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Evidence for an effective defence against ocean acidification in the key bioindicator pteropod *Limacina helicina*

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The pteropod *Limacina helicina* has become an important bioindicator species for the negative impacts of ocean acidification (OA) on marine ecosystems. However, pteropods diversified during earlier high CO₂ periods in Earth history and currently inhabit regions that are naturally corrosive to their shells, suggesting that they possess mechanisms to survive unfavourable conditions. Recent work, which is still under considerable debate, has proposed that the periostracum, a thin organic coating on the outer shell, protects pteropods from shell dissolution. Here, we provide direct evidence that shows that damage to the *L. helicina* periostracum results in dissolution of the underlying shell when exposed to corrosive water for ~8 d, while an intact periostracum protects the shell from dissolution under the same conditions. This important first line of defence suggests that pteropods are more resistant to OA-induced shell dissolution than is generally accepted.

Keywords: aragonite, bioindicator, *Limacina helicina*, ocean acidification, periostracum, pteropod, shell dissolution.

Introduction

The ocean takes up an estimated 26% of all anthropogenic carbon dioxide (CO₂) emissions (Friedlingstein *et al.*, 2022), which slows the warming effects of climate change but also alters seawater carbonate chemistry through a process known as anthropogenic ocean acidification (OA) (Caldeira and Wickett, 2003; IPCC, 2011). This process has already decreased average open ocean surface pH by ~0.1 and carbonate ion concentration ([CO₃²⁻]) by >10% since preindustrial times (Caldeira and Wickett, 2003; Orr *et al.*, 2005; Franco *et al.*, 2021). Decreasing [CO₃²⁻] results in decreases in the calcium carbonate (CaCO₃) mineral saturation state (Ω). When $\Omega > 1$, abiogenic precipitation is thermodynamically favoured, and when $\Omega < 1$, abiogenic dissolution is favoured. As a result, OA has implications for species that build CaCO₃ shells or skeletons (e.g. bivalves, corals, and calcifying plankton such as pteropods) and is expected to have negative consequences for marine ecosystems in general (Fabry *et al.*, 2008; Kroeker *et al.*, 2013; Haigh *et al.*, 2015; Marshall *et al.*, 2017; Doney *et al.*, 2020). Ocean acidification constitutes an additional stressor to other challenges marine organisms face in a changing ocean (e.g. warming), and its effect will only increase as human activity continues to increase atmospheric CO₂ (Caldeira and Wickett, 2003; Sabine *et al.*, 2004; Orr *et al.*, 2005; Feely *et al.*, 2009).

The thecosome pteropod *Limacina helicina* is the focus of a large body of literature on the negative effects of OA (Bednaršek *et al.*, 2016b; Manno *et al.*, 2017). The thin (<10 µm) shells of pteropods are composed of the metastable

CaCO₃ polymorph aragonite (Lalli and Gilmer, 1989), which is >50% more soluble than the polymorph calcite in seawater (Mucci, 1983). As OA increases, seawater will become increasingly undersaturated with respect to aragonite ($\Omega_{Ar} < 1$) before calcite (Orr *et al.*, 2005), making it “corrosive” to that mineral. As such, pteropods are particularly sensitive to OA, and their decline could have broad impacts (Orr *et al.*, 2005; Fabry *et al.*, 2008; Manno *et al.*, 2017). Pteropods constitute important prey for juvenile pink salmon (Armstrong *et al.*, 2008), contribute to the diets of other species of fish, marine mammals, and seabirds (Lalli and Gilmer, 1989; Hunt *et al.*, 2008), and are the only prey of some non-shelled gymnosome pteropods (sea angels) (Lalli and Gilmer, 1989). Recent work has also shown that thecosome pteropods may dominate pelagic calcification on a global scale (Buitenhuis *et al.*, 2019).

Studies investigating the response of pteropods to lower Ω_{Ar} have generally found increases in shell dissolution, reductions in calcification, growth, and survival, and the precipitation of thinner, less-dense shells (Bednaršek *et al.*, 2016b; Manno *et al.*, 2017; Mekkes *et al.*, 2021a, b). As a result, pteropods have become model organisms for OA research and are deemed important bioindicators of the negative impacts of OA on marine ecosystems (Bednaršek *et al.*, 2016b, 2017). Some studies have even suggested that pteropods may be at risk of local extinctions within the century due to OA (Comeau *et al.*, 2012a; Bednaršek *et al.*, 2016b).

