Biogeosciences
Discussions
Open Access
EGU

Assessing annual variability in the shell thickness of the pteropod *Heliconoides inflatus* in the Cariaco Basin using micro-CT scanning

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Abstract

Pteropods have been nicknamed the 'canary in the coal mine' for ocean acidification because they are predicted to be among the first organisms to be affected by future changes in ocean chemistry. This is due to their fragile, aragonitic shells and high abundances in polar and sub-polar regions where the impacts of ocean acidification will manifest first. For pteropods to be

5 used most effectively as indicators of ocean acidification, their natural variability in the modern ocean needs to be quantified and understood. Here, we measured the shell condition (i.e., the degree to which a shell has dissolved) and shell characteristics, including size, number of whorls, shell thickness, and shell volume (i.e., amount of shell material) of nearly fifty specimens of the pteropod species *Heliconoides inflatus* from a sediment trap in the Cariaco Basin, Venezuela sampled over an 11-month period. The water in the Cariaco Basin is supersaturated with respect to aragonite year-round, and hydrographic and chemical

10 properties vary seasonally due to the movement of the Inter Tropical Convergence Zone (ITCZ). Shell condition was assessed using with two methods: the *Limacina* Dissolution Index (LDX) and the opacity method. The opacity method captured changes in shell condition only in the early stages of dissolution, whereas the LDX recorded dissolution changes over a much larger range. Shell condition did not deteriorate with the length of time in the sediment trap. Instead, the most altered shells occurred in samples collected in September and October when water temperatures were warmest, and the amount of organic matter

15 degradation in the water column was likely to have been the greatest. Shells of *H. inflatus* varied in size, number of whorls, and thickness throughout the year. The number of whorls did not correlate with shell diameter, suggesting that shell growth is plastic. *H. inflatus* formed shells that were 40% thicker and 20% larger in diameter when nutrient concentrations were high during times of upwelling, compared to specimens sampled from the oligotrophic rainy season. This study produces a baseline dataset of the variability in shell characteristics of *H. inflatus* in the Cariaco Basin and establishes a methodology for generating

20 similar baseline records for pteropod populations globally.

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1 Introduction

The global ocean has absorbed over a third of anthropogenic carbon dioxide emissions since the industrial revolution (Sabine et al., 2004, Gruber et al., 2009). This has caused the chemistry of the oceans to change, decreasing both the pH and the concentration of carbonate ions in seawater. The impact of this decrease in carbonate ion concentration on mineral formation

5   can be expressed using the saturation state equation of Broecker and Peng (1982):

$$\Omega = \frac{[Ca^{2+}]_{SW} \times [CO_3^{2-}]_{SW}}{[Ca^{2+}]_{saturation} \times [CO_3^{2-}]_{saturation}}$$

where Ω is the calculated saturation state, $[Ca^{2+}]$ is the concentration of calcium ions, $[CO_3^{2-}]$ is the concentration of carbonate ions, and *SW* is seawater. At Ω of greater than one, the seawater is supersaturated with respect to the mineral, and at values

10   less than one, seawater is undersaturated with respect to the mineral, causing it to be chemically unstable.

Recent studies have proposed that biological indicators of carbonate undersaturated waters can be used to monitor future changes in ocean chemistry (Bednaršek et al., 2017, 2019; Gaylord et al., 2018; Marshall et al., 2019). Establishing biological indicators is complicated because organisms are exposed to a multitude of variability in oceanic conditions, from temperature

15   and salinity to carbonate saturation levels and nutrient concentrations, on diurnal, seasonal, and annual timescales. All of these variables have been shown to impact shell growth in calcareous organisms (e.g. Comeau et al., 2009, 2010; Hettinger et al., 2013; Hiebenthal et al., 2011; Joubert et al., 2014; Meinecke and Wefer, 1990; Melzner et al., 2011) and it is therefore crucial that organisms' natural variability in response to environmental fluctuations is understood prior to their use as indicators of changes in ocean chemistry.

20   ### 1.1 Understanding natural pteropod variability

Pteropods are a group of pelagic molluscs that have been proposed as biological indicators of ocean acidification (Bednaršek et al. 2014a, 2017, 2019). They form their thin (10–15 µm) shells from the mineral aragonite, a more soluble form of calcium carbonate (Mucci, 1983), and therefore are at a greater risk from ocean acidification than organisms with calcitic shells (Fabry, 2008, Orr et al., 2005). Pteropods are protandric hermaphrodites, meaning they transition from juveniles, to mature males, to

25   females during ontogeny (Lalli and Wells, 1978). Their lifespans are thought to be between 0.5 and 2 years (Gannefors et al., 2005; Hunt et al., 2008; Kobayashi, 1974; Wang et al., 2017; Wells, 1976a). Isotopic studies have found that pteropods calcify between 50 and 650 m depth (Fabry and Deuser, 1992; Juranek et al., 2003; Keul et al., 2017) suggesting that they are exposed to a wide range of water chemistries as they migrate through the water column diurnally. Pteropods are also key components of the marine food web, feeding on phytoplankton and small zooplankton (Gilmer and Harbison, 1986, 1991; Lalli and Gilmer,

30   1989), and being consumed by zooplankton, krill, fish, and seabirds (Doubleday and Hopcroft, 2014; Foster and Montgomery, 1993; Hunt et al., 2008; Karnovsky et al., 2008; Pakhomov et al., 1996; Willette et al., 2001).

Because of their sensitivity to ocean acidification, there has been a significant increase in research on this group over the past decades, including incubation experiments, studies on natural CO_2 gradients, and descriptions of the genetic variability within natural populations (c.f. Manno et al., 2017). The impact of predicted future conditions on live specimens has been assessed using wide variety of parameters, including calcification (Comeau et al., 2009, 2010; Maas et al., 2018; Moya et al., 2016),

5   shell degradation (Bednaršek et al., 2012b; Bergan et al., 2017; Lischka and Riebesell, 2012), metabolic rates (Maas et al., 2011; Seibel et al., 2012), respiration (Comeau et al., 2010; Maas et al., 2018; Moya et al., 2016), and gene expression patterns (Koh et al., 2015; Maas et al., 2015, 2018; Moya et al., 2016; Thabet et al., 2017). Generally, previous studies have found that as the aragonite saturation state decreases, pteropod calcification rates decrease (Comeau et al., 2010, 2009; Lischka and Riebesell, 2012). This decreased calcification may be manifested in the formation of smaller, thinner, or more porous shells

10   (Bednaršek et al., 2017, 2019; Roger et al., 2012).

Although much has been learned about the response of pteropods to acidification, there are still fundamental processes that remain incompletely understood, including how shell characteristics, such as shell thickness or shell diameter, change through ontogeny, and whether these parameters are affected by ocean chemistry. This work is hampered because pteropods are

15   difficult to culture (Howes et al., 2014), with only one study reporting successfully rearing a captive generation (Thabet et al., 2015). Understanding how shell shape and size change through ontogeny is instead based on measurements from repetitive sampling of natural populations (Hsiao, 1939; Redfield, 1939; Wells, 1976b), and on the diversity of shells in the sedimentary record (Janssen, 1990).

