Oakes and Sessa – Response to Reviewers

Reviewer comments have been copied below in plain text. Author responses are listed under the reviewer comments in bold and italics. Page and line numbers refer to the tracked changes version of the manuscript.

Review #1 - Anonymous Referee #1

Reviewer # 1 - General Comments:

The aim of this study is to describe annual variability of different shell growth parameters (thickness, diameter, number of whorls, amount of shell material) of the pteropod Heliconoides inflatus in the Cariaco Basin (Venezuelan Shelf). Additionally, shell condition was analyzed applying the Limacina Dissolution Index (LDX). Pteropod samples were collected over a year period from a sediment trap and compared to prevailing carbonate chemistry and nutrient conditions with the goal to entangle driving abiotic or respectively biotic factors of the various measures. The authors found that food availability has a greater control on shell formation than aragonite saturation and that shell condition was not altered with time spent in the sediment trap cup. Hence, the results can serve as baseline data to better quantify the response of this highly vulnerable organism group to ocean acidification (OA) by disentangling abiotic from biotic factors that impact on shell formation.

I think this study is very interesting and addressing a very important question in relation with consequences of OA on highly vulnerable thecosome pteropods. It gives strong in situ evidence that food availability and energy constraints have a major potential to mitigate abiotic stress and shows nicely that various shell parameters indicative for growth and calcification did not depend on the saturation state of aragonite, at least not in the range observed (always above 2).

From my understanding, the purpose of the study was twofold: 1) How does length of time (preservative) in the trap impact shell condition and potentially lead to false conclusions in the OA context? 2) Do changes in water column properties affect shells and how or which? Hence, point 1 looks at dead organisms, point 2 affects live organisms in the water column (including the carbonate chemistry history pteropods experienced in the past). In this context, my main criticism is that the author did not distinguish between processes that happened when pteropods were still alive (in the water column) and already dead (in the water column and the sediment) particularly with respect to potential shell degradation they observed on the preserved samples. Did the authors simply assume that shell integrity was intact as long as organisms dwelled in the water column alive? Might indeed be reasonable to assume but the authors need to state clearly in their ms what their opinion on that is and whether/when they talk about live or dead organism. Furthermore, one important issue with sediment trap samples is that pteropods might have entered them as “swimmers” not as dead individuals that simply sank into the trap. This problem should be mentioned in the introduction and picked up later in the discussion again, would that impact the conclusions to draw from the Results?

Author response: We agree with the general comment about the need to discuss processes affecting pteropod shells when the organism is live, dead, and in the preservative. We have added information to the abstract and introduction, and have reformatted our discussion to better outline these processes and the likelihood of shell alteration occurring at each of these stages.

Abstract: Pg. 2, lines 10 – 13, Introduction: Pg. 4, line 33 – Pg. 5, line 11, Discussion: Pg. 12, lines 5 – 24). We have also discussed the potential impact of swimmers on the samples in the introduction (Pg. 5, lines 1 – 4) and discussion (Pg. 12, lines 24 – 30) sections.

Reviewer #1 - Specific Comments

Reviewer comment: The title does not reflect the study content well enough. For example micro-CT is not even mentioned in the abstract and shell thickness is only one out of a set of measured parameters mentioned in the abstract. LDX is much more prominent in the abstract instead. Also, I think the title should reflect that the ms is about sediment trap samples of H. inflatus. Please change title accordingly to maybe something like this: "Assessing abiotic and biotic impact on annual variability of shell condition of the pteropod Heliconoides inflatus in the Cariaco Basin: shell dissolution index, size and thickness as revealed from sediment trap samples."

Author response: Thank you for pointing that out. We have changed our title to, "Determining how biotic and abiotic variables affect the shell condition and parameters of Heliconoides inflatus pteropods in the Cariaco Basin", based on your recommendation.

Reviewer comment: L4–6: This study does not deal with natural variability of pteropods (in terms of abundance of which "variability" is usually understood if not stated otherwise), neither is it discussed. Either remove this sentence or rephrase to harmonize with the variability you are actually focusing on (shell growths parameters).

Author response: We agree that the use of 'variability' could have been interpreted in multiple ways throughout the text. We have changed the text in the abstract to clarify our focus on shell growth parameters. It now reads, "… the biotic and abiotic factors influencing their shell formation and dissolution in the modern ocean need to be quantified and understood." (Pg. 2, lines 5 – 7). We have either changed the wording, or expanded on the meaning of our use of the word 'variability' through the manuscript.

Reviewer comment: L11: remove "with"

Author response: done

Reviewer comment: L14/15: Are the authors talking about dead or live individuals?

Author response: We agree that the differentiation between processes that affected live and dead individuals was unclear. We have restructured the abstract to clarify the mechanisms that can alter shell condition when the animals are live, dead, and in the preservative. "The shell condition of pteropods from sediment traps have the potential to be altered at three stages: 1) when the organisms are live in the water column associated with ocean acidification, 2) when organisms are dead in the water column associated with biotic decay of organic matter, 3) and when organisms are in the sediment trap cup associated with the abiotic alteration by the preservation solution." (Pg. 2, lines 10 – 13).

L19: : : : in shell characteristics of H. inflatus of trapped pteropods: : :

Author response: We acknowledge the importance of conveying the fact these samples are from a sediment trap in the abstract. We have added a section on mechanisms affecting shell condition in

specimens from sediment traps (Pg. 2, lines 10 – 13) and have added additional mentions in the abstract.

Reviewer comment: Section 1.2: The authors should shortly mention the problem of collecting live pteropods ("swimmers") in sediment traps and how that could have affected their work approach and results. (Alternatively it might be mentioned on P4 last paragraph).
Author response: Good point - we have added discussion of swimmers entering sediment trap samples in the introduction (Pg. 5, lines 1 – 4), and have mentioned how our results may have been impacted by swimmers in the discussion (Pg. 12, lines 24 – 30).
Introduction: "A further complication of sediment trap data is that interpretation can be skewed by the presence of 'swimmers', i.e., specimens that were alive when they entered the trap (Harbison and Gilmer, 1986). This is a particular concern with pteropods as they sink to avoid predation (Harbison and Gilmer, 1986) and therefore may enter into the trap while still alive."
Discussion: "These results could have been further complicated by the presence of swimmers, which would have entered the trap live and therefore would not have undergone any dissolution in the water column. If there was an increase in swimmers entering the traps at one time of year relative to another, it could be interpreted as less water column breakdown during these months. The most pristine shells in this study entered the trap in June and December, suggesting that there was not a seasonal pattern to swimmer frequency. We therefore assume that the number of swimmers entering the sediment trap is constant throughout the year and therefore does not affect the seasonal trends reported above."

Reviewer comment: Throughout the ms, they need to make clear whether they talk about live or dead organisms.
Author response: We agree that the distinction between processes that affect live and dead shells was not clear in the original manuscript. We have worked to clarify how different processes affect live, dead, and preserved shells: Abstract: Pg. 2, lines 10 – 13, Introduction: Pg. 4, line 33 – Pg. 5, line 11, Discussion: Pg. 12, lines 5 – 24.

Reviewer comment: P4L6: Lischka and Riebesell 2017 (Polar Biol, Volume 40) also studied metabolic response of pteropods (oxygen consumption).
Author response: Thank you for reminding us of this work - we have added the reference (Pg. 4, lines 11 – 12)

Reviewer comment: P4L24: through misses the "r"
Author response: corrected

Reviewer comment: P5L33: remove comma between body and whorl
Author response: In molluscs, the final whorl is also known as the body whorl. The sentence is grammatically correct as it was, "...the final, or body, whorl." (Pg. 6, line 17)

Reviewer comment: P6L10: Please detail at what temperature and for how long shells were dried.
Author response: We have added details about drying to the methods section (Pg. 6, lines 28 – 29): "Calcareous plankton were wet-picked, and left to dry in a 40°C oven for 24 hours, before being, dried, and stored for faunal analysis (E.Tappa pers.comm.)."