Yet, all modern pteropod clades diversified during previous high CO₂ periods in Earth history that caused the oceans

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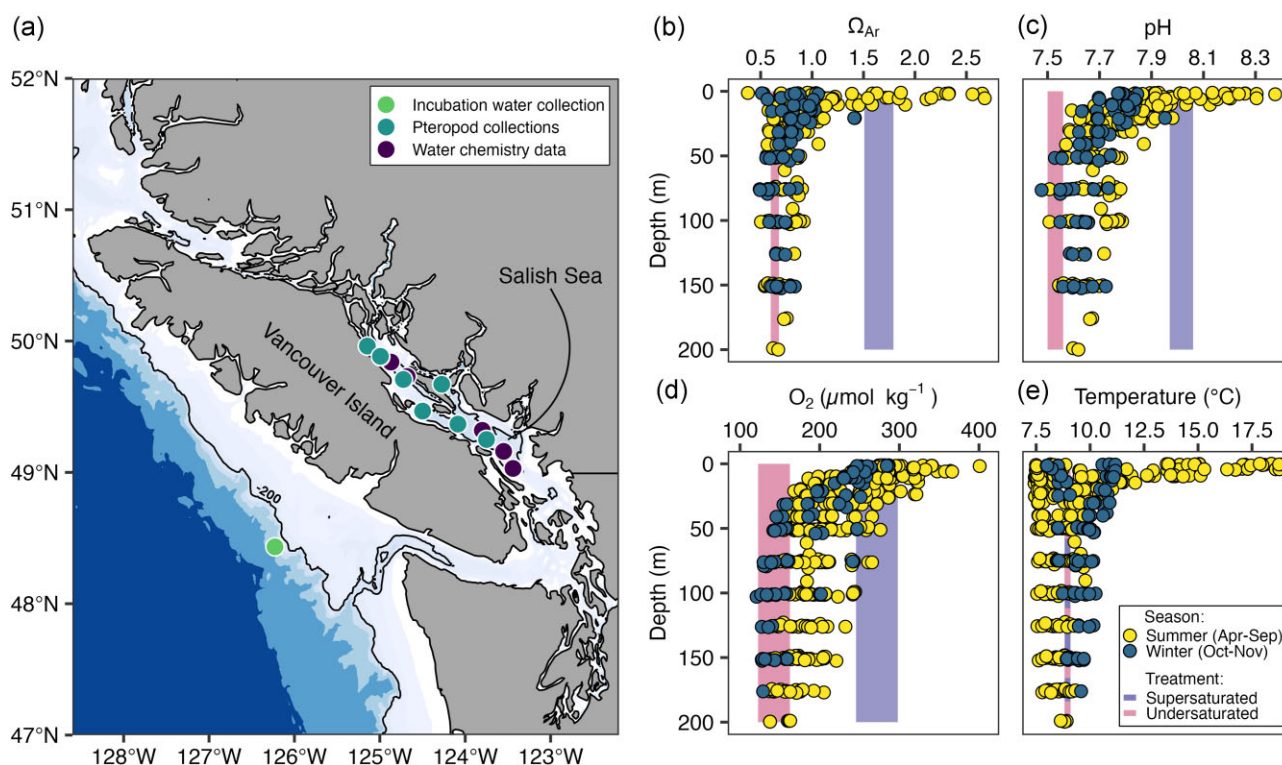


