source and *Nautilus* tissue differs between aquaria and wild specimens (Fig. 3).

Elemental Analyses—ICP-MS of Tank Water

Instant Ocean[®], used by the National Zoo, was compared to San Francisco Bay Sea Salt which was used as a proxy to the *Nautilus* natural habitat. The ICP-MS of the solutions of dissolved Instant Ocean[®] and tank waters at the National Zoo showed low levels of all elements studied except for Br, which was high in Instant Ocean[®] (Fig. 4). This indicates that tank water is most likely not responsible

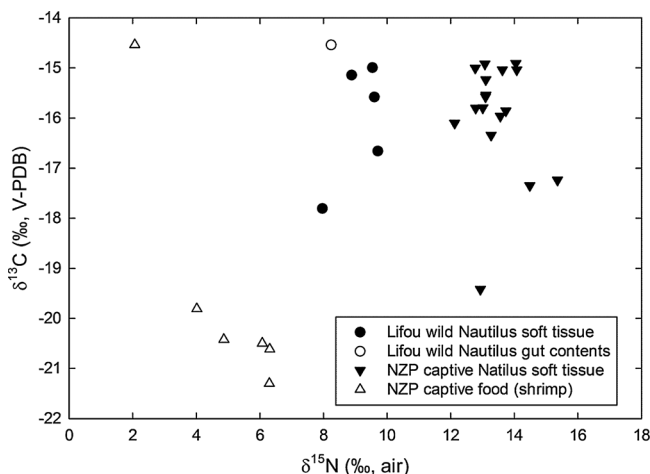


Fig. 3. Carbon and nitrogen isotope values of soft tissue from wild *Nautilus* samples from Lifou Island, National Zoo aquaria *Nautilus*, and their food sources indicate any dietary differences may not be transmitted to the organic shell material.