20   Pteropod samples can be collected live, using plankton nets, or dead, in sediment traps. Although net catches have the advantage of sampling pteropod populations at the time of collection, they only represent a snapshot in time. Pteropods have patchy distributions (Bednaršek et al., 2012a; Thibodeau and Steinberg, 2018; Wang et al., 2017), and therefore pteropod yields in net samples are highly variable. Sediment traps use a large, upward facing cone to collect the flux of organic and inorganic particles that sink though the water column into collection cups containing preservative. These collection cups are

25   automatically closed and switched out on a regular basis (i.e., every two weeks or every month) which enables the flux of particles in the water column, including dead plankton, to be continuously sampled over a longer period than is possible via net catches. The disadvantage of sediment traps is that there can be alteration to the shells in the sediment trap cup, due to decay and the preservation solution. For example, a study by Oakes et al. (2018) found that when left in mercuric chloride or formalin, the most common solutions used in sediment trap studies (e.g., Collier et al., 2000; Manno et al., 2007; Meinecke

30   and Wefer, 1990; Mohan et al., 2006; Singh and Conan, 2008), pteropod shells underwent dissolution over the study period of 15 months. The condition of shells from sediment traps must, therefore, be interpreted in the context of both water column properties and potential breakdown during the time they are in the collection cup.

1.2 The CARIACO Time Series

The Cariaco Basin is a tectonic depression on the Venezuelan shelf (Fig. 1) separated from the Caribbean Sea by a shallow sill (~140 m) meaning the deep waters of the basin are permanently anoxic (Muller-Karger et al., 2001). The surface water conditions in the Cariaco Basin vary seasonally with the migration of the Inter Tropical Convergence Zone (ITCZ). During

5   the winter and spring (Dec – Apr), the ITCZ moves south, the Easterly trade winds are strong (>6 m s^{-1}), and Ekman transport causes coastal upwelling, bringing cold, high salinity water to the surface (Astor et al., 2003, 2013). During the summer and fall (Aug – Nov), the ITCZ moves north, causing winds to weaken and rainy conditions to become pervasive; there is no upwelling, and surface waters are warm, oligotrophic, and lower salinity relative to the upwelling season (Astor et al., 2013; Muller-Karger et al., 2019).

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The CARIACO (Carbon Retention In A Colored Ocean) project was a time-series study that ran from 1995–2017 to measure the relationships among physical and biological processes in the Cariaco Basin, Venezuela. The CARIACO time series coupled bi-weekly sediment trap samples with monthly oceanographic cruises to measure hydrography, nutrient concentrations, and biogeochemical parameters (c.f. Muller-Karger et al., 2019). There have been numerous studies of planktonic foraminifera

15   from the sediment trap samples, investigating their flux, their seasonal and interannual variability, and their ability to record changes in the oxygen isotopic composition and carbonate chemistry of seawater (e.g., Marshall et al., 2013, 2015; McConnell et al., 2009; Tedesco et al., 2007; Tedesco and Thunell, 2003). Despite this focus on calcareous plankton, there have not been any studies of the pteropods from the Cariaco sediment trap records.

20   The wealth of data collected during the CARIACO time series, and the seasonal variability in water column properties, makes the Cariaco Basin an ideal place to study the abiotic and biotic controls on the shell characteristics of *Heliconoides inflatus* pteropods. Temperature, salinity, nutrient concentration, and carbonate chemistry of the water column were collected as part of the CARIACO time series. To determine how changes in these water column properties affect the shells of pteropods, we assessed 50 specimens from eight sediment trap samples over an 11-month period, using a combination of light microscopy

25   and CT scanning. Light microscope images were used to assess the shell condition, i.e., the amount that the shell has dissolved. Three-dimensional CT scan data were used to analyze shell properties such as shell diameter, number of whorls, shell thickness, and amount of shell material, i.e., the total volume of carbonate in the shell. Shell diameter was used as a metric for size, and shell thickness and amount of shell material were used as metrics for calcification. Shell thickness has been used as a metric for calcification in previous studies, initially calculated from point measurements on the shell aperture from scanning electron

30   microscope images (Bednaršek et al., 2014b; Roger et al., 2012), and later measured across entire shells from CT reconstructions (Howes et al., 2017; Oakes et al., 2019; Peck et al., 2018). Here we use modal shell thickness to compare calcification among samples following the methods of Oakes et al. (2019). Although this method analyses shell thickness across the entire shell, the final, or body, whorl, composed of the most recently calcified material, is the largest portion of the

shell (Fig. S1) (Fabry and Deuser, 1992; Keul et al., 2017). This final whorl therefore comprises the majority of the shell volume and hence will dominate the modal shell thickness measurement.

2 Materials and Methods

2.1 Sediment trap collections and water column properties

5   The samples for this study come from the CARIACO Time Series trap deployed at 150 m water depth (also known as the Z trap) (10° 30.0' N, 64° 38.5' W) (Fig. 1). Sediments were collected continuously for two-week intervals in collection cups that were filled with a borate-buffered formalin solution prior to trap deployment to preserve the sample (Thunell et al., 2000). There were 13 cups in the trap and the trap was retrieved and redeployed every six months (Thunell et al., 2000). On recovery, the contents of the sediment trap cups were washed and split as described in Thunell et al. (2000) and Tedesco and Thunell

10 (2003). A quarter split was washed over a 150-micron sieve with deionized water, dried, and stored for faunal analysis.

2.2 Specimen selection

We analyzed 50 specimens of *Heliconoides inflatus* (Mollusca, Gastropoda, Euthecosomata, Limacinidae) from eight collection cup samples spanning March 2013 through February 2014 (Table 1). All whole pteropod specimens were picked from the washed and dried faunal samples by B. Marshall and C. Davis (University of South Carolina). Specimens are

15 deposited in the Malacology collection at the Academy of Natural Sciences of Drexel University, Philadelphia, PA (ANSP). Catalogue numbers can be found with sample information in Table 1.

2.3 Light microscopy

Forty-nine of the 50 shells were imaged under the light microscope in order to assess shell condition (i.e., the degree to which shells have undergone dissolution); one specimen broke after CT scanning and therefore was not imaged via light microscopy.

20 Thirty-eight of these 49 shells were imaged on a Zeiss Stemi 2000-C microscope with a Canon G9 camera in SCN mode, in the Paleoceanography Lab at the Pennsylvania State University; 11 shells were imaged on a Leica S8APO microscope with a Leica DFC HD Camera at the Academy of Natural Sciences of Drexel University. All images are available in the supplemental materials (Fig. S1).