Figure 1. (a) Locations of seawater sampling and *Limacina helicina* collections. Seawater for pteropod incubations was collected on the outer coast of Vancouver Island at 50 and 300 m. Pteropods were collected by vertical net tows in the Salish Sea. The 200-m depth contour (black line) indicates the deep basins in the Salish Sea and roughly represents the shelf break on the outer coast. Deeper bathymetry is indicated by darker blue contours. The map was generated in the programme R v4.0.2 (R Core Team, 2020) using the CRAN packages “rnaturalearth” v0.1.0 (South, 2022), “sf” v0.9.7 (Pebesma, 2018), “marmap” v1.0.5 (Pante and Simon-Bouhet, 2013), “ggplot2” v3.3.2 (Wickham, 2016), and bathymetry data from the NOAA ETOPO1 database (<https://www.ngdc.noaa.gov/mgg/global/>). (b–e) Typical present-day seasonal *in situ* ranges of aragonite saturation state, pH, dissolved oxygen, and temperature experienced by *L. helicina* throughout their vertical habitat (upper 200 m) in the northern Salish Sea, determined using water chemistry data collected in 2010–2012 (Ianson et al., 2018), 2014 (Chandler et al., 2021a), and 2015 (Chandler et al., 2021b) (see Ianson et al. (2016) for dataset interpretation) during summer and winter (Supplementary Methods). Coloured vertical bands represent the range of conditions in the undersaturated and supersaturated incubation bottles over the course of the experiment (Ω_{Ar} , pH, and O_2 decreased, while temperature was constant).

to become more acidic (e.g. ~56 ma during the Paleocene–Eocene Thermal Maximum) (Hönisch et al., 2012; Janssen et al., 2016; Peijnenburg et al., 2019). Moreover, some species currently inhabit regions that are naturally corrosive to their aragonite shells, such as the northern Salish Sea (Mackas et al., 2013; Keil et al., 2021), where they are exposed to undersaturated conditions across 90–100% of their vertical habitat (upper 200 m) (Lalli and Gilmer, 1989) throughout the year (Ianson et al., 2016; Evans et al., 2019; Jarníková et al., 2022), and where there are minimal seasonal and annual differences in Ω_{Ar} and pH below ~20 m (Figure 1).

Pteropod shell dissolution rates have also been observed to be lower than those predicted for abiogenic aragonite (Bednaršek et al., 2014), and many calcifying organisms (including pteropods) are able to precipitate shells in undersaturated waters ($\Omega_{Ar} < 1$), albeit at reduced rates (Lischka et al., 2011; Comeau et al., 2012b; Waldbusser et al., 2015; Frieder et al., 2017; Mekkes et al., 2021a). Taken together, this evidence suggests that pteropods may possess mechanisms to counter thermodynamically unfavourable carbonate chemistry.

Recent evidence that the periostracum, the thin organic proteinaceous coating found on the outer layer of the shell of all shelled molluscs (Taylor and Kennedy, 1969; Harper, 1997; Johnson et al., 2019), may protect the pteropod shell from dissolution (Peck et al., 2016a), and that pteropods can

pair breaches in the shell caused by dissolution damage on the outer layer by thickening the shell from the inside (Peck et al., 2016a, 2018), provides insight into their potential resistance to OA-induced dissolution. However, the general understanding of the role of the pteropod periostracum remains limited, and considerable debate followed these recent findings (Bednaršek et al., 2016a; Peck et al., 2016b). Prior to work by Peck et al. (2016a), the *L. helicina* periostracum had generally been assumed to offer little to no protection from corrosive water (Bednaršek et al., 2012a, b, 2016b), and was not mentioned in the most comprehensive review of the pteropod literature to date (Manno et al., 2017). One factor that has likely contributed to this oversight is the fact that the pteropod periostracum is extremely thin (<1 μm) (Sato-Okoshi et al., 2010) relative to the shell and is challenging to discern even in scanning electron microscope (SEM) images. Given that pteropods have emerged as important bioindicators for OA (Bednaršek et al., 2012a, 2016b, 2017), there is a clear need to increase our understanding of this important organic layer and its ability to protect pteropod shells.

The periostracum of many other mollusc species protects the shell from dissolution by acidic water (Taylor and Kennedy, 1969; Saleuddin and Petit, 1983), for example, in deep-sea mussels living in acidic conditions (pH 5.36–7.29) near hydrothermal vents (Tunnicliffe et al., 2009) and in unionoidean bivalves living in acidic freshwater (Tevesz and

Carter, 1980). The primary function of the mollusc periostracum is to seal off the site of calcification from the outside environment and provide the scaffolding onto which the shell is secreted (Taylor and Kennedy, 1969; Harper, 1997; Johnson *et al.*, 2019). This strategy allows the animal to drastically increase the saturation state of the calcifying pallial fluid to levels that are highly supersaturated ($\Omega \gg 1$) compared to ambient conditions, creating a microenvironment conducive to precipitating CaCO_3 (Taylor and Kennedy, 1969; Saleuddin and Petit, 1983; Harper, 1997; Spalding *et al.*, 2017; Johnson *et al.*, 2019). Thus, once formed and intact, it acts as a physical barrier separating the outside of the CaCO_3 shell from the ambient environment.