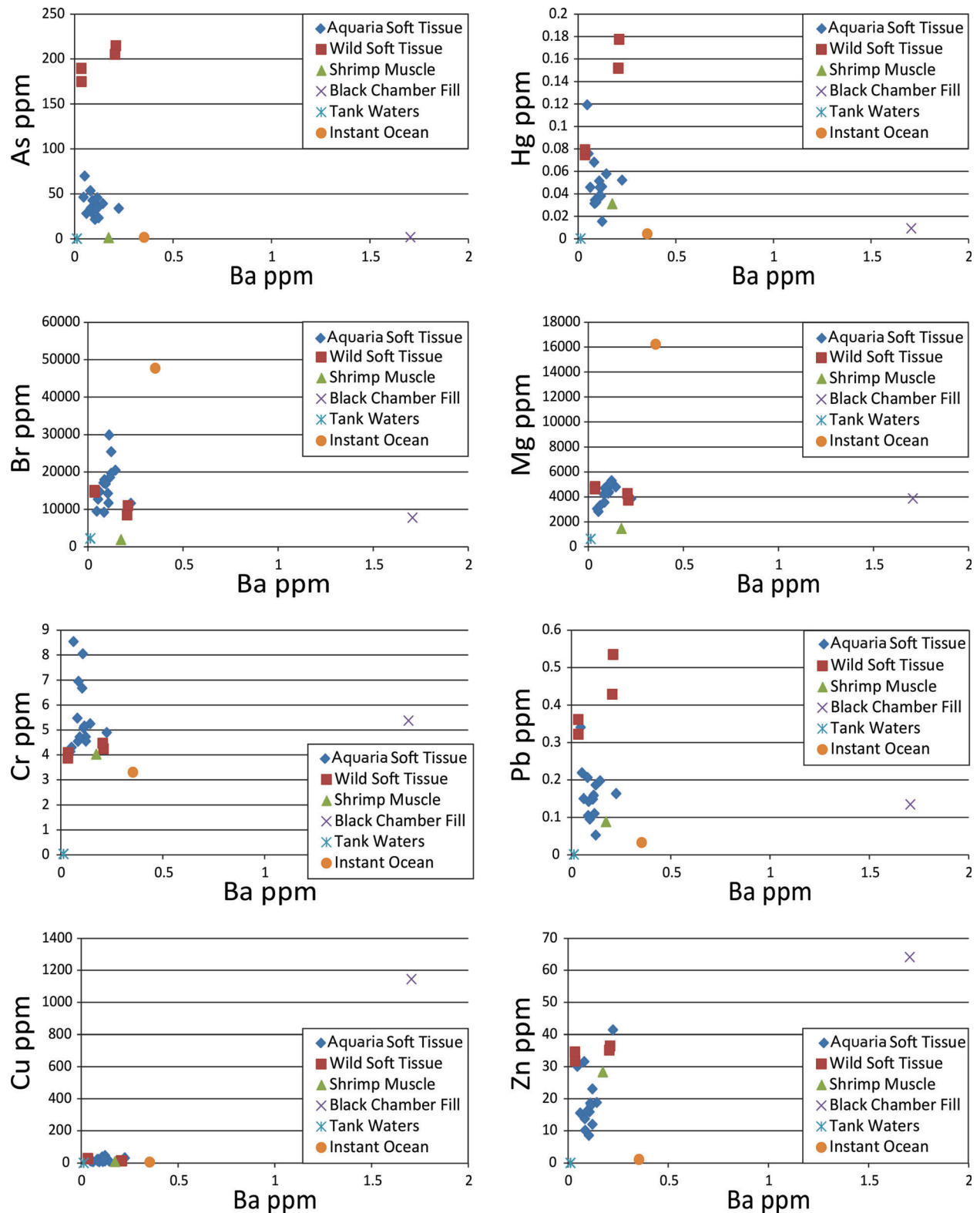


Fig. 4. Inductively coupled plasma mass spectrometry (ICP-MS) of aquaria soft tissue, wild soft tissue, shrimp muscle, black chamber fill, tank waters, and Instant Ocean[®] shows high levels of Cu, and Zn in black shell formed in aquaria (Black Chamber Fill). Mg, Cu, and Zn concentrations were similar between wild and aquaria grown soft tissue.

for high levels of Zn and Cu that are observed in the aquaria vs. wild black shell (see subsequent sections).

Elemental Analyses—ICP-MS of Soft Tissues

Hg, As, and Pb had higher ($\sim 2\times$) concentrations in wild ocean grown soft tissue, while aquaria grown tissue only had slightly higher concentration of Cr (Fig. 4). However, since aquaria *Nautilus* diet (food, tank water, and Instant Ocean) did not show high levels of Cr, a physiological biomagnification effect could be the cause of the increased levels of Cr in aquaria grown *Nautilus* tissue. Mg, Cu, and Zn concentrations were similar between wild and aquaria *Nautilus* tissues (Fig. 4). Compared to wild ocean grown tissue, Br showed a slightly increased concentration ($\sim 2\times$) in some of the aquaria grown *Nautilus* tissue. However, in some other tissue Br concentration is about the same in aquaria and the wild tissues. Therefore, it seems unlikely that Br poisoning was occurring due to the Instant Ocean[®] salt mix used at the National Zoo.

Elemental Analyses—ICP-MS of Black Chamber Fill

ICP-MS analysis of the black material found in a *Nautilus* chamber showed high levels of Cu, moderately high levels of Zn, and low levels of all other elements examined (Fig. 4).

Elemental Analyses— μ XRF Analysis of Instant Ocean

The μ XRF analysis of pure (solid) Instant Ocean[®] showed higher levels of Ca, Br, and Sr, with the Br concentration the highest, compared to the Bay Sea Salt.

Elemental Analyses— μ XRF Analyses of Wild White Shell, Aquaria White, and Aquaria Black Shells

The μ XRF analyses of wild white non-pigmented shell areas, aquaria white non-pigmented areas, and abnormal black shell areas showed that S, Cl, Fe, Cu, Zn, and Br concentrations were much higher in the aquaria-formed shell (Fig. 5). Since relatively low levels of Cu and Zn were obtained for food and water, the food source is not the likely cause of elevated Cu and Zn in aquaria-formed shell. The previously discussed stable isotope evidence supports the hypothesis that diet is not the source of elemental anomalies in the captive shell. Rather, a physiological biomagnification process is responsible. The case for Br is not as straight forward. While the μ XRF showed a high level of Br in aquaria shells, ICP-MS showed low levels of Br in these shells. Moreover, the level of Br was also high in Instant Ocean[®] water. To confirm the existence of high levels of Cu and Zn and to resolve the discrepancy between the ICP-MS and the μ XRF data regarding Br levels in black aquaria-formed shell, fragments of the three following samples were analyzed by X-ray microanalysis in the SEM: 09-0216 (blackened material from aquaria-formed shell specimen), 60-0f-C (aquaria-formed shell specimen), and 60-0f-W (wild-formed shell specimen). Blackened material from 09-0216 has a spectrum typical for CaCO_3 , but a number of elements are present at minor-trace concentration levels (Fig. 6). Figure 6 (insets) shows the expanded 1–3 keV range of X-ray peaks for Mg, Br, Sr, P, S, and Cl, and a set of deconvolved Gaussian peaks fitted to a background