Reviewer comment: P10L31/32: Could any changes detected originate in the time prior collection in the trap during live in the water column?
Author response: This is an interesting question. We assume that there was no in-life dissolution as the water is permanently supersaturated with respect to aragonite, and because we don't see any evidence of patchy dissolution, such as is seen when pteropods undergo dissolution in-life (e.g. Peck et al., 2016, 2018). We have clarified this in the text (Pg. 12, lines, 7 – 11): "The water in the Cariaco Basin was supersaturated with respect to aragonite throughout the study. The thin aragonite shells of the pteropods are therefore chemically stable in the water column so it is unlikely that they underwent in-life dissolution. Furthermore, there is no evidence of patchy dissolution in pristine shells, or those which have undergone dissolution, such as has been observed in pteropod shells undergoing in-life dissolution in naturally undersaturated environments (Peck et al., 2016; 2018)."

Reviewer comment: P11L7/8: How can the authors know, pteropods were dead already? How likely is it that shell deterioration happened on the live organism? The assumption that any shell degradation took place only when organisms were dead already, is this simply based on the assumption that under aragonite supersaturated conditions no shell deterioration happened? If so, state clearly and support your view.
Author response: See response above

Reviewer comment: P11L20: : : : in the overall trend: : : (remove "is no")
Author response: done

Reviewer comment: Fig. 4: It would help the clarity of the figure if September, June, December (mentioned in the text) could be indicated on the x-axis.
Author response: Good point – see updated figure (now figure 5)

Reviewer comment: Fig. 8: Italics for Heliconoides inflatus
Author response: changed

We thank Reviewer #1 for their thorough and constructive review of our manuscript. Their comments have helped us to improve the scope and clarity of our work

Review #2 - K. Kimoto (Referee) - kimopy@jamstec.go.jp

This manuscript describes the biometrics of pteropod shell and its degradation based on sediment trap samples in the Cariaco Basin. This kind of works of pteropod shells using the sediment trap samples are insufficient, so it is very important to trace the biological responses to the ocean acidification and related ocean environmental changes. Especially the information of tropical species is less. In this sense, this work has the potential to become the base and develop criteria for this kind of study. Below I pointed out some concerning issues for this ms and make the comments

Reviewer comment: The relationship between shell length and whorls. According to photos in supplemental document, the aperture of some shells was damaged or showed bad preservation. The author inferred that shell diameter and whorl does not show clear relationship, but if a part of aperture had lost

by dissolution and/or physical damage, its relationship between shell length and whorl might become uncertainty. If the plankton tow samples are available, the authors should use those plankton samples, not sediment trap ones. Or at all possible, the authors should use the only perfect shell in order to interpret length-whorl relationship.

Author response: Thank you for pointing out this potential source of bias. The fragility of H. inflatus shells make them particularly susceptible to breakage, often during collection (Pg. 7, lines 7 – 8). Shell diameter and shell whorl measurements were made on CT-scanned specimens but analyses were also run on a subset of 29 unbroken specimens. There was a weak but statistically significant relationship between shell diameter and the number of whorls. The FDR corrected p value was identical in both the whole and the subset dataset, although the R^2 was higher in the subset dataset (whole dataset: R^2 = 0.074, p Bon. = 0.415, p FDR = 0.057; subset dataset (Table S1): R^2 = 0.101, p Bon. = 0.513, p FDR, 0.057). We have added this information into the methods (Pg. 7, lines 8 – 9), and results (Pg. 10, lines 27 – 30) sections of our manuscript. Since there was no difference between the two analyses, Figure 6 in the main manuscript contains the full dataset. The same figure with only the unbroken specimens is reproduced in the supplemental materials (Fig. S4).

Methods: "Because H. inflatus shells are fragile, they often break at the aperture during collection and processing. Although shell diameter and number of whorls were measured on all CT-scanned specimens, a subset of 29 shells with complete apertures was created for further analyses (Table S1)." Results: "There was a weak, but statistically significant correlation between shell diameter and the number of whorls which remained when analyzing the subset of complete shells (Table S1) (whole dataset: R_2 = 0.074, p Bon. = 0.415, p FDR = 0.057; subset dataset (Table S1, Fig. S4): R_2 = 0.101, p Bon. = 0.513, p FDR, 0.057)."

Reviewer comment: Shell dissolution: How and when? The authors described that preservation states of shells in the sediment trap was not related to the duration time, so it might be negligible dissolution in the sediment trap collection cups. If this is correct, shell dissolution occurred at the water column, and it was associated with microzoo/bacterial activity which was decomposing organic tissues. My questions are that 1) in this case, does shell dissolution occurred at the inside, and outside of shell is sufficiently preserved? Can the authors show this evidence? Based on the photographs on the supplement material, surface texture of some shells looks like cloudy and lost their luster, indicating dissolution of outer shell. I am wondering that the decision of less dissolution in the sediment trap collection cups based on the result of relationship between residence time and LDX might be insufficient. In other words, I infer that shell dissolution occurs not only by microorganisms/bacterial activity but also post depositional oxidization in the sediment trap cups, as authors mentioned. I am understanding that this certification is very difficult, but the authors are using SEM, so please show some possibilities from the direct observations of materials.

Author response: We agree that this is an important question and decided to investigate a subset of seven shells, ranging from the best to the worst shell condition, under the scanning electron microscope (Page 8, section 2.4, lines 1 – 7). Our investigations revealed that the majority of the dissolution at LDX scores below 2.5 occurs on the outside of the shell. At LDX scores of 2.5 or higher, there is dissolution on both the inside and the outside of the shell. The external dissolution could be

attributed to either dissolution associated with decaying organic matter in the water column, or alteration associated with the preservative in the sediment trap cup. Internal dissolution is associated with the decaying organic body of the pteropod and/or alteration in the sediment trap cup. We have added discussions of these possible scenarios in the manuscript (Pg. 12, lines 18 – 19): "Scanning Electron Microscopy reveals that the majority of this dissolution occurred on the outside of the shells (Fig. 2, Fig. S2).", and (Pg. 13, lines 2 – 3): "SEM images reveal that the internal shell walls were only impacted by dissolution at LDX values of 2.5 and higher (Fig. 2 d, h, l), indicating that the preservative did not cause dissolution." We have added a figure of a selection of the SEM images to the main manuscript (Fig. 2) and all the SEM images to the supplemental (Fig. S2).

Reviewer comment: Another possibility, is it available the comparison between organic carbon of the samples and LDX? Highly input of organic carbon flux induce carbonate dissolution at the inside of sediment trap cup.

Author response: Unfortunately, there are no measurements of the organic carbon content of the samples from the sediment trap cups. Particulate Organic Carbon was measured as part of the CARIACO time series hydrographic measurements; however, these values are only available for the first half of this study so we cannot make this comparison.

Reviewer comment: Relating to above, I understood this study is the first, and make the baseline of this kind of pteropod study, but it is bit unclear the main subject and purposes. If the authors interpretation is correct, does the pteropod shell of this species /or in this region not become an index of ocean acidification? I suppose that the authors want to make the criteria as OA index by using pteropod shell, but in this case, I think that shell preservation states indicate microorganisms activity in the pteropod shell.

Author response: Hopefully the restructuring of the abstract, and introduction have served to clarify the purpose of this study. From an ocean acidification perspective, we make the point that although many studies have focused solely on aragonite saturation, the availability of food and the collection and preservation methods used may also affect shell condition (Pg. 14, section 4.3). We hope this research leads to a more holistic view of shell condition interpretations. We have rephrased the conclusion to clarify this point (Pg. 15, lines 27 – 32): "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 shell parameters and dissolution in the context of multiple biotic and abiotic factors, not just aragonite-saturation. We hope that the baseline dataset of pteropod shell parameters presented in this study is the first of many focused regional studies around the world. These datasets will enable the quantification of the response of this sentinel group to ocean acidification."