To empirically test the protective nature of the pteropod periostracum, we intentionally damaged a small, specific area of the periostracum (while leaving the remaining periostracal surface undamaged) on 46 live *L. helicina* collected from the northern Salish Sea (Figure 1a). We then incubated them for 8 d in glass bottles (three to four individuals per bottle, seven replicate bottles per treatment) containing natural seawater that was either undersaturated or supersaturated with respect to aragonite (hereafter referred to as the undersaturated or supersaturated treatments). We compared post-incubation shell condition between the two treatments using a combination of light and scanning electron microscopy (SEM). SEM images of the shells from the supersaturated treatment were used to determine whether the process of damaging the periostracum caused unintentional physical damage to the underlying aragonite shell.

Methods

Water sampling and analyses

Water for incubations and analyses was collected during a research cruise aboard the CCGS *John P. Tully* (Sastri *et al.*, 2022) at the end of the coastal upwelling season (September) in 2019 in the northern California Current System, adjacent to the Salish Sea (Figure 1a), at 300 and 50 m depths on the continental shelf break, in addition to a full conductivity–temperature–depth profile. We anticipated that this water would have aragonite saturation states of ~ 0.5 (300 m, undersaturated) and 1.5 (50 m, supersaturated), based on previous data (Haigh *et al.*, 2015). Seawater collection and analysis for DIC and A_T followed standard methods (Dickson *et al.*, 2007). Incubation and control seawater were stored unpreserved in 500-ml borosilicate glass bottles sealed with Apiezon® L grease. Initial *in situ* water conditions (DIC, A_T , phosphate, and silicic acid) and final (post-incubation) DIC and A_T were measured at the Institute of Ocean Sciences in Sidney, BC, while initial and final DO concentrations were estimated from measured *in situ* DO adjusted for headspace gas exchange and pteropod respiration (Supplementary Methods). DIC and A_T were used to determine the carbonate system and associated uncertainties, along with phosphate, silicic acid, temperature, salinity, and pressure (Supplementary Methods). The cruise data are available as a published dataset (Sastri *et al.*, 2022), and all water chemistry data for the incubation samples are included in this article's Supplementary Materials as Supplementary Dataset 1.

Pteropod collection

Although pteropods are found routinely in the region where we collected the incubation water (West Coast Vancouver Island) (Mackas and Galbraith, 2012) (Figure 1a), abundances were unusually low during the research cruise. We encountered sufficient numbers in the northern region of the adjacent Salish Sea 6 d after incubation water collection and opted to collect live *L. helicina* from seven locations there (Figure 1a). Pteropods were collected by vertically towing a bongo-style plankton net with a 236 μm black nylon mesh from maximum depths ranging from 395 to 150 m (depending on station bottom depth) to the surface. Adult *L. helicina* were gently picked from the samples using a wide-mouth plastic pipette and observed under a stereo microscope. A few of the collected individuals (~ 4) appeared dead, had broken shells, or had large patches of shell dissolution, and were discarded. Individuals with small patches of dissolution confined to the centre one or two whorls were accepted since this condition is commonly observed on wild-caught specimens (and because pteropod abundance was limited). Due to its location on the shell, this type of dissolution is unlikely to interfere with the area where the manual damage was induced (the outermost one or two whorls) or influence dissolution on the rest of the shell.

Manual scratching of shells

Live pteropods were carefully held in place under the microscope using a fine-tipped paintbrush, while mechanical damage to the periostracum was induced by gently scratching a small, easily identifiable area on the outermost one or two whorls, directly opposite to the shell aperture, using a thin (00-gauge) black enamelled insect pin. Minimal pressure was applied while scratching to avoid damaging the aragonite beneath the periostracum, and scratching was performed for five seconds on each individual. All scratching was conducted by the same investigator to ensure as much consistency as possible, and the entire process (from collection to placement in the incubation bottles) was done within minutes to minimize animal stress. All individuals were scratched, and the remainder of each shell was used as an unscratched internal control, which ensured that both the control and scratched portions of the shell experienced exactly the same conditions.