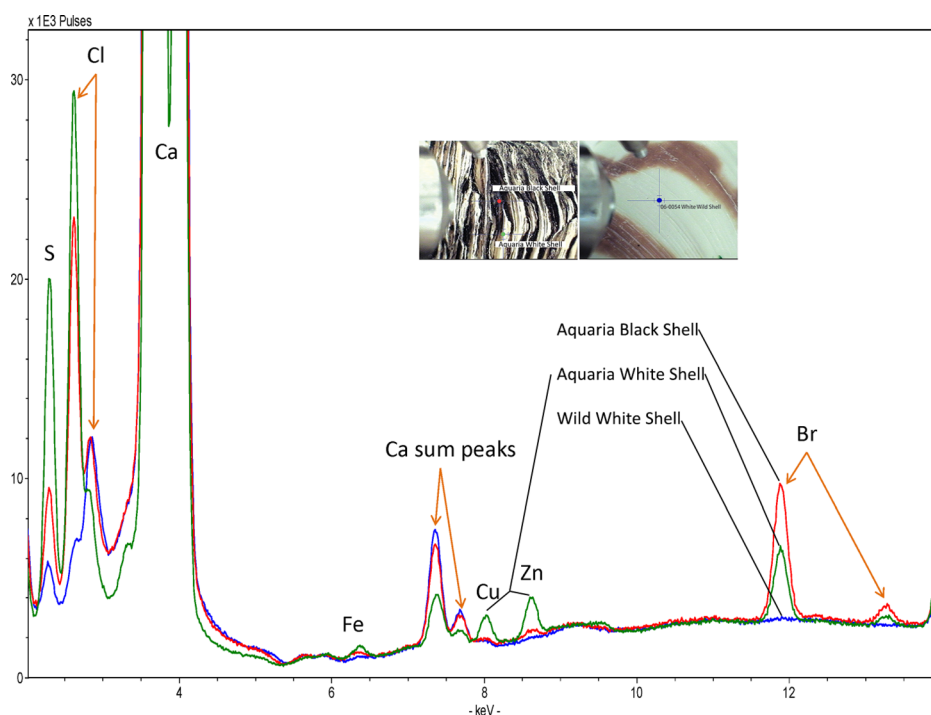


Fig. 5. Micro x-ray fluorescence (μ XRF) show levels of copper (Cu), Zinc (Zn), and Bromine (Br) in three different areas of the *Nautilus*: white wild formed shell, white aquaria formed shell, and blackened aquaria formed shell. Insets show the spots where the analyses were performed.

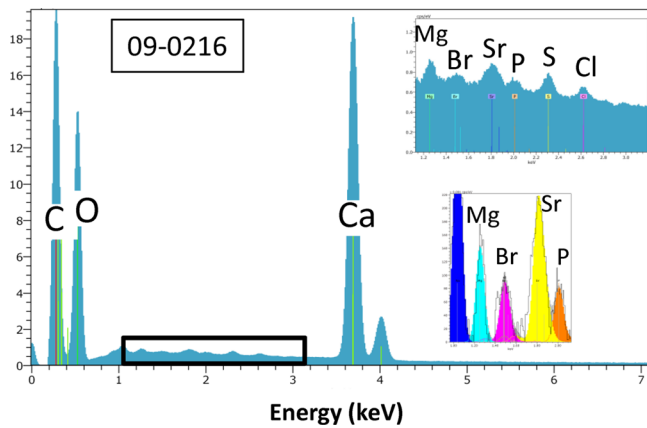


Fig. 6. X-ray microanalysis spectrum for specimen 09-0216 show elements present in Nautilus shell with high levels of Mg and Sr, and the presence of Br. Upper inset is an expanded view for minor to trace element peaks of interest in the 1–3 keV range. Lower inset shows minor and trace elements after subtracting background and Gaussian deconvolution to enhance peaks.