Reviewer comment: I think it is very important finding that shell thickness does not have relationship with surrounding omega value (but still supersaturated). I strongly agree with the authors that they have resistance characteristics to small changes of saturation states and depends on available food to build their shells. However, the authors did not show what kind of food is important for their prey. If their main food

is phytoplankton, please show their annual variations through a year instead of nutrient concentrations (Or is it possible to show the number of diatom bulbs in the sediment trap cups?) Because their food is particulate matters, not chemical component. It might be a good evidence to indicate their food availability.

Author response: Yes, good point. Although we don't have any diatom counts from these sediment trap cups, we have added references to two other studies conducted in the same basin which find that both organic carbon production (Thunell et al., 2000) and diatom populations (Romero et al., 2009) show strong increases at times of upwelling. Nutrient concentrations are good proxies for upwelling and therefore showing the nutrient changes through the year is a reasonable approximation for food availability (Introduction: Pg. 5, lines 21 – 24, Discussion: Pg. 14, lines 13 – 16).
Introduction: "Organic carbon fluxes in the basin vary in response to these hydrographic changes, with one study reporting a tripling of primary productivity in response to upwelling (Thunell et al., 2000). Diatoms, a known food source for pteropods (Lalli and Gilmer, 1989), contribute to over 50% of this organic carbon flux, with their blooms coinciding with hydrographic and nutrient changes during times of upwelling (Romero et al., 2009)."
Discussion: "These upwelling-related nutrient changes in the Cariaco Basin have been shown to correspond with increases in organic carbon flux and diatom blooms (Thunell et al., 2000; Romero et al., 2009), indicating that pteropod food supply (Lalli and Gilmer, 1989) increases during upwelling conditions."

Pteropods eat diatoms, as well as dinoflagelletes and tintinnids. We have added a reference about this to the introduction (Lalli and Gilmer, 1989) (Pg. 4, lines 3 – 4): "Pteropods are also key components of the marine food web, feeding on phytoplankton and small zooplankton, such as diatoms, dinoflagellates, and tintinnids (Gilmer and Harbison, 1986, 1991; Lalli and Gilmer, 1989)".

Reviewer comment: The authors did not touch the phylogenetic variation of the species, but I am wondering the possibility of mixture of some lineages of this species. H. inflatus is certificated as a single-genetic species around the Cariaco basin? Or exists some cryptic species? If the author has this kind of information, please mention it for just confirmation. It is possible that the plasticity of shell (shell length, number of whorls) of this species that author mentioned is related with the phylogenetic variations.

Author response: There has not been any genetic work done on H. inflatus. Both Van der Spoel (1967) and Janssen (2004) noted that there was variability in the shape and location of the rib, however, there is no data to determine whether this in intra- or inter-specific variability. We have added this information to the discussion (Pg. 13, line 34 – Pg. 14, line 4): "Both Van der Spoel (1967) and Janssen (2004) have described variability in the shape and position of the aperture tooth in H. inflatus, which could be attributed to intraspecific or interspecific variations. As there has not been any genetic work conducted on H. inflatus from the Caribbean, we cannot be sure that the variability we see in shell shape cannot be attributed to two or more genetically-defined species."

Reviewer comment: Can the author interpret about morphological implication from the microXCT analysis Because it is very powerful tool and shows huge possibility for morphological information. If the

authors want to indicate some suggestive issues, please make comment for following researchers and future study.

Author response: We agree that the CT data offer the opportunity to do some really interesting geometric morphometric work. We have added a section entitled "Further Work" (Pg. 15, lines 1 – 9) where we discuss the challenges of the field of gastropod geometric morphometrics, and discuss two recent studies. Our CT data will be available on publication and so can be used in future geometric morphometric studies.

We would like to thank Dr. Kimoto for his constructive review. It has helped us improve the completeness and clarity of this work.

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

Determining how biotic and abiotic variables affect the shell condition and parameters of *Heliconoides inflatus* pteropods in the Cariaco Basin

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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~~changing 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~~are most pronounced. For pteropods to be used most effectively as indicators of ocean acidification, the biotic and abiotic factors influencing their shell formation and dissolution ~~ir natural variability~~ in the modern ocean need~~s~~ 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* sampled from a sediment trap in the Cariaco Basin, Venezuela ~~sampled~~ over an 11-month ~~period.~~ period. The shell condition of pteropods from sediment traps have the potential to be altered at three stages: 1) when the organisms are live in the water column associated with ocean acidification, 2) when organisms are dead in the water column associated with biotic decay of organic matter, 3) and when organisms are in the sediment trap cup associated with the abiotic alteration by the preservation solution. Shell condition was assessed using two methods: the *Limacina* Dissolution Index (LDX) and the opacity method. The opacity method was found to capture changes in shell condition only in the early stages of dissolution, whereas the LDX recorded dissolution changes over a much larger range. Because the water in the Cariaco Basin is supersaturated with respect to aragonite year-round, we assume no dissolution occurred during life, and there is no evidence that shell condition deteriorated with the length of time in the sediment trap. Light microscope and SEM images show the majority of alteration happened to dead pteropods while in the water column associated with the decay of organic matter. The most altered shells occurred in samples collected in September and October when water temperatures were warmest, and the amount of organic matter degradation, both within the shells of dead specimens and in the water column, was likely to have been the greatest.

~~The water in the~~Changes in the hydrography and chemical properties in the Cariaco Basin ~~is supersaturated with respect to aragonite year-round, and hydrographic and chemical 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 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. There was not a strong correlation between the number of whorls ~~did not correlate with~~and the shell diameter, suggesting that shell growth is plastic. *H. inflatus* formed shells that were 40% thicker and 20% larger in diameter during nutrient rich, upwelling times when food supply was abundant,~~when nutrient concentrations were high during times of upwelling, compared to specimens sampled from the oligotrophic rainy season.~~ indicating that shell growth in this aragonite-supersaturated basin is controlled by food

availability. This study produces a baseline dataset of the variability in shell characteristics of *H. inflatus* pteropods in the Cariaco Basin and documents the controls on alteration of specimens captured via sediment traps. The methodology outlined for assessing shell parameters ~~and~~ establishes a ~~methodology~~ protocol for generating similar baseline records for pteropod populations globally.

1 Introduction

The global ocean has absorbed over a third of anthropogenic carbon dioxide emissions since the industrial revolution (Gruber et al., 2009; 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 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 values greater than one, the seawater is supersaturated with respect to the mineral, and at values 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 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 the natural variability of organisms' shell parameters~~variability~~ in response to environmental fluctuations is understood prior to their use as indicators of changes in ocean chemistry.

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 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 during their diurnal migration ~~as they migrate through the water column diurnall~~. Pteropods are also key components of the marine food web, feeding on phytoplankton and small zooplankton, such as diatoms, dinoflagellates, and tintinnids (Gilmer and Harbison, 1986, 1991; Lalli and Gilmer, 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), shell degradation (Bednaršek et al., 2012b; Bergan et al., 2017; Lischka and Riebesell, 2012), metabolic rates (Lischka and Riebesell, 2017; 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 (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 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).

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 through the water column into collection cups containing preservative. These collection cups are 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. Organisms falling through the water column may decay en-route to the sediment trap, which can cause dissolution

in calcareous organisms (Lohmann, 1995; Milliman et al., 1999; Oakes et al., 2019), and therefore specimens from sediment traps do not perfectly capture in-life shell conditions. A further complication of sediment trap data is that interpretation can be skewed by the presence of 'swimmers', i.e., specimens that were alive when they entered the trap (Harbison and Gilmer, 1986). This is a particular concern with pteropods as they sink to avoid predation (Harbison and Gilmer, 1986) and therefore may enter into the trap while still alive. ~~The disadvantage of~~ Additionally, sediment trap samples ~~is that there~~ can be subjected to alteration ~~to the shells~~ in the sediment trap cup, due to decay of the organic matter and degradation associated with 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 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 when the individuals are alive, post-mortem decay before specimens reach the sediment trap, and potential breakdown during the time they are in the ~~collection~~ sediment trap 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 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). Organic carbon fluxes in the basin vary in response to these hydrographic changes, with one study reporting a tripling of primary productivity in response to upwelling (Thunell et al., 2000). Diatoms, a known food source for pteropods (Lalli and Gilmer, 1989), contribute to over 50% of this organic carbon flux, with their blooms coinciding with hydrographic and nutrient changes during times of upwelling (Romero et al., 2009).