2.4 Assessment of shell condition

25 Dissolution visibly affects the shells of pteropods, altering them from glassy and transparent when pristine, to milky-white, and then white and opaque as they dissolve (Almogi-Labin et al., 1986). The visible changes in pteropod shells have been used as a metric of dissolution. Here we assess the amount of dissolution the pteropod shells have undergone, hereafter referred to as the shell condition, using two methods: the *Limacina* Dissolution Index, and the opacity method. The *Limacina* Dissolution Index (LDX) is designed to assess the extent of dissolution in pteropods from the fossil record via a scale from 0 (pristine

shell) to 5 (highly dissolved shell) based on observations made using a light microscope (Gerhardt et al., 2000; Gerhardt and Henrich, 2001). The opacity method (Bergan et al., 2017) was designed to quantify small changes in shell dissolution by measuring the greyscale values of light microscope images of a shell relative to a black background to determine how much light is able to pass through the shell. A pristine shell will have a low opacity (~0 – 0.25), as the background will be visible

5   through transparent shell, and a highly altered shell will have a high opacity score (~0.5 – 0.7) as the opaque shell will block light from travelling through the shell. The shells in this study were analyzed by Oakes using both the LDX and opacity methods.

2.5 CT scanning

2.5.1 CT data collection

10   Forty-four of the 50 *H. inflatus* specimens were CT scanned (Table 1). The remaining six shells fragmented or broke completely prior to CT scanning. CT scanning was conducted using two different CT scanners due to scanner availability (Table S1). Thirty-one specimens were scanned at General Electric Inspection Technology, Lewistown, PA using a GE phoenix v|tome|x m micro-CT system (General Electric, Fairfield, CT, USA). Specimens were scanned at a resolution of 1–2 µm/voxel using the 180kV nanofocus tube with a diamond target and a beam energy of 65 kV and 230 µA. X-ray radiographs

15   were collected with 500 ms exposure times and five radiographs were collected and averaged (average 5, skip 1) at 1000 projections around the specimen, yielding an overall scan time of 50 minutes. Because of the closure of the GE facility, the remaining 13 specimens were scanned at the Microscopy and Imaging Facility and the American Museum of Natural History, New York, NY using a GE phoenix v|tome|x s 240 dual tube 240/180 kV system (General Electric, Fairfield, CT, USA). Specimens were scanned at resolution of 1–2 µm/voxel using the 180 kV nanofocus tube with a diamond target and a beam

20   energy of 65 kV and 230 µA. X-ray radiographs were collected with 400 ms exposure times and three radiographs were collected and averaged (average 3, skip 1) at 1500 projections around the specimen yielding an overall scan time of 40 minutes.

Ideally, all scans would have been conducted with the same equipment and parameters, but the GE facility closure, and limited scan time availability at the AMNH, resulted in reducing the total scan time from 50 minutes at the GE facility to 40 minutes

25   at the AMNH in order to scan the greatest number of shells possible. To assess the impact of using both different scanners and scan parameters on the calculated modal shell thickness, a key measurement used in this study, one specimen was scanned four times: 1) original scan at GE; 2) scan at AMNH; 3) re-scan at AMNH; 4) rescan at AMNH using scan parameters from GE (Table S2; see supplemental materials for further details). Although there were minor variations among scans (Fig. S2), the modal shell thickness calculated for all four scans was 0.008 mm. This demonstrates that modal shell thickness is a robust

30   metric and was not impacted by the different scanners, scan parameters, or scan times used in this study.

2.5.2 CT data processing

All CT data were reconstructed using *datos|x* v. 2 (General Electric, Wunstorf, Germany) and analyzed using the *VGStudio MAX* v. 3.1 (Volume Graphics, Heidelberg, Germany). Shell material was differentiated from background using the automatic surface determination module. Some shells were filled with other material, such as foraminifera tests or sediment. To ensure

5 that only the shell of the pteropod was analyzed, a region of interest (ROI) was created from the surface and non-pteropod shell material was manually removed from the ROI. The resulting surface was exported as a *.DICOM image stack. The volume, or amount of pteropod shell material, was calculated using the properties tool in VG Studio MAX v. 3.1.

Data were visualized in Avizo v. 9.4.1. Shell diameter was measured at the widest part of the shell following the methods of

10 Lischka et al., (2011) using the caliper tool in Avizo v. 9.4.1 (Fig. 2). The number of whorls were counted to the nearest eighth of a whorl following the method of Janssen (2007) (Fig. 2). Shell thickness was measured using the BoneJ plugin (Doube et al., 2010; Hildebrand and Rüegsegger, 1997) in ImageJ (Schneider et al., 2012) following the methods of Oakes et al. (2019).

2.6 Seawater Chemistry

Water chemistry was analyzed monthly as part of the Cariaco Basin ocean time series program. These data are publicly

15 available at *http://imars.marine.usf.edu/WebPageData_CARIACO/Master_Hydrography/*. Water samples were collected at discrete intervals to measure nutrient concentrations and carbonate chemistry parameters, the details of which can be found in Astor et al. (2011). There are 12 water sampling datasets that span the duration of this study (March 2013 – February 2014). Aragonite saturation (Ω_{arag}) was calculated indirectly from the pH and total alkalinity (TA) data from the timeseries using CO2SYS (Pierrot et al., 2006). Carbonate dissociation constants were used from Mehrbach et al. (1973) as refitted by Dickson

20 and Millero (1987).

2.7 Statistical analyses

Relationships among shell parameters (whorls, diameter, amount of shell material, and shell condition via LDX) were examined relative to each other using a simple linear model in the computing language R, version 3.6.0 (R Core Team, 2019) using the RStudio interface (RStudio Team, 2016). To account for running multiple comparisons, *p*-values were corrected

25 using both the more conservative Bonferroni correction, and the less conservative false discovery rate (FDR) (Benjamini and Hochberg, 1995). The R^2, Bonferroni-adjusted p-value (*p* Bon.), and FDR-adjusted p-value (*p* FDR) are reported for each comparison in the text and in Table S5.

3 Results

Pteropod shell condition varied throughout the course of the experiment, with LDX rankings ranging from 0 (pristine, transparent and lustrous shell) to 4 (shell highly altered, opaque-white and lusterless shell with surface layer dissolution) and shell opacity values ranging between 0.17 (pristine, transparent shell) and 0.74 (highly altered opaque, white shell) (Table S3).

5 Shell condition did not deteriorate with the amount of time spent in the trap (Fig. 3). Although there was a statistically significant relationship ($R^2 = 0.357$, p Bon. $= 5.17*10^{-5}$, p FDR $= 1.29*10^{-5}$) between time in trap and shell condition, the trend suggests shell condition improves with the time in the trap (Fig. 3), which is opposite from the expectation that more time in trap would result in more degradation. The least well-preserved specimens came from the September and October 2013 samples (Fig. 4), and had spent a maximum of 2–6 weeks in the sediment trap cup (Fig. 3). The most well-preserved specimens

10 came from June and December 2013 and had spent a maximum of 20 – 22 weeks in the sediment trap cup (Figs. 3, 4).