subtracted spectrum. Mg and Sr were likely substitutes for Ca in the structure of carbonate. It is less clear what the disposition of other elements (e.g., Br, P, S, and Cl) was and how they were sited in the material. While the abundance of some elements was rather low for an SEM-EDS measurement, careful background fitting and peak deconvolution yielded a Br concentration of 0.14 ± 0.06 wt%, 2σ for example. This experiment, therefore, confirmed the existence of Br during aquaria shell formation. However, the concentration levels and their uncertainties are further regarded as broad estimates, given the three-dimensional nature of the fragments analyzed and the lack of a mechanically polished surface typically used for high accuracy quantification of SEM-EDS spectra.

SEM Structural Analysis of Shells

SEM images obtained from the aquaria and wild portions of *Nautilus* shell are shown in Figure 7. The wild-formed shell showed an ordered crystalline structure while the aquaria-formed shell had a less ordered, poorly defined, seemingly amorphous crystalline structure. SEM-EDS analyses of these areas also revealed significant differences in elemental composition. Aquaria-formed shell areas exhibited 2–3 times higher Na, P, and K and much lower concentrations of Ca and Mg, which play a significant role in shell formation.

DISCUSSION

The results of the stable isotope analyses documented $\delta^{18}\text{O}$ enrichment of water at lower latitudes (i.e., Philippines) compared to higher latitudes (i.e., Washington, DC) could be responsible for the observed depletion upon entering the aquaria assuming the tanks used a local water source [Fricke and O'Neil, 1999; Bowen and Wilkinson, 2002]. Aquaria

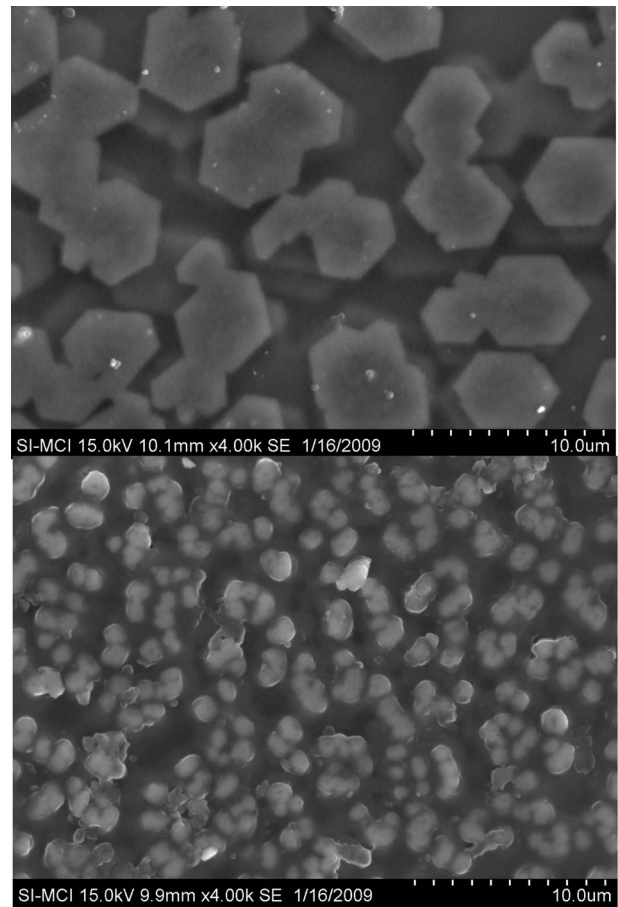


Fig. 7. Scanning electron microscopy (SEM) images show the orderly crystalline structure of the shell in the wild formed Nautilus shell (Top) and the disorderly crystalline structure of the aquaria formed Nautilus shell (Bottom).

waters would then have different baseline $\delta^{18}\text{O}$ values and possibly also different baseline $\delta^{13}\text{C}$ values in the dissolved inorganic carbonate (DIC) which are used to form the shell aragonite. Specific measurements of $\delta^{13}\text{C}_{\text{DIC}}$ in both natural environments and aquaria would be necessary to confirm this.