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 plankt~~on~~ic foraminifera from the sediment trap samples, investigating their flux, the variability of assemblages both seasonally and interannually, ~~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 on~~f the~~ pteropods from the Cariaco sediment trap records.

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 concentrations, 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, scanning electron microscopy, 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.~~ To compare among and between pteropod shells from different samples, ~~S~~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 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~~; Oakes et al., 2019~~). 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 in *Heliconoides inflatus* pteropods (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

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 (2003). A quarter split was washed over a 150-micron sieve with deionized water. Calcareous plankton were wet-picked, and left to dry in a 40°C oven for 24 hours, before being~~, dried, and~~ stored for faunal analysis (E.Tappa, pers.comm.).

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 pteropod specimens were picked from the washed and dried faunal samples by B. Marshall and C. Davis (University of South Carolina). Light microscope images were used to assess shell condition (Fig. S1), scanning electron microscopy was used to assess the extent to which dissolution had occurred on the internal and external areas of the shell, and CT scans were used to determine shell diameter, number of whorls, shell thickness, total shell volume. Because *H. inflatus* shells are fragile, they often break at the aperture during collection and processing. Although shell diameter and number of whorls were measured on all CT-scanned specimens, a subset of 29 shells with complete apertures was created for further analyses (Table S1). Specimens are 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 *H. inflatus* 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. 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).

2.3.14 Assessment of shell condition

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~~ was designed to assess the extent of dissolution in pteropods from the fossil record using~~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 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.

2.4 Scanning Electron Microscopy

A subset of seven specimens, spanning pristine to highly altered shell conditions, were imaged using a scanning electron microscope (SEM) to determine the extent of internal and external shell dissolution. Specimens were imaged using an FEI Quanta 600 ESEM at the Nanoscale Characterization Facility at the Singh Center for Nanotechnology at the University of Pennsylvania, Philadelphia, USA. Samples were mounted on carbon tape and were imaged uncoated. All specimens were imaged at 200-, 500-, 50,000-, and 100,000-times magnification on the external wall, and 50,000- and 100,000-times magnification on the internal wall where possible (Fig.2. Fig. S2).

2.5 CT scanning

2.5.1 CT data collection

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 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 a resolution of 1–2 µm/voxel using the 180 kV nanofocus tube with a diamond target and a beam 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 at the AMNH in order to scan the greatest number of shells possible. To assess the impact of using both different scanners, and different 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~~S3), the modal shell thickness calculated for all four scans was 0.008 mm. This demonstrates that modal shell thickness is a robust 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 materials, such as foraminifera tests or sediment. To ensure 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.

2.5.3 Quantifying shell parameters

Data were visualized and measured in Avizo v. 9.4.1. The s~~S~~hell diameter was measured at the widest part of the shell following the methods of Lischka et al., (2011) using the caliper tool in Avizo v. 9.4.1 (Fig. ~~2~~3). The number of whorls were counted to the nearest eighth of a whorl following the method of Janssen (2007) (Fig. ~~2~~3). 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 available at *http://imars.marine.usf.edu/WebPageData_CARIACO/Master_Hydrography/*. Water samples were collected at discrete depth 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 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 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). Scanning electron microscopy showed the majority of dissolution was concentrated on the outside of the shell up to LDX rankings of 2.5. At higher values, both internal and external walls display evidence of dissolution, and in some cases, the external surface has dissolved completely revealing the prismatic shell layer (Fig. 2, Fig. S2). The impact of preservation method on pteropod shell condition in this study was determined by comparing the time spent in the sediment trap with the condition of the shells (Fig. 4). Shell condition did not deteriorate with the amount of time spent in the trap (Fig. 34). 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 time in the trap (Fig. 34), 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. 45), and had spent a maximum of 2–6 weeks in the sediment trap cup (Fig. 34). The most well-preserved specimens came from June and December 2013 and had spent a maximum of 20–22 weeks in the sediment trap cup (Figs. 34, 45).

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. 56, 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 6 a; Table S1). SThe relationship between shell diameter 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 (Fig. 6 b). 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 (Fig. 6 c). The modal thickness of the shells of *Heliconoides. inflatus* also varied through the year (Fig. 5 6 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 6 d). There was a weak, but statistically significant correlation between shell diameter and the number of whorls which remained when analyzing the subset of complete shells (Table S1) (whole dataset: R_2 = 0.074, *p* Bon. = 0.415, *p* FDR = 0.057; subset dataset (Table S1, Fig. S4): R_2 = 0.101, *p* Bon. = 0.513, *p* FDR, 0.057). As shell diameter, thickness, and amount of shell material are related to size, unsurprisingly, thereunsurprisingly there were significant correlations between shell diameter and amount of shell material (R^2 = 0.819, *p* Bon. = 2.201.98*10^{-15}, *p* FDR = 2.2086*10^{-15}), shell diameter and shell thickness (R^2 = 0.582, *p* Bon. = 1.7961*10^{-8}, *p* FDR = 5.9737*10^{-9}), and shell

thickness and amount of shell material (R^2 = 0.680, *p* Bon. = 6.095.48*10^{-11}, *p* FDR = 3.052.74*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. 67; Table S4). Water chemistry measurements from 55 m depth were used because 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 7 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), using a Welch's t-test. 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 conditions prevailed (Welch's *t*-test: $p = 4.41 \times 10^{-4}$; Table S6; Figs. 67, 78). 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 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 S3S5; Table S6), indicating that water column properties impact calcification regardless of shell size.

4 Discussion

4.1 Shell condition

This study focuses on how the interplay of biotic and abiotic factors impacts the shell characteristics of the pteropod *Heliconoides. 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. 89). 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. 89). Since the opacity method

was designed to assess pteropods from an incubation experiment, it was ~~designed~~ intended to capture the earliest stages of dissolution (Bergan et al., 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 shell condition of specimens from sediment trap samples has the potential to be altered via three mechanisms: 1) dissolution in the water column when the organism is alive; 2) dissolution in the water column when the organism is dead; 3) alteration in the sediment trap cup associated with the preservative. The water in the Cariaco Basin was supersaturated with respect to aragonite throughout the study. The thin, aragonite shells of the pteropods would therefore have been chemically stable in the water column and thus it is unlikely that they underwent in-life dissolution. Furthermore, there is no evidence of patchy dissolution in pristine shells, or those which have undergone dissolution (Fig. 2, Figs. S1, S2), such as has been observed in pteropod shells undergoing in-life dissolution in naturally undersaturated environments (Peck et al., 2016; 2018).

Once a pteropod dies, the degradation of the organic body and associated acid production has been found to cause significant dissolution on the internal walls of the pteropod shell, even in an aragonite-supersaturated water column (Oakes et al., 2019). Dissolution can occur on the outside of the shells from the breakdown of free-floating organic matter in the water column creating aragonite-undersaturated microenvironments in an otherwise aragonite-supersaturated water column (Milliman, 1999). LDX rankings show the greatest amount of shell alteration occurred in specimens from the September – October 2013 samples (Fig. 5). Scanning Electron Microscopy reveals that the majority of this dissolution occurred on the outside of the shells (Fig. 2, Fig. S2). During September and October, water temperatures at 55 m were at their highest (Fig. 7 b). These warm temperatures would have increased the rate of microbial breakdown of both the organic body within the shell (Oakes et al., 2019), and in the free-floating decaying organic matter in the water column (Lohmann, 1995; Milliman et al., 1999; Schiebel et al., 2007). The shells of the organisms that died during the warmer months likely encountered more aragonite-undersaturated microenvironments associated with this organic matter breakdown as they fell through the water column and into the trap, increasing the rates of dissolution of these shells relative to those trapped during cooler months. These results could have been further complicated by the presence of swimmers, which would have entered the trap live and therefore would not have undergone any dissolution in the water column. If there was an increase in swimmers entering the traps at one time of year relative to another, it could be interpreted as less water column breakdown during these months. The most pristine shells in this study entered the trap in June and December, suggesting that there was not a seasonal pattern to swimmer frequency. We therefore assume that the number of swimmers entering the sediment trap is constant throughout the year and therefore does not affect the seasonal trends reported above.