Incubations and shell preservation

Immediately after scratching, the 46 pteropods were randomly divided amongst the 500-ml incubation bottles containing the undersaturated and supersaturated water, with five bottles containing three individuals and two bottles containing four individuals in each treatment (a total of seven incubation bottles containing pteropods in each treatment and 23 pteropods in total per treatment). Incubation bottles were immediately sealed and maintained in the dark in a chilled water bath at $9.0 \pm 0.42^\circ\text{C}$ for ~ 8 d, checked once each day to confirm that the pteropods were alive and swimming, and filmed at the end of the incubations (Supplementary Videos). Previous pteropod studies have shown 8-d incubations to be sufficient to observe shell dissolution (Manno *et al.*, 2012; Bednaršek *et al.*, 2012a, b; Comeau *et al.*, 2012b; Peck *et al.*, 2016a). As recommended by Peck *et al.* (2016b), only surviving and actively swimming individuals were used for further shell analysis since the internal dissolution of dead pteropods caused

Table 1. The number of individual *L. helicina* specimens that were incubated in either aragonite undersaturated or supersaturated water, survived the ~8 d incubation period, and were subsequently analysed for shell condition, as well as the occurrence rates of each type of dissolution observed on the analysed shells.

		Undersaturated treatment	Supersaturated treatment
Dissolution type observed on analysed shells:	Incubated:	23	23
	Survived:	18/23 (~78%)	20/23 (~87%)
	Analysed:	17/23 (~74%)	20/23 (~87%)
	Scratched region:	17/17 (100%)	0/20 (0%)
	Axial ridges:	17/17 (100%)	0/20 (0%)
	Radial peaks:	17/17 (100%)	0/20 (0%)
	Centre whorls:	9/17 (~53%)	12/20 (60%)

The shell of one of the 18 surviving individuals from the undersaturated treatment was accidentally crushed during imaging and was excluded from further analysis.

by the breakdown of organic tissue creates a cloudy appearance that may mask external dissolution in light microscope images. Bottles were filmed at the end of the incubations, and then individual pteropods were removed from the bottles and filmed under a stereomicroscope to further document their survival (Supplementary Videos). Videos for bottles #7 and #8 were not recorded, but videos of each individual from these bottles are provided in the Supplementary Videos. Bottle #24 was not filmed since none of the four pteropods survived. The incubation water in the bottles was immediately preserved with 100 µL of supersaturated HgCl₂ for DIC and A_T analyses. To preserve the shells and minimize salt crystallization on their surface, live individuals were quickly (<1 min) euthanized in a buffered deionized water rinse (saturated with CaCO₃) to avoid shell dissolution, air-dried in front of a small space heater for 24-h, and stored in individual slide wells until further analysis (Peck *et al.*, 2016b; Oakes *et al.*, 2018). The survival rate between treatments was tested using a generalized linear mixed-effects model with a binomial error distribution and bottle number as a random effect (Supplementary Methods).

Control water samples

Three control water samples were preserved and analysed for each treatment; the first was preserved immediately after the incubation water was collected to represent the initial conditions (Table 2). There was then a 6-d gap between water collection and encountering adult *L. helicina*, so a second control sample from each treatment was preserved once pteropods were added to the incubation bottles in order to determine whether the initial carbonate chemistry had changed during those 6 d. A third control sample not containing any pteropods was also incubated for each treatment in the same way as the pteropod incubations and was preserved when the incubations were ended to determine whether background microbial respiration had influenced the carbonate system during the incubations (Supplementary Figure S1).