The pteropod shells varied in number of whorls, diameter, amount of shell material, and modal shell thickness both within and among samples throughout the year in the Cariaco Basin (Fig. 5, Table S1). The number of whorls varied between 2 1/4 and 2 7/8, and displayed no overall trend through the 11-month study (Fig. 5 a; Table S1). The relationship between shell diameter

15 varied in samples collected through the year: specimens from March 2013 had the greatest shell diameters (average 1.70 mm), and shells in the rest of the study period (June 2013 – February 2014) ranged from 0.68 to 1.40 mm in diameter with an average of 0.98 mm. The amount of shell material followed a similar pattern to shell diameter, with specimens from March 2013 sample containing the greatest amount of shell material (0.104 mm^3) and specimens from the June 2013 – February 2014 ranging from 0.005 to 0.038 mm^3, with an average amount of 0.021 mm^3. The modal thickness of the shells of *H. inflatus* also varied through

20 the year (Fig. 5 d; Table S1). The thickest shells were sampled in March 2013, with an average modal shell thickness of 0.018 mm, and the thinnest shells were sampled in September 2013, with an average modal shell thickness of 0.009 mm (Fig. 5 d). As shell diameter, thickness, and amount of shell material are related to size, unsurprisingly there were significant correlations between shell diameter and amount of shell material ($R^2 = 0.819$, p Bon. $= 1.98*10^{-15}$, p FDR $= 2.86*10^{-15}$), shell diameter and shell thickness ($R^2 = 0.582$, p Bon. $= 1.61*10^{-8}$, p FDR $= 5.37*10^{-9}$), and shell thickness and amount of shell material ($R^2 =$

25 0.680, p Bon. $= 5.48*10^{-11}$, p FDR $= 2.74*10^{-11}$). These results highlight that larger shells are generally thicker and contain more shell material.

The modal shell thickness of the specimens, used in this study as a calcification metric, was analyzed with respect to the water column properties in the Cariaco Basin (Fig. 6; Table S4). Water chemistry measurements from 55 m depth were used because

30 this was the closest water sample to the most recent of *H. inflatus* calcification depth estimate of 75 m (Keul et al., 2017). The Cariaco Basin was supersaturated with respect to aragonite throughout the studied interval (Ω_{arag} range 2.28 – 3.59), and the thickest shells formed when the aragonite saturation was the lowest (March 2013, Dec 2013 – Feb 2014; average Ω_{arag} 2.49) (Fig. 6 c; Table S4). Specimens collected during the upwelling season (December – April) were compared to those from the

rainy season when there was no upwelling (August – November). A Welch's *t*-test was used to compare specimens from the upwelling season to those from the rainy season, because the two groups had different variances and unequal sample sizes, prohibiting the use of a Student's *t*-test (Revelle, 2018). Pteropod shells were 40% thicker during the upwelling season, when water temperatures were lower and nutrient concentrations were higher, than during the rainy season when oligotrophic

5   conditions prevail (Welch's *t*-test: $p = 4.41 \times 10^{-4}$; Table S6; Figs. 6, 7). Pteropod shell diameters were also 20% larger during the upwelling season than during the rainy season (Welch's *t*-test: $p = 0.0080$; Table S6).

Because shell diameter and shell thickness are related to the overall size of a specimen, the influence of shell diameter on shell thickness was removed using a simple linear regression model of thickness as a function of diameter. Analysis of the residuals

10   of this model, hereafter referred to as 'residual thickness', found that specimens sampled during the upwelling season had significantly higher residual thicknesses than those sampled during the rainy season (Welch's *t*-test: $p = 0.0260$; Figure S3; Table S6), indicating that water column properties impact calcification regardless of shell size.

4 Discussion

4.1 Shell condition

15   This study focuses on how the interplay of biotic and abiotic factors impacts the shell characteristics of the pteropod *H. inflatus* in the Cariaco Basin. The specimens used in this study were collected using a sediment trap, adding a third variable, taphonomy. Pteropod shell condition was assessed using both the LDX (Gerhardt et al., 2000; Gerhardt and Henrich, 2001) and opacity (Bergan et al., 2017) methods. By comparing the results from these two methods, we found that the opacity scale lacked sensitivity to changes in shell condition at LDX values of 2 (opaque white shells with lustrous surface) and higher (Fig.

20   8). When pteropod shells dissolve, the shell transparency changes first, from transparent, to milky-white, to opaque-white, followed by the surface texture (Gerhardt and Henrich, 2001). Because the opacity method is based on greyscale values of the light microscope images, it quantifies the change in shell color but not texture, meaning this method is only sensitive to shell condition changes in the early stages of dissolution (LDX stages 0 – 2; Fig. 8). Since the opacity method was designed to assess pteropods from an incubation experiment, it was designed to capture the earliest stages of dissolution (Bergan et al.,

25   2017). Because of the wide range of shell conditions of the specimens in this study, spanning both changes in color and texture, all shell condition analyses are based on LDX measurements.

The borate-buffered formalin solution used to preserve sediment trap samples has been shown to influence the condition of pteropod shells (Oakes et al., 2018). The impact of preservation method on pteropod shell condition in this study was

30   determined by comparing the time spent in the sediment trap with the condition of the shells (Fig. 3). We found that shell condition did not deteriorate with time spent in the sediment trap cups. This suggests that the preservative in the collection cups effectively minimized post-collection sample degradation and that any changes in shell condition likely happened on

timescales of 2 weeks or less, the amount of time the specimens were in the final sediment trap collection cup before trap recovery.

Pteropod shell condition did vary among samples, with the most alteration occurring in specimens from the September – October 2013 samples (Fig. 4). As the time in trap did not deteriorate the condition of the shells (Fig. 3), shell condition was likely influenced by water column conditions. During September and October, water temperatures at 55 m were at their highest (Fig. 6 b). These warm temperatures would have increased the rate of microbial breakdown of organic matter within the shell, linked to the decaying body of the pteropod (Oakes et al., 2019), and in free floating decaying material in the water column (Lohmann, 1995; Milliman et al., 1999; Schiebel et al., 2007). This decay may have created undersaturated microenvironments in an otherwise aragonite saturated water column. The shells of the organisms that died during the warmer months likely encountered more of these aragonite undersaturated microenvironments as they fell through the water column and into the trap, increasing the rates of dissolution of these aragonitic shells of *H. inflatus* relative to those trapped in cooler months. Coincidentally, the specimens from the warmer months with poorer shell conditions were in the sediment trap cups for a short amount of time, explaining the significant trend suggesting that shell condition improves with the time in the trap (Figs. 3, 4).

4.2 Pteropod development

Assessing the number of whorls, shell diameter, amount of shell material, and shell thickness provides an integrated view of *H. inflatus* shell growth in the Cariaco Basin. The number of whorls varies both within and among samples throughout the year. The number of whorls does not correlate with shell diameter (Fig. 5 a, b), implying that *H. inflatus* displays plasticity during growth. These measurements support the observations of Janssen (1990) who found that both the number and diameter of the whorls of *H. inflatus* increase irregularly. There are no patterns showing changes in is no overall trend in *H. inflatus* shell diameter through the year in the Cariaco Basin (Fig. 5), which suggests there are no cohorts. This corroborates the work of Wells (1976a) who found that *H. inflatus* collected off the coast of Barbados reproduced throughout the year.