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 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 (Fig. 4). Preservation-associated dissolution would have affected both the internal and external walls of the shell. SEM images reveal that the internal shell walls were only impacted by dissolution at LDX values of 2.5 and higher (Fig. 2 d, h, l), indicating that the preservative did not cause dissolution. Specimens that had undergone the most dissolution were sampled during the warmest months, which happened to coincide with the shortest amount of time in the trap. This produced an apparent trend of improving shell condition with time in the trap (Figs. 4, 5). This suggests that the preservative in the sediment trap collection cups effectively minimized post-collection sample degradation and that any sediment trap-associated 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 has a weak but statistically significant correlation with~~does not correlate with shell diameter (Fig. ~~5 a, b), implying that~~ Although there is a weak relationship between the number of whorls and shell diameter (Fig. 6 a, b), *H. inflatus* displays considerable 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 in the~~showing changes in is no~~ overall trend of~~in~~ *H. inflatus* shell diameter through the year in the Cariaco Basin (Fig. ~~5~~6 b), which suggests there are no cohorts. Another low latitude study found that *H. inflatus* collected off the coast of Barbados reproduced throughout the year (Wells, 1976a), although *H. inflatus* from off Bermuda in the Sargasso Sea have been shown to spawn in the spring (Almogi-Labin et al., 1988).~~this finding~~This corroborates ~~with~~. Both Van der Spoel (1967) and Janssen (2004) have described variability in the shape and position of the aperture tooth in *H. inflatus*, which could be attributed to intraspecific or

interspecific variations. ~~his variability could be intraspecific (Van der Spoel, 1967), or representative of two or more individual species. T~~As there has not been any genetic work conducted on *H. inflatus* from the Caribbean, we cannot be sure that the variability we see in shell shape cannot be attributed to two or more genetically-defined species.

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 aragonite-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~~ 7 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~~8), 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, ~~7~~8; Table S6) (Muller-Karger et al., 2001, 2019). These upwelling-related nutrient changes in the Cariaco Basin have been shown to correspond with increases in organic carbon flux and diatom blooms (Thunell et al., 2000; Romero et al., 2009), indicating that pteropod food supply (Lalli and Gilmer, 1989) increases during upwelling conditions. 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~~S5, 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.

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

4.4 Further work

Micro-CT scanning enables pteropod shells to be digitized in three dimensions, creating the opportunity for more complex quantitative analyses of shell shape and parameters than presented in this study. Despite their geometrically simple shapes, gastropod shells are particularly challenging to perform geometric morphometric analyses on because of their lack of fixed landmark points (Liew et al., 2016). There has been recent progress in the field of gastropod 3D geometric morphometrics, to understand variability in shell form (Liew et al., 2016) and changes in shell calcification associated with ocean acidification (Harvey et al., 2018). These analyses are beyond the scope of this study; however, the CT data are available on Morphosource and therefore can be used for morphometric analyses.

5 Conclusions

In this study, we analyzed the shell diameter, number of whorls, thickness, amount of shell material, and shell condition of *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~~ and therefore 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 trap samples collected when seawater temperatures were the highest, suggesting that dead specimens were affected by dissolution from ~~potentially linked to~~ increased rates of microbial breakdown of organic matter both in the water column, and 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 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 shell parameters and dissolution~~s~~ in the context of multiple biotic and abiotic factors, not just aragonite-saturation. We hope that the baseline dataset of pteropod shell parameters presented in this study is the first of many focused regional studies around the world. These datasets will enable the quantification of the response of this sentinel group to ocean acidification.

6 Data availability

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.

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)

9 Competing interests

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

10 Acknowledgements

The authors would like to thank B. Marshall for picking the first batch of specimens whilst in the midst of finishing her Ph.D, and C. Davis for picking the second set of samples, and for helpful discussion about sample processing. Light microscopy was performed in the Paleobotany Lab at the Pennsylvania State University thanks to P. Wilf, and at the Academy of Natural Sciences thanks to R. Thomas and C. Vito. Scanning electron microscopy was performed at the Nanoscale Characterization Facility at the Singh Center for Nanotechnology at the University of Pennsylvania, Philadelphia thanks to J. Ford. CT scanning was performed at GE in Lewistown thanks to J. Urbanksi and W. Yetter, and the American Museum of Natural History in New York thanks to M. Hill and M. Siddall. Thanks to T. Woodger and J. Foster for logistical support. We thank E. Tappa for information about the CARIACO sediment trap and for providing a map, and T. Bralower, M. Potapova and G. Rosenberg for discussions that helped to shape this manuscript. Thanks to K. Kimoto, A. Almogi-Labin, and an anonymous reviewer for their thoughtful, constructive comments which helped to improve this manuscript.~~improve this manuscript~~. This work was funded by the Deike Research Grant awarded to T. Bralower and R. Oakes, and R. Oakes was supported by the John J. & Anna H. Gallagher Fellowship, The Academy of Natural Sciences of Drexel University.

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12 Figures

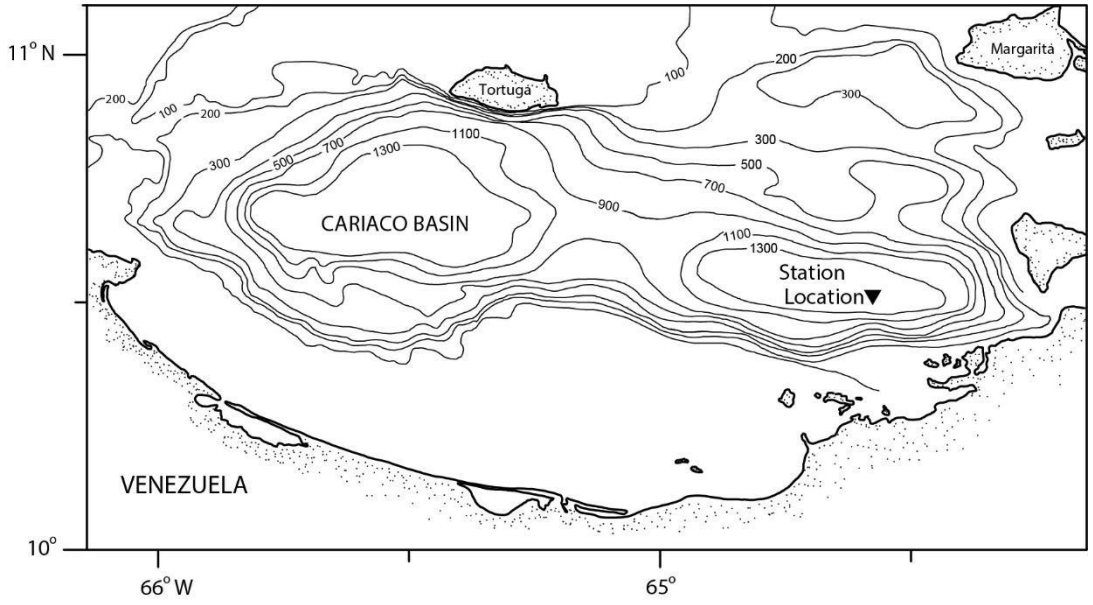


Figure 1: Bathymetric map of the Cariaco Basin. The location of the sediment trap (10° 30.0' N, 64° 38.5' W) is marked with a triangle (modified from Marshall et al., 2013).