An alternate explanation for the observed oxygen isotope depletions within individuals' aquaria-formed and wild-formed shells is the potential occurrence of temperature-dependent kinetic fractionation effects during shell formation. *Nautilus* may experience different temperatures or temperature fluctuation patterns in the wild versus aquaria habitats resulting in different isotopic fractionations. The extreme nature of an estimated temperature increase of $\sim 22^\circ\text{C}$ upon entering the aquaria demonstrates a shift between wild and aquaria habitats not expected since aquarist aim to maintain physical conditions that mimic the range found in nature. This suggests any effects from differing temperatures between the wild environment and the regulated aquaria may be overshadowed by the previously mentioned changes in the baseline water isotope values.

The variable $\delta^{18}\text{O}$ values in wild shell are noticeably more cyclic than the observed pattern in aquaria-formed shell.

Our sampling protocol captures a homogenized value within each septa deposited over the course of several weeks to almost a year [Cochran et al., 1981; Ward, 1985; Landman et al., 1989; Westermann et al., 2004]. Changes in the baseline $\delta^{18}\text{O}$ value of the ocean water during this limited timeframe are unlikely, thus rendering temperature change as the most probable factor mitigating the observed variations in wild shell. The wild-formed shell $\delta^{18}\text{O}$ value range indicates a $\sim 9^\circ\text{C}$ variation over various periods in the animals' lives in the wild (minimum 4.9°C variation observed in specimen 09-0250; maximum 11.5°C variation observed in specimen 10-0053). It is not clear whether the temperature changes in natural shell are due to daily migrations to shallower waters, latitudinal movement, or seasonal changes experienced by the *Nautilus*. *Nautilus* is well suited to vertical movement and to a lesser degree geographical dispersal [Saunders, 1987]. Curiously, *Nautilus* incubated in aquaria for nearly 1 year under constant, moderate water temperatures (ca. 22°C) emerge from their egg capsules with apparently normal shells lacking any obvious black lines (Bruce Carlson, personal communication). The frequency and timing of the cyclic temperature change patterns will require a separate study to uncover the details of the conditions *Nautilus* naturally experience.

Mollusca typically do not demonstrate any kind of metabolic fractionation effect [Auclair et al., 2004; Lecuyer et al., 2004]. It is, however, possible that the physiological mechanism controlling fractionation is affected by environmental changes in the aquaria such that the overall metabolic fractionation changes after entering aquaria. This hypothesis may be valid, considering that the variability of $\delta^{18}\text{O}$ values in aquaria is approximately equal to that in the wild, despite tank temperatures that are more constant than temperatures experienced in the wild. Furthermore, the shifts in $\delta^{18}\text{O}$ in aquaria are apparently random suggesting a human-induced influence as opposed to a cyclic, natural environmental fluctuation. Further controlled studies comparing water temperature, $\delta^{18}\text{O}$ water, and $\delta^{18}\text{O}$ of *Nautilus* shell material is needed to examine the effects of aquarium residency on kinetic and metabolic fractionation.

The results of the stable isotope analysis and elemental analysis of the tank water and food sources support the hypothesis that *Nautilus* diet is not the major cause of shell deformation in aquaria. Changes in nitrogen and carbon isotopic values during an animal's lifespan typically indicate a change in diet or changing position in the food web [DeNiro and Epstein, 1978; DeNiro and Epstein, 1981; Peterson and Fry, 1987; Ambrose, 1993; Koch et al., 1994]. The consistent $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values in the sequential samples thereby indicate that this *Nautilus* most likely did not transition to a vastly different diet in aquaria. However, the $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ offset of isotope values between food source and *Nautilus* tissue is $\sim 8\text{‰}$ and $\sim 5\text{‰}$, respectively, for aquaria *Nautilus* which is greater than typically observed in most natural aquatic ecosystems [Minagawa and Wada, 1984; Fry, 1988; Post, 2002]. The offsets for wild *Nautilus* in this study are $< 2\text{‰}$, a value which is more typical for marine animals. This

suggests that the aquaria diet has a different baseline isotope signature than that consumed in the wild, but the *Nautilus* tissue may not be turning over quickly enough to record the change or such changes do not transmit to the organic shell material. If dietary changes between wild and aquaria environments are not transmitting to the organic shell material, this may imply that other elemental anomalies in the shell, such as elevated heavy metal levels, do not originate from the captive diet either. In the case of heavy metals, the observed increases in aquaria-formed shell could not, therefore, be attributed to the concentrating of metals in higher levels of the food chain, a phenomenon which is common in marine ecosystems.