4.3 Pteropod growth and water column properties

Because of their shell chemistry, pteropods have been proposed as biological indicators of aragonite saturation (Bednaršek et al., 2017, 2019). In this study we used shell thickness as a metric of calcification. In the Cariaco Basin, the water is permanently supersaturated with respect to aragonite (i.e., $\Omega_{arag} > 1$). In this supersaturated setting, the thickness of pteropod shells does not correlate with aragonite saturation, and the thinnest shells were found when the aragonite saturation was the highest (Aug – Nov 2013 – average Ω_{arag} 3.26) (Fig. 6 c). Instead, the shell thickness of *H. inflatus* varies with the physical oceanographic conditions in the Cariaco Basin, with median shell thickness increasing by 40% during times of upwelling (Fig. 7), when nutrient rich waters are brought to the surface, relative to shells forming during the rainy season when there is no-upwelling and oligotrophic conditions prevail (Figs. 6, 7; Table S6) (Muller-Karger et al., 2001, 2019). The diameters of pteropod shells sampled during times of upwelling were 20% larger than those formed during the rainy season (Table S6) and the trend of

increased shell thickness during times of upwelling still holds once the influence of shell diameter on shell thickness is removed (Fig. S3, Table S6). The observed changes in *H. inflatus* modal shell thickness and diameter are therefore likely linked to changes in nutrients, and therefore food supply, in the Cariaco Basin through the year.

5   The link between food availability and shell growth has been proposed for another species of pteropod in the same family as *H. inflatus*, *Limacina retroversa*, which was found to form smaller shells when food resources were limited (Meinecke and Wefer, 1990). Furthermore, the availability of food has been found to offset, or even negate, the negative effects of increased pCO_2 levels or low pH in other groups of marine calcifiers such as mussels, oysters, and corals (Heinemann et al., 2012; Hettinger et al., 2013; Kroeker et al., 2016; Ramajo et al., 2016; Thomsen et al., 2013; Towle et al., 2015), presumably because

10   organisms require energy for biomineralization (Palmer, 1992). Feeding rates in calcifiers can also be affected by acidified conditions. The effects vary according to phylum, feeding style, life stage, and exposure time, with the feeding rates of suspension-feeding molluscs particularly susceptible to decrease with increased CO_2 (Clements and Darrow, 2018). There have not been any studies conducted on the response of pteropods to varying acidification and food availability conditions, however, we assume that as in other groups of marine calcifiers, food availability plays an important role in calcification. This

15   body of research supports the inference made from the finding of this study that when water is supersaturated with respect to aragonite, such as in the Cariaco Basin, food availability is the main control of *H. inflatus* shell growth.

5 Conclusions

In this study, we analyzed the shell diameter, number of whorls, thickness, amount of shell material, and shell condition of

20   *Heliconoides inflatus*, a species of pteropod from the Cariaco Basin, over an 11-month period. Because specimens in this study came from a sediment trap, the impact of time in the sediment trap on shell condition was analyzed. Shells were assessed using both the LDX and opacity methods, however, as the opacity method was only sensitive to changes in shell condition at LDX scores of two or lower, the LDX was used for all analyses. Although all shells had undergone some alteration, shell condition did not deteriorate with increased time in the sediment trap cup. The most poorly preserved specimens came from sediment

25   trap samples collected when seawater temperatures were the highest, potentially linked to increased rates of microbial breakdown of organic matter both within the pteropod shell, and in the water column.

The size, number of whorls, thickness and amount of shell material in the shells of *H. inflatus* vary throughout the year, and therefore are likely to be influenced by external factors. Water chemistry in the Cariaco Basin is controlled by the movement

30   of the ITCZ and has two distinct phases: an upwelling phase and a non-upwelling, oligotrophic phase. We find that *H. inflatus* produces larger, thicker shells during times of upwelling, when food availability is greater. The Cariaco Basin was supersaturated with respect to aragonite throughout the study period (i.e. $\Omega_{arag} > 1$) and shell thickness does not correlate with Ω_{arag}. This demonstrates that in this aragonite supersaturated setting, the availability of food has a greater control on shell formation than aragonite saturation. This pattern has been seen in other groups of molluscs, such as oysters and mussels and

underlines the necessity of assessing pteropod shells in the context of multiple biotic and abiotic factors. We hope that the baseline dataset of pteropod shell parameters presented in this study is the first of many. These datasets will enable the quantification of the response of this sentinel group to ocean acidification.

6 Data availability

5   The data which support the conclusions in this manuscript are available in the tables, figures, references, and supplemental materials. CT data will be made available on MorphoSource (https://www.morphosource.org) once the manuscript is accepted.

7 Sample availability

Specimens have been deposited in the Malacology collection at the Academy of Natural Sciences of Drexel University, Philadelphia, PA, USA (ANSP). A sample list, including the ANSP catalog numbers, can be found in Table 1.

10   **8 Author contributions**

Following CRediT: Conceptualization (RLO), Data curation (RLO, JAS, PC), Formal analysis (RLO), Funding acquisition (RLO, JAS, TJB), Investigation (RLO), Methodology (RLO), Project administration (RLO), Resources (BM, RT, CD – University of South Carolina, JU, WY, MH), Software (RLO), Supervision (RLO, JAS, TJB), Validation (RLO, JAS), Visualization (RLO), Writing – original draft (RLO), Writing – reviewing and editing (RLO, JAS)

15   **9 Competing interests**

This is an original submission and the authors do not declare any conflicts of interest.

10 Acknowledgements

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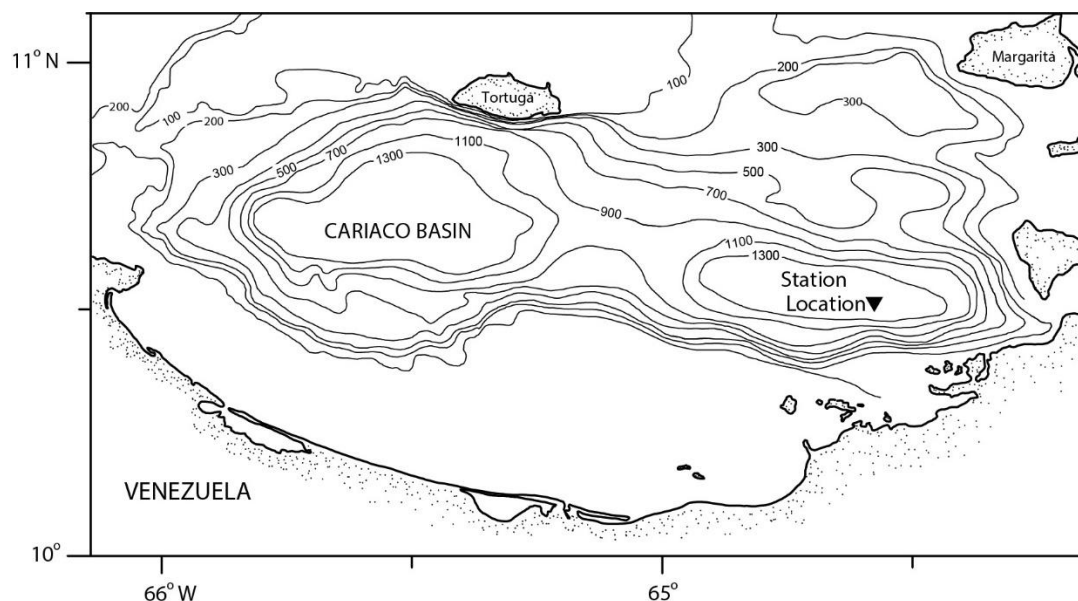15 14–41, doi:10.1046/j.1054-6006.2001.00043.x, 2001.