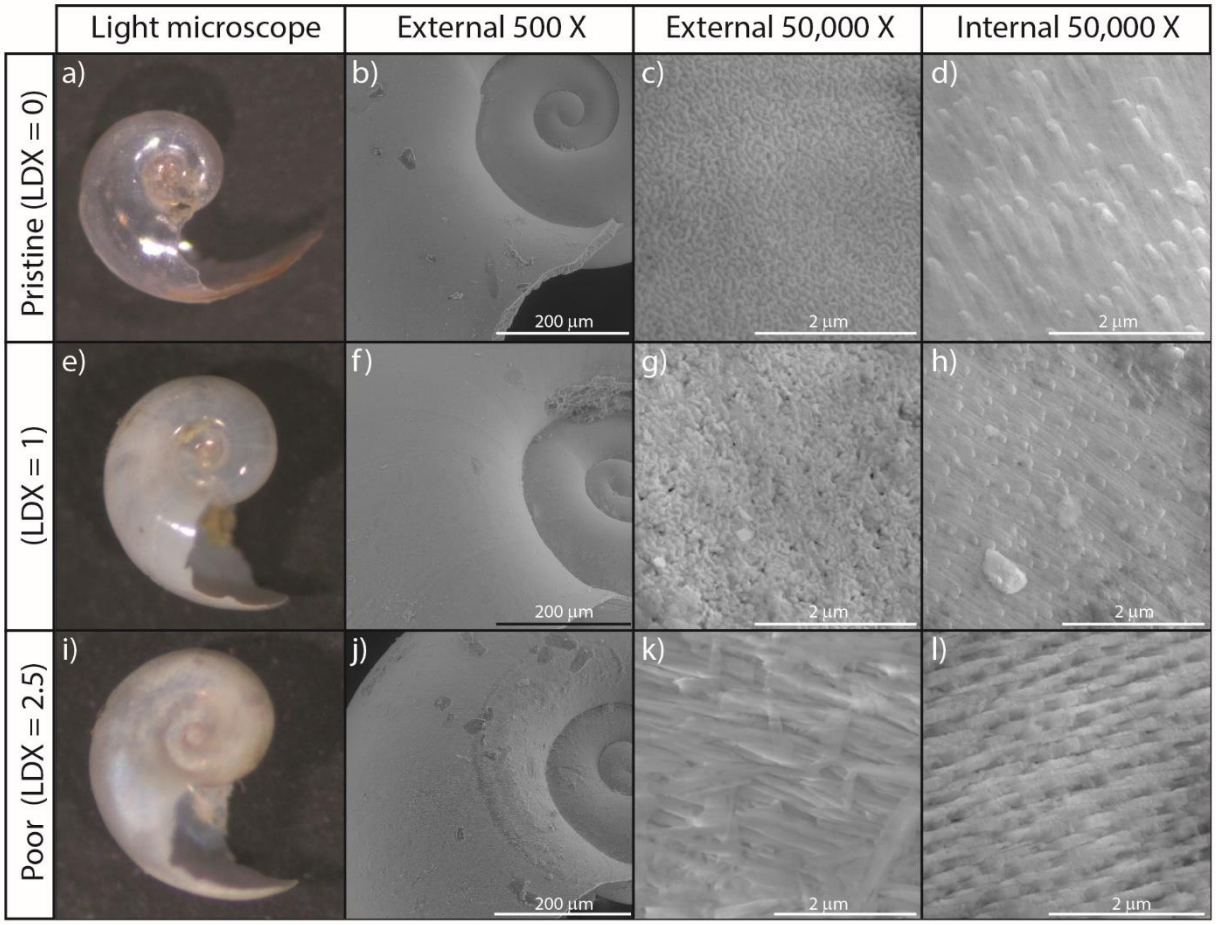


Figure 2: Light microscope (a, e, i), and scanning electron microscope images of the external (b, c, f, g, j, k), and internal (d, h, l) faces of *H. inflatus* shells from the Cariaco Basin. Light microscope images show the shell changing from pristine and glassy to opaque and white with increasing dissolution. This change is accompanied by an increase in pocking on the shell surface to reveal the tops of the prismatic crystals (c, g) and then the whole prismatic layer (k). The topography on the internal face is due to the terminations of the cross-lamellar crystals intersecting with the internal face (d, h). These become more distinct as dissolution increases the porosity of the internal face (l).

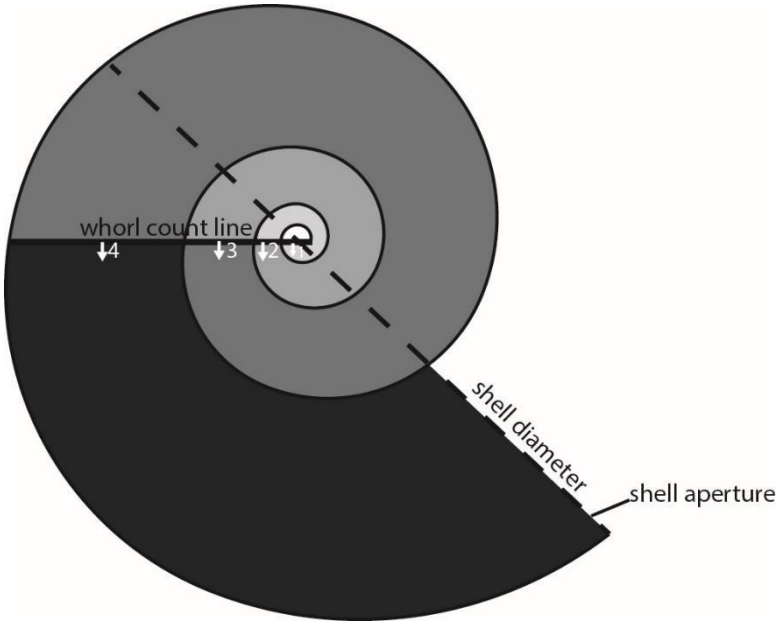


Figure ~~2~~3: Schematic diagram of a pteropod shell demonstrating how shell diameter (the metric used for size) was measured, and how the number of whorls was counted. Following the methods outlined in Janssen (2007), a straight line is drawn across the shell separating the semi-circular nucleus (center) 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.