Moreover, the results of ICP-MS, μXRF , and SEM based x-ray microanalysis confirm higher levels of Na, P, K, Cu, Zn, and Br, and lower levels of Ca and Mg in aquaria shell formation relative to wild shell formation. Elevated levels of Cu, Zn, and Br in aquaria-formed shell are of interest. Bromine has been shown to play an important role in cuticles [Robert et al., 2009]. Br-rich tips of calcified crab claws are less hard but more fracture resistant, and brominated cuticles can withstand a greater deformation before fracture [Robert et al., 2009]. One can also consider the role of metals in bone mineralization, which has been examined more to provide insight into deformation during concurrent deposition of organic protein and inorganic mineral phases in the presence of elevated Br, Zn, and Cu in Mollusca. Brominated tyrosine has been demonstrated to play a role in protein cross-linking in bones [Robert et al., 2009]. This crosslinking strengthens the bone and may do the same for shell formation. Zinc is an essential trace element required for bone formation [Seo et al., 2010] and possibly shell formation [Mai et al., 2003]. Zn has a stimulatory effect on bone formation and mineralization both in vivo and vitro. It stimulates collagen production, decreases bone resorption, and stimulates alkaline phosphatase (ALP) activity [Seo et al., 2010], all of which enhances the biomineralization process. Overgrowth of *Nautilus* shells has been observed in aquaria such that the leading edge of the shell begins to curve back towards the posterior of the animal. Similarly, copper-containing proteins are also involved in bone formation and are critical for proper cross-linkages in bone proteins [Opsahl et al., 1982]. SEM-based microanalysis data demonstrate lower Ca and Mg concentrations in aquaria-formed shell. In light of the poorly-defined crystalline structure of the aquaria-formed shell this is of particular interest and may imply that, under aquaria conditions these organisms are simply not able to process these essential elements for proper shell formation. Alternately, these elements may not be bioavailable in the quantities necessary to sustain normal shell formation within the aquaria organism. As a result, trace elements such as Br, Zn, and Cu may be biomagnified by the organism in an attempt to compensate for these other chemical deficiencies. The trace metals are a response to deformation rather than being causative. The insufficient levels of Ca and Mg may trigger a response whereby the organism attempts to strengthen its shell by other means, resulting in a

biomineralization process that is out of physical and chemical equilibrium, resulting in a deformed shell structure. Future work in this area includes the analysis of shell proteins (proteomics) to determine if irregularities arise in aquaria-formed shell during biomineralization.

The source of the elevated Cu, Zn, and Br, and decreased Ca and Mg in aquaria-formed shell does not appear to be related to diet nor tank water chemistry. It is reasonable to suspect that environmental conditions in aquaria such as temperature, pressure, and UV light, not studied here, may play an important role in shell deformation in aquaria *Nautilus*. Future work will examine the effect of these environmental stresses on the shell deformation process.

CONCLUSIONS

Results presented here indicate that the irregular black material deposits forming shell lines contain excess copper, zinc, and bromine. Environmental stress such as changed pressure, temperature, and UV light patterns may cause the animal to reinforce its aquaria formed shell owing to failure to achieve the robust crystalline structure formed under wild natural conditions. Moreover, high copper, zinc, and bromine contents in the black material found in aquaria shell formation point to differences in the role that proteins play in the construction of the shell. Aquaria conditions that have developed to maintain *Nautilus* will better approximate wild conditions as new information about the natural temperature fluctuations and patterns are revealed by building upon this work. Measuring impacts of changing environmental conditions will help us determine the variability that *Nautilus* naturally experience and how changes in the wild may affect their survival in the future. To address these questions, we plan to study the effect of environmental factors such as temperature and light on the production of deformed black areas of *Nautilus* shell formation in aquaria. We also intend to compare the proteomics of *Nautilus* shell areas formed in wild versus aquaria conditions.

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