Light microscope and SEM imaging

Dried shells were imaged and their diameters measured using a Leica MZ6 stereo microscope with a Zeiss CL 4500 LED light source, a Leica MD 170 HD camera, and Leica Application Suite X[®] version 3.0.11 software. Both the objective lens and the light source were fitted with polarized filters to minimize glare that would otherwise create overexposed areas in images. Shell diameter was measured following Oakes and

Sessa (2020) using a line tool from the widest part of the shell at the aperture, transecting the apex of the shell, to the opposite edge. Additionally, all shells were imaged using a Hitachi S-4800 field emission scanning electron microscope (SEM) at an acceleration voltage of 1.0 kV and 8.00 mm working distance at the University of Victoria's Advanced Microscopy Facility. No chemical treatment of shells was performed (as recommended by Peck *et al.* 2016a), and shells were SEM imaged without coating to avoid any potential further alterations to the extremely thin and delicate periostracum. Three images were taken of each shell: one at 30× showing the entire shell, one at 80× showing the scratched region, and one at 150× to enhance the view of the scratches. Higher magnification images of the scratches were taken on some individuals and to document interesting features such as patches of centre whorl dissolution. Light and SEM images of all specimens can be found in Supplementary Figures S3 and S4.

Results

Survival and behaviour

Pteropod incubations lasted 8.3 ± 0.7 d (mean $\pm$ SD). Survival during the incubations was high and did not significantly differ between treatments ($p = 0.88$) (Supplementary Figure S5, Supplementary Table S2), with 18 of 23 individuals (~78%) and 20 of 23 individuals (~87%) surviving the undersaturated and supersaturated treatments, respectively (Table 1). Four of the five mortalities in the undersaturated treatment were from the same incubation bottle. Only the surviving individuals were included in the subsequent analysis. The mean diameter of all analysed shells was 3.0 ± 0.3 mm ($n = 37$).

Pteropod behaviour was typical of that observed in previous incubation experiments (Howes *et al.*, 2014), consisting of repeated periods of upward swimming and downward sinking, followed by periods of remaining on the bottom. Except for the few mortalities, all other individuals were observed swimming each day throughout the incubation period (Supplementary Videos). One surviving specimen from the undersaturated treatment was accidentally crushed during light microscope imaging and was excluded from further analysis.

Scratched region of shell

Post-incubation, all 17 shells analysed from the undersaturated treatment showed dissolution in the small area where the periostracum had been manually scratched prior to incubations, which appeared opaque in light microscope images

Table 2. Comparison of the water chemistry between two natural seawater types (undersaturated and supersaturated with respect to aragonite) collected for *L. helicina* incubation experiments.

	Undersaturated			Supersaturated		
	Initial (<i>n</i> = 1)	Final (<i>n</i> = 6)	Δ	Initial (<i>n</i> = 1)	Final (<i>n</i> = 7)	Δ
DIC (μmol kg ⁻¹)	2264 ± 6	2292 ± 8	28 ± 10	2003 ± 6	2039 ± 11	36 ± 13
A _T (μmol kg ⁻¹)	2273 ± 5	2283 ± 4	10 ± 7	2163 ± 5	2167 ± 3	4 ± 6
pCO ₂ (μatm)	1339 ± 84	1563 ± 108	224 ± 134	370 ± 18	467 ± 44	97 ± 48
pH	7.56 ± 0.03	7.50 ± 0.03	-0.06 ± 0.04	8.06 ± 0.02	7.97 ± 0.04	-0.09 ± 0.04
Ω _{Ar}	0.68 ± 0.04	0.60 ± 0.04	-0.08 ± 0.06	1.8 ± 0.1	1.5 ± 0.1	-0.3 ± 0.1
O ₂ (μmol kg ⁻¹)	163 ± 1	122 ± 11	-41 ± 11	298 ± 1	245 ± 17	-53 ± 17
O ₂ sat (%)	57.5 ± 0.4	43 ± 4	-14 ± 4	104.1 ± 0.4	86 ± 6	-19 ± 6

Initial conditions [measured values ± standard uncertainties for dissolved inorganic carbon (DIC), and total alkalinity (A_T), and calculated values ± combined standard uncertainties for pCO₂, pH, and Ω_{Ar}] represent the conditions of the incubation water at the time of collection, except for O₂ which is the adjusted initial value ± standard uncertainty (Supplementary Methods). Two additional water samples from each treatment, one preserved at the start and the second at the end of the pteropod incubations act as control samples (Supplementary Figure S1). These samples fell within the standard uncertainties of the initial conditions of their respective treatments. Final conditions are expressed as the mean ± standard deviation of all incubation bottles within each treatment after the ~8 d pteropod incubations (undersaturated: *n* = 6, supersaturated: *n* = 7), excluding one bottle from the undersaturated treatment containing four pteropods that experienced 100% mortality (Supplementary Figure S1). The change in water chemistry parameters over the incubation period (Δ ± propagated uncertainty) was calculated as the difference between initial and final measurements. O₂ saturation, pCO₂, pH, and Ω_{Ar} were calculated at incubation temperature (9.0°C), ambient lab pressure (1 atm), and practical salinities of 34.0 and 32.4 for the undersaturated and supersaturated treatments, respectively.