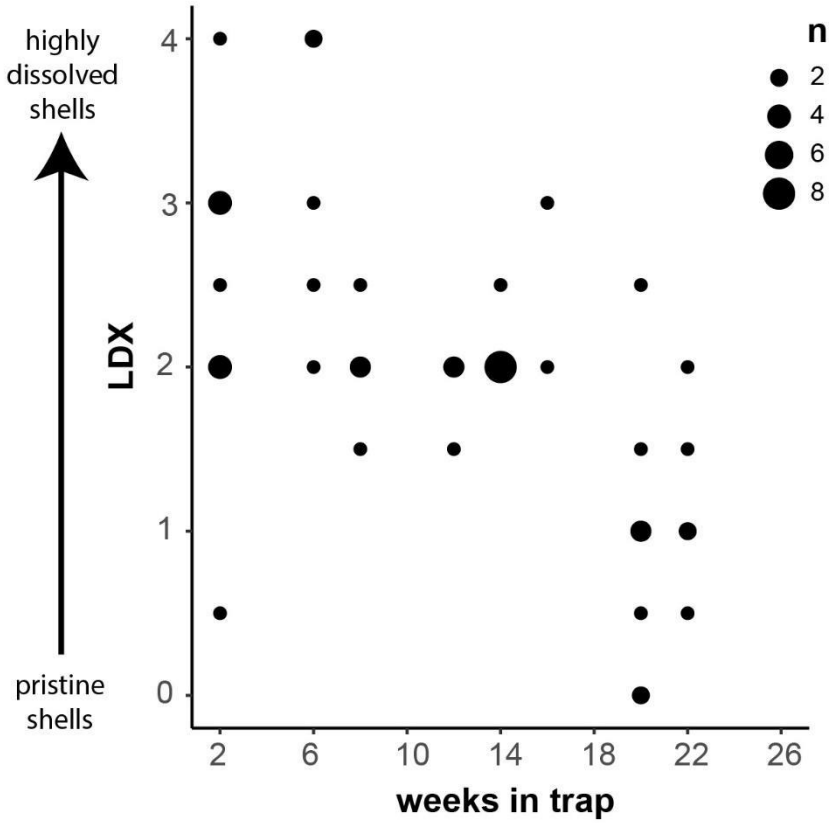
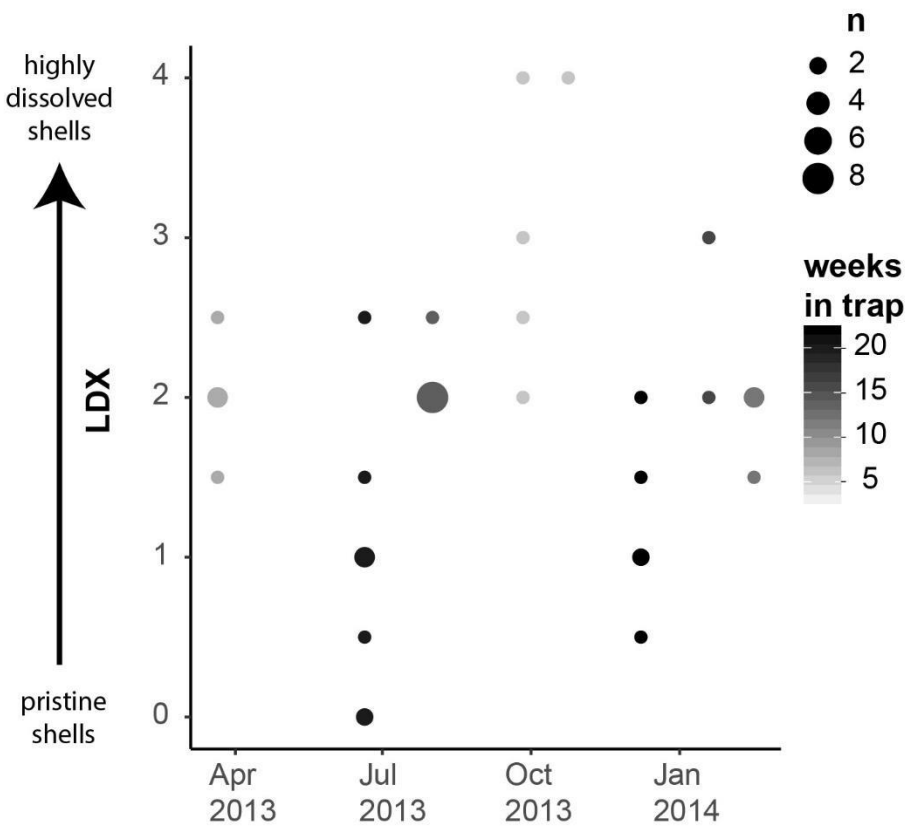


Figure 34: Shell condition of *Heliconoides inflatus*, ranked on the *Limacina* Dissolution Index (LDX) scale, plotted against the maximum amount of time specimens spent in the sediment trap (i.e., the number of weeks from the trap opening time). The size of the symbols corresponds to n, the number of specimens plotted at a given point.


highly
dissolved
shells
LDX
pristine
shells
4
3
2
1
0
Apr
2013
Jul
2013
Oct
2013
Jan
2014
n
2
4
6
8
weeks
in trap
20
15
10
5

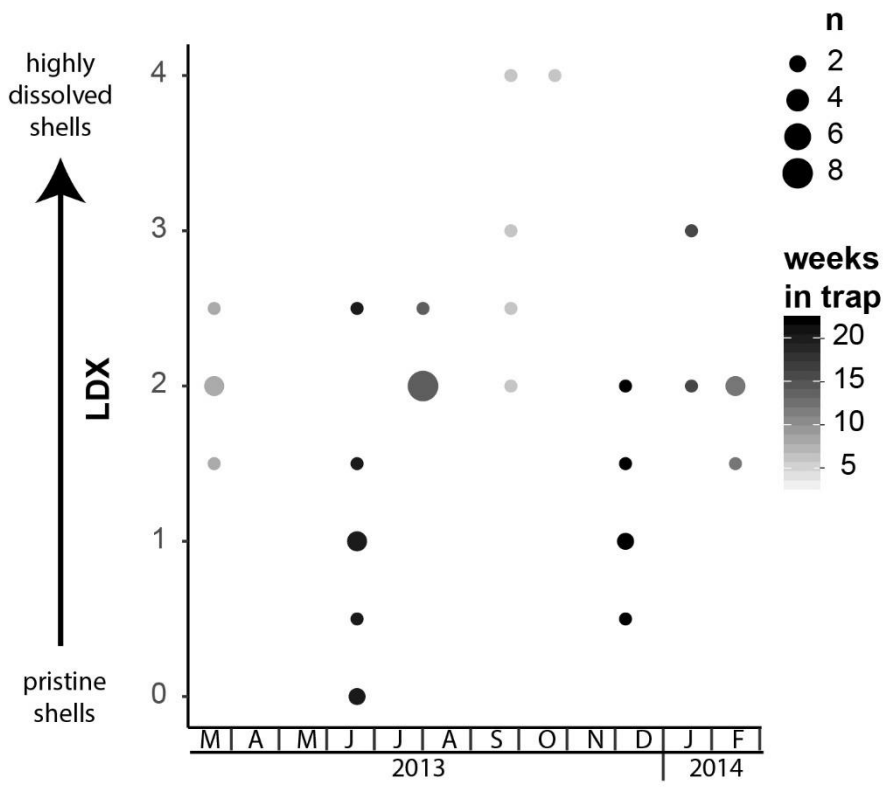


Figure 45: Shell condition of *Heliconoides inflatus*, ranked using the *Limacina* Dissolution Index (LDX) scale, over the study period. The samples with the poorest preservation are from September and October 2013 when water temperatures were the highest. The size of the circles corresponds to n, the 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.