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12 Figures

Figure 1: Bathymetric map of the Cariaco Basin. The location of the sediment trap (10° 30.0' N, 64° 38.5' W) is marked with a triangle (modified from Marshall et al., 2013).
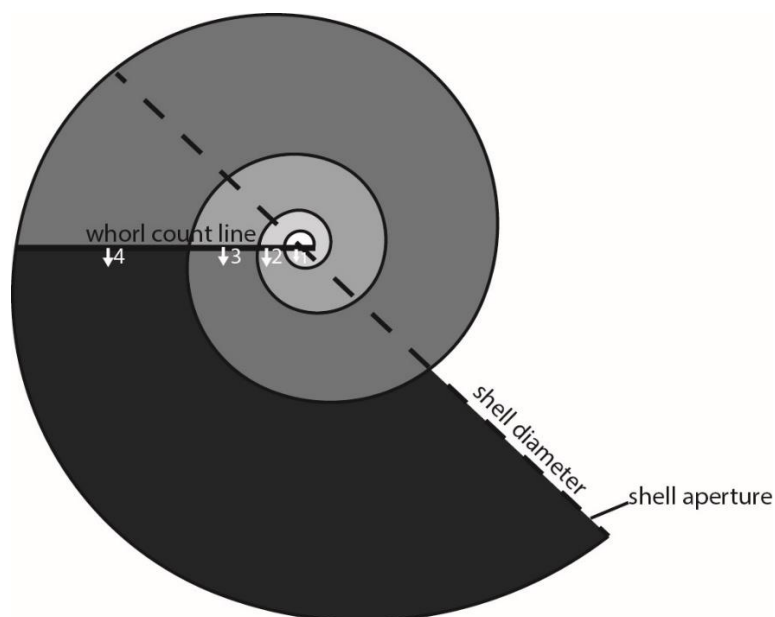

Figure 2: Schematic diagram of a pteropod shell demonstrating how shell diameter (the metric used for size) was measured, and how the number of whorls was counted. Following the methods outlined in Janssen (2007), a straight line is drawn across the shell separating the semi-circular nucleus (center) from the rest of the shell. Whorls are then counted as 360 ° rotation from the straight line, marked in progressively darker shades of grey, until the aperture of the shell is reached. The number of whorls is recorded with an accuracy of an eighth of a whorl. The shell in the schematic diagram has 3 3/8 whorls.
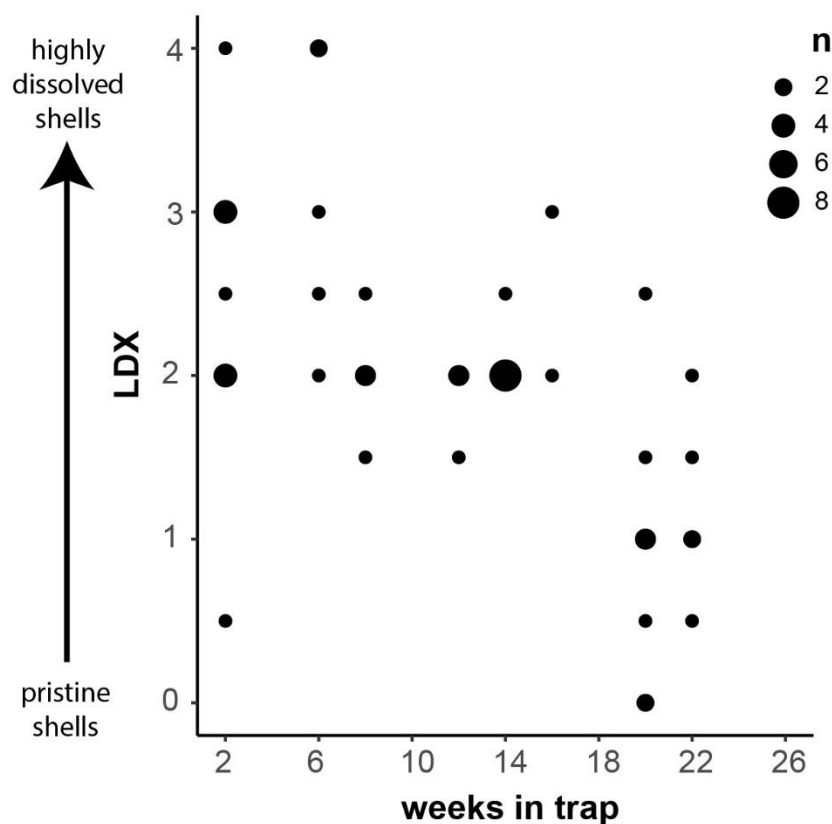
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Figure 3: Shell condition of *Heliconoides inflatus*, ranked on the *Limacina* Dissolution Index (LDX) scale, plotted against the maximum amount of time specimens spent in the sediment trap (i.e., the number of weeks from the trap opening time). The size of the symbols corresponds to n, the number of specimens plotted at a given point.
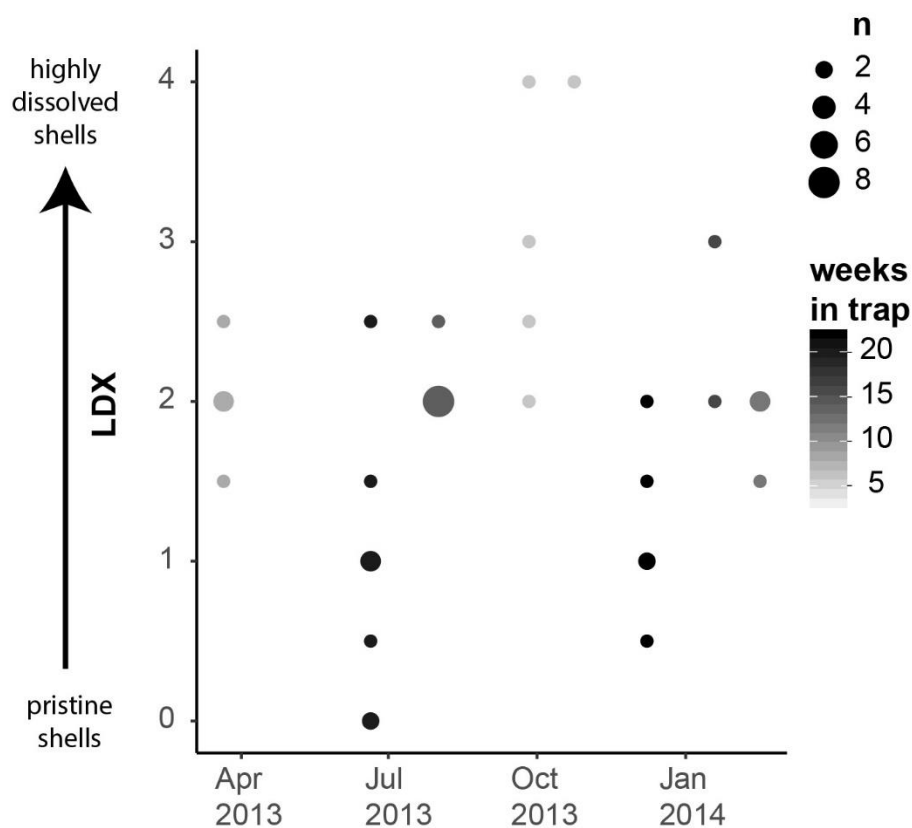
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Figure 4: Shell condition of *Heliconoides inflatus*, ranked using the *Limacina* Dissolution Index (LDX) scale, over the study period. The samples with the poorest preservation are from September and October 2013 when water temperatures were the highest. The size of the circles corresponds to n, the number of specimens plotted at a given point, and the color of the circles corresponds to the maximum number of weeks specimens were in the trap.
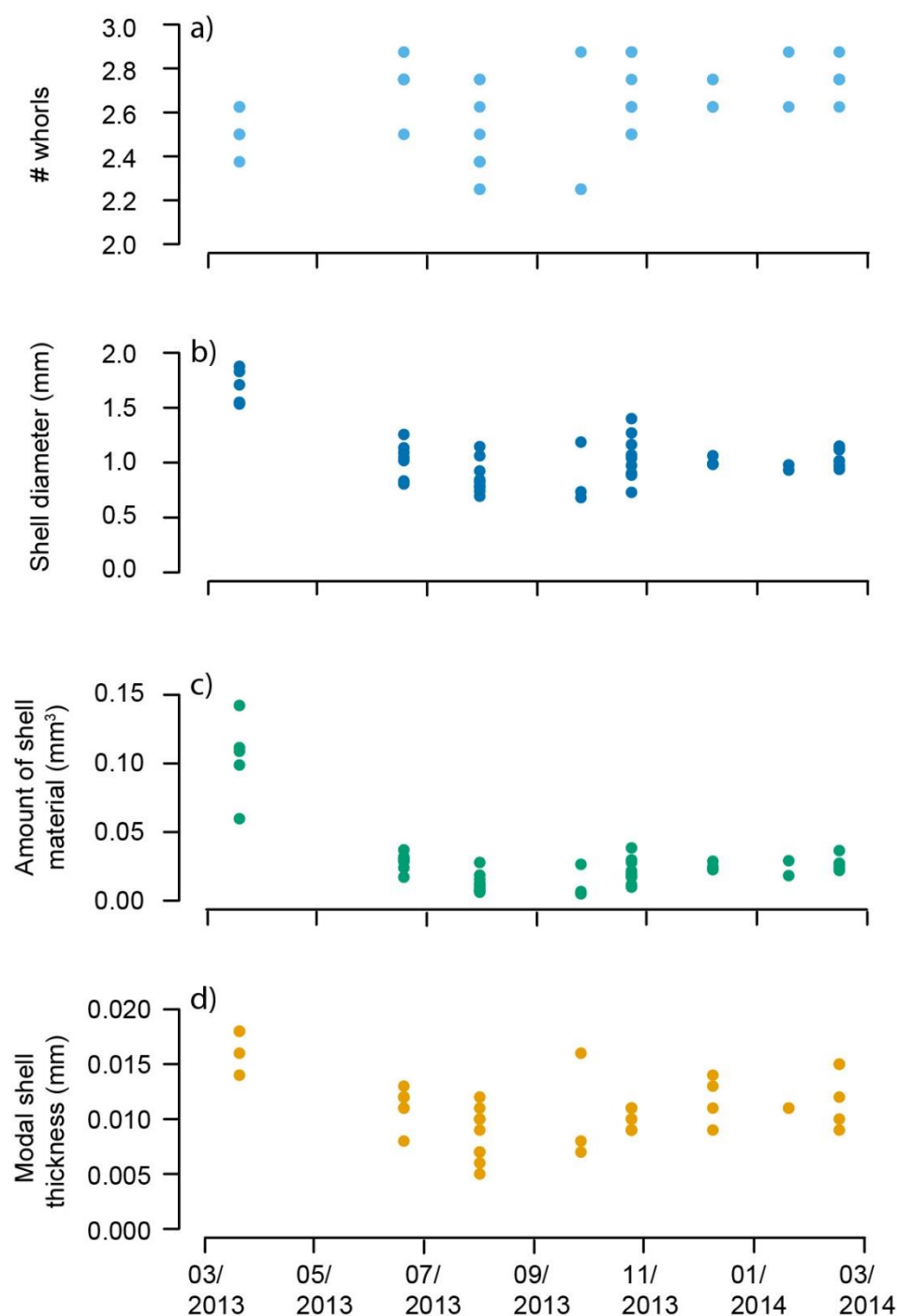
Figure 5: *Heliconoides inflatus*: **a) number of whorls; b) shell diameter; c) amount of shell material; and d) modal shell thickness throughout the year in the Cariaco Basin. Each point represents an individual specimen.**
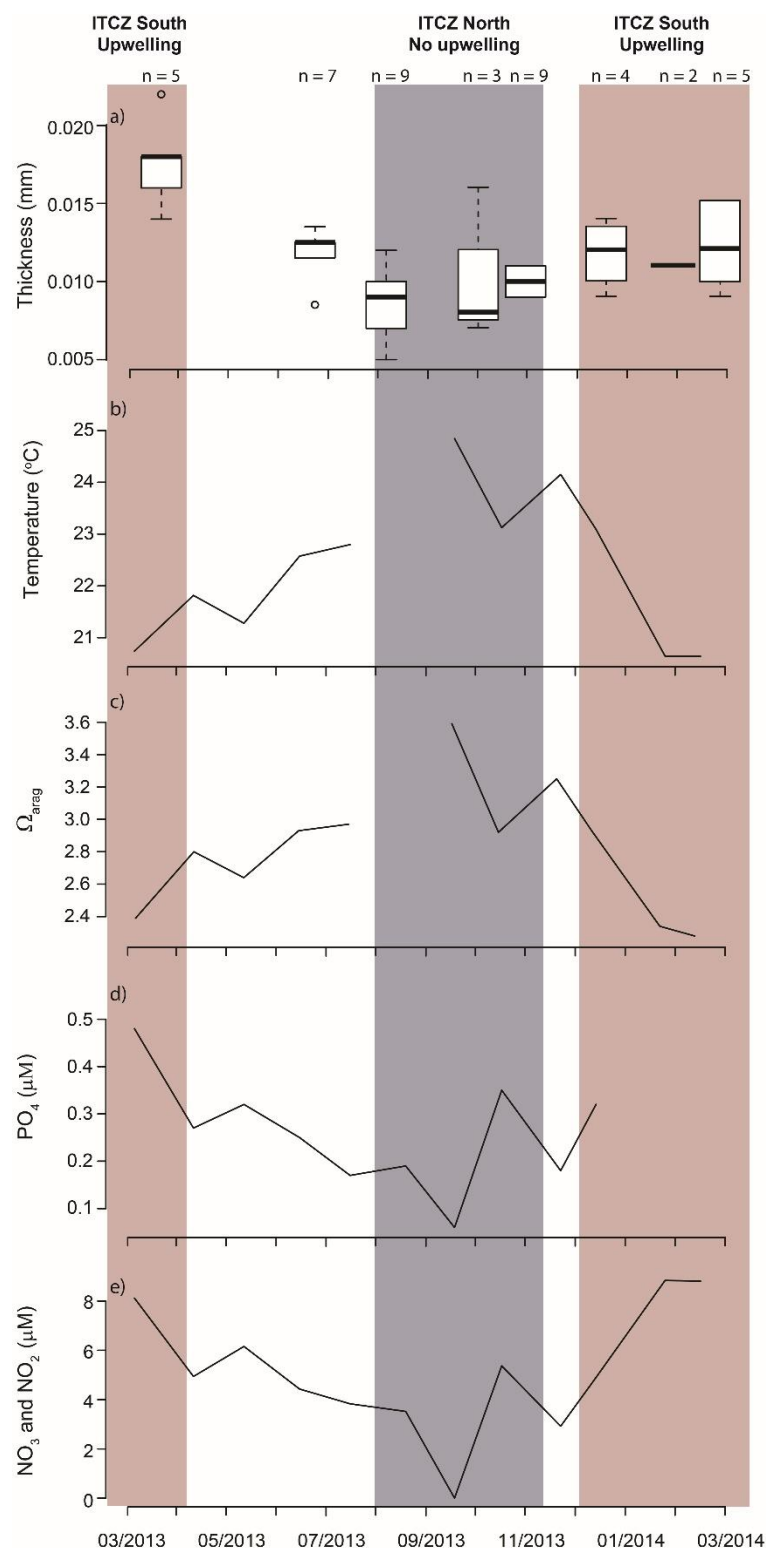