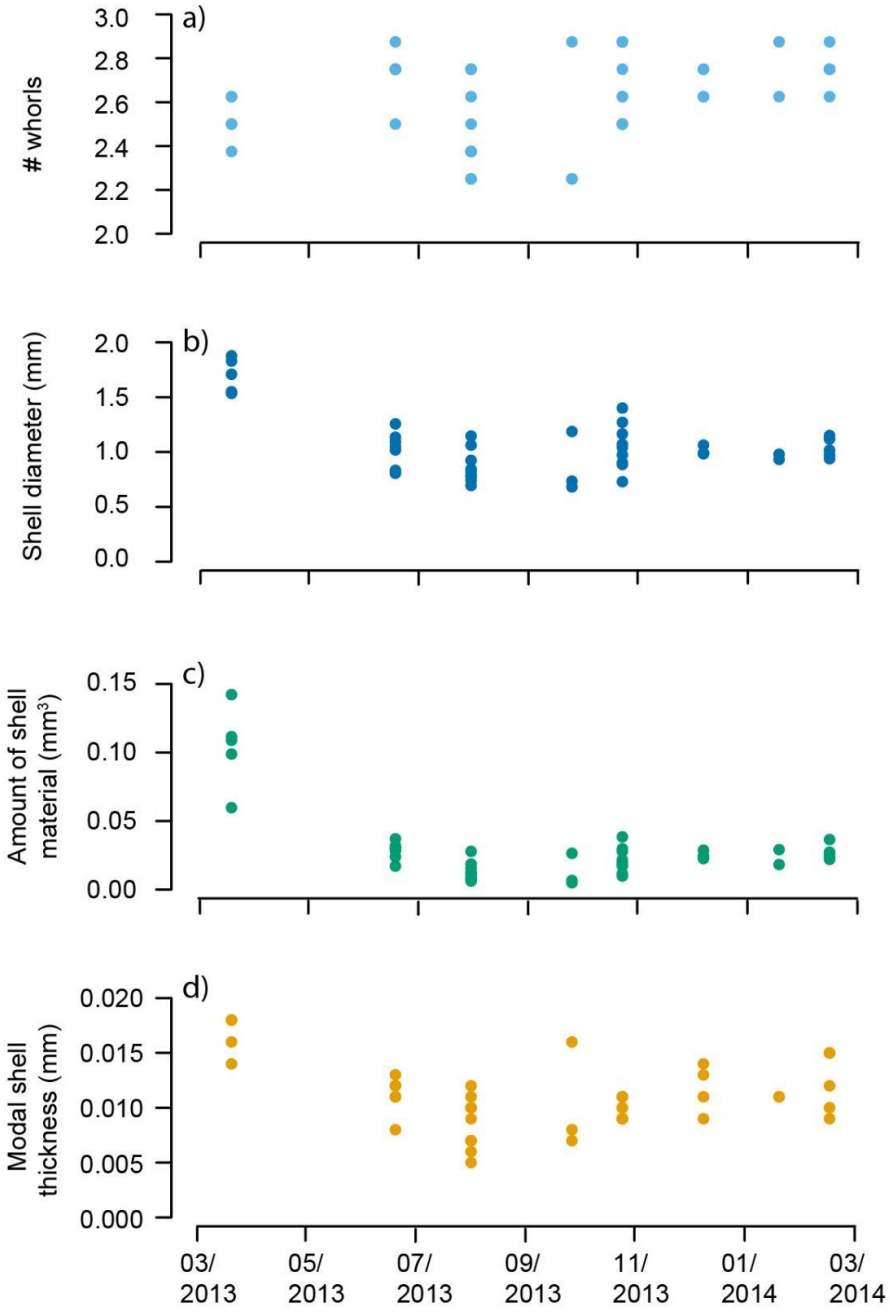


Figure ~~5~~6: *Heliconoides inflatus*: a) number of whorls; b) shell diameter; c) amount of shell material; and d) modal shell thickness throughout the year in the Cariaco Basin. Each point represents an individual specimen.

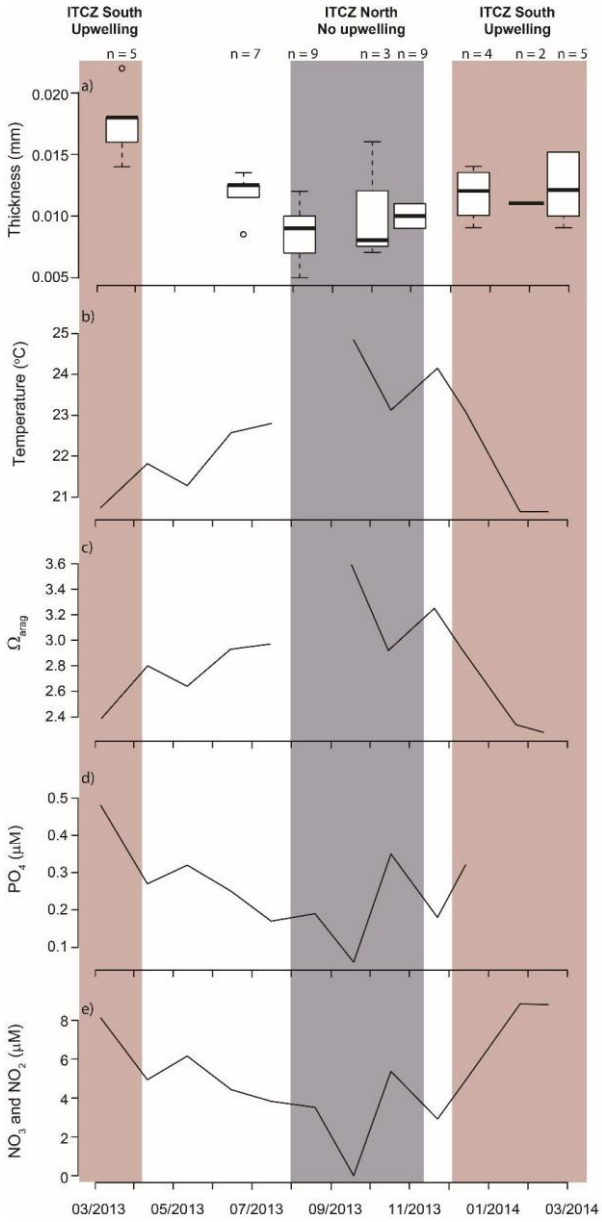


Figure ~~6~~7: Shell thickness and water column properties plotted over the study period: a) Heliconoides inflatus modal shell thickness, b) seawater temperature, c) Ω_{arag}, d) PO_4, and e) NO_2 and NO_3. Nutrient concentrations (d and e) are plotted as proxies for upwelling and food availability (Romero et al., 2009; Thunell et al., 2000). 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.

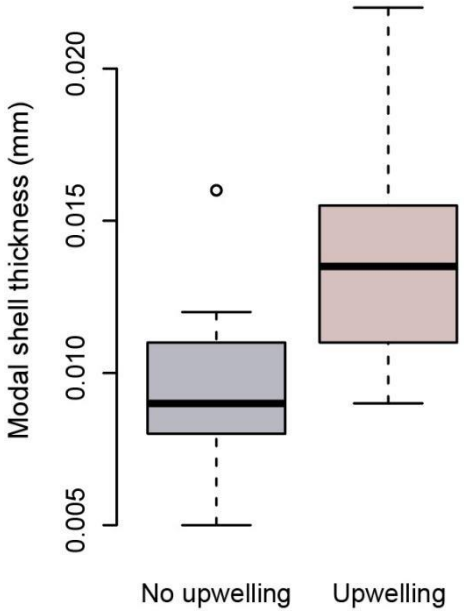


Figure ~~7~~8: Modal shell thicknesses of specimens from times of upwelling (red) and times of no upwelling (grey) in the Cariaco Basin. Specimens collected during times of upwelling are significantly thicker than those which formed at times with no upwelling (Welch's t-test: $p = 4.4 \times 10^{-4}$).

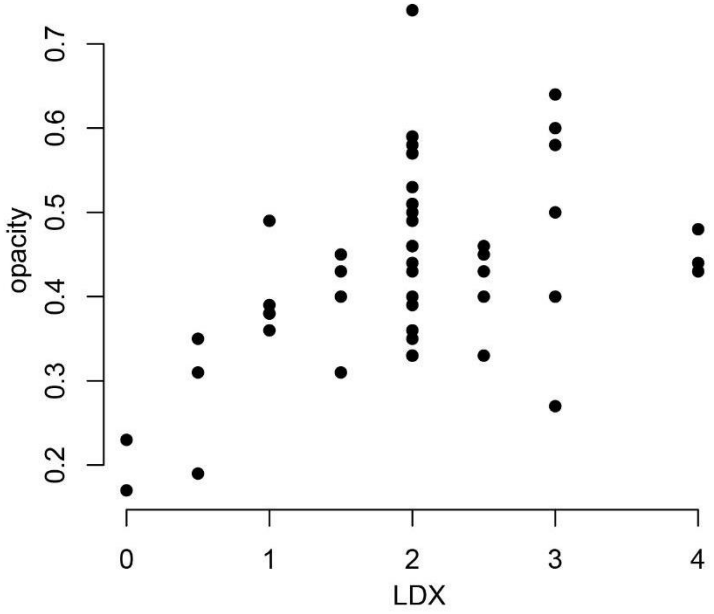


Figure ~~8~~9: Shell condition of *Heliconoides inflatus*, ranked on the *Limacina* Dissolution Index (LDX), plotted against shell condition of the same shells quantified using the opacity scale. LDX and opacity are positively correlated until LDX scores of 2, at which point there is no correlation between 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 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.