Figure 6: Shell thickness and water column properties plotted over the study period: a) *Heliconoides inflatus* **modal shell thickness, b) seawater temperature, c)** Ω_{arag}**, d) PO$_4$, and e) NO$_2$ and NO$_3$. All water column measurements (b-e) are from 55 m depth because this is the water sample closest to the predicted calcification depth of** *Heliconoides inflatus* **(Keul et al., 2017). The upwelling season is indicated by a red box, and the rainy season, when there is no upwelling, is indicated by a grey box.**
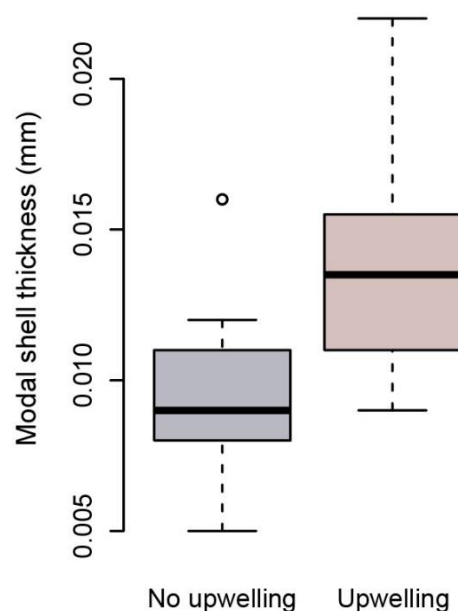
Figure 7: Modal shell thicknesses of specimens from times of upwelling (red) and times of no upwelling (grey) in the Cariaco Basin. Specimens collected during times of upwelling are significantly thicker than those which formed at times with no upwelling (Welch's t-test: *p* = 4.4 x 10⁻⁴).
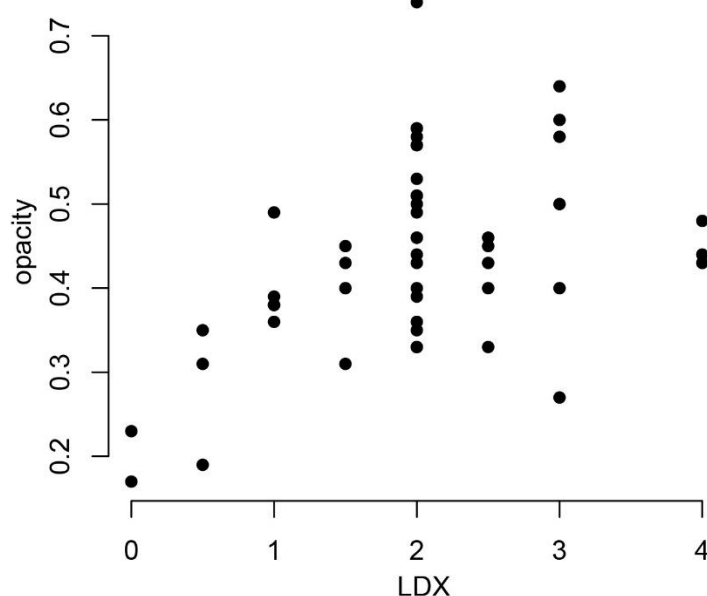

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Figure 8: Shell condition of Heliconoides inflatus, ranked on the Limacina Dissolution Index (LDX), plotted against shell condition of the same shells quantified using the opacity scale. LDX and opacity are positively correlated until LDX scores of 2, at which point there is no correlation between LDX and opacity. This breakdown is likely due to the changes in surface texture of the pteropod shell from shiny to matte. The texture change linked to dissolution is a factor when assigning values on the LDX scale but as the
10   **color and opacity do not change, it is not detected by the opacity scale.**

13 Tables

Sample	Trap date	Light microscope imaged	CT scanned	ANSP catalogue no.
CAR34Z#10	21/03/2013	5	5	477912
CAR35Z#04	20/06/2013	8	7	477913
CAR35Z#07	01/08/2013	9	9	477914
CAR35Z#11	26/09/2013	5	3	477915
CAR35Z#13	24/10/2013	11	9	477916
CAR36Z#03	08/12/2013	5	4	477917
CAR36#06	19/01/2014	2	2	477918
CAR36#08	16/02/2014	4	5	477919
		49	**44**	

Table 1: Number of specimens imaged and CT scanned from each sediment trap cup.

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