and revealed deep damage in the SEM images (Table 1, Figure 2a–c, Supplementary Figure S4). In contrast, none of the 20 shells from the supersaturated treatment showed any dissolution in the manually scratched region (Table 1, Figure 2d–f, Supplementary Figure S3). SEM images of shells from the supersaturated treatment (where no dissolution occurred) indicate some variability in the strength of the scratches. As such, these shells were divided into three categories based on the visibility of the scratches: the scratches were not visible on four individuals (Figure 2d), almost undetectable on five individuals (Figure 2e), and visible on the remaining 11 individuals (Figure 2f) (Supplementary Figure S3). Inspection of the visible scratches under higher magnification SEM revealed that they had almost no depth (e.g. Figure 2f, Supplementary Figure S3), indicating that manually induced damage to the shell (if any) was confined to the upper prismatic layer.

Dissolution along the axial ridges and radial peaks of whorls

Shell dissolution was also observed along the raised axial ridges (perpendicular to whorls) between shell growth bands on the outermost whorl (Figure 3a–c), and along the radial peaks (i.e. most elevated point of the convex whorl surface, running parallel to whorl direction) of the inner whorls (Figure 3g–i) on all individuals from the undersaturated treatment (Table 1). The axial ridge dissolution followed almost the entire length of these raised ridges but terminated where the whorl starts to curve back down towards the suture between the two outermost whorls (Figure 3a–c). The pattern of dissolution along the radial peaks can be connected with a straight line drawn on a side-profile SEM image of the shell (Figure 4). As such, the raised axial ridges and the radial peaks would have been the main points of contact between the shell and the walls of the glass incubation bottles as the pteropods bumped into the sides while swimming or remaining on the bottom. The depth of this dissolution is similar to that of the dissolution observed along the manually induced scratches. In contrast, SEM images of the shells from the supersaturated treatment revealed no visible damage along any of the axial ridges (Figure 3d–f) or radial peaks (Figure 3j–l) (Table 1), indicating that the contact with the glass did not cause visible damage to the shell even though it may have worn away

the periostracum. In the undersaturated treatment this damage to the periostracum allowed undersaturated water to directly contact the shell and cause dissolution.

Centre whorl dissolution and remainder of shell

Upon collection, roughly half of the individuals in each treatment displayed small opaque white patches of dissolution confined to the centre one or two shell whorls (e.g. Figure 5) (Table 1). The locations of these patches did not overlap with the manually scratched regions. Apart from the manually induced scratches, axial ridges, radial peaks, and centre whorls, no dissolution was evident on the remaining shell surfaces of the individuals from the undersaturated treatment, and much of the shell surface between axial ridges and on the slopes of the inner whorls appeared pristine [i.e. translucent under light microscope, smooth under SEM (Oakes *et al.*, 2018; Peck *et al.*, 2018; Mekkes *et al.*, 2021a)] (e.g. Figure 3a–c, g–i).

Seawater chemistry conditions in the incubations

Dissolved inorganic carbon (DIC) and total alkalinity (A_T) in control incubation bottles not containing any pteropods did not change from the initial conditions (Δ within standard uncertainties) (Supplementary Figure S1), indicating that background microbial respiration prior to and during the 8-d incubation period was negligible. DIC in the pteropod incubation bottles increased from the initial conditions (Δ > standard uncertainty) by similar amounts in both treatments (Table 2, Supplementary Figure S1), which we assume was due to pteropod respiration. A_T in the pteropod incubation bottles did not change from the initial conditions (Δ within standard uncertainty) in both treatments (Table 2, Supplementary Figure S1). The changes in DIC in the incubation bottles caused pH and Ω_{Ar} to decrease from the initial conditions (Δ > combined standard uncertainties) in both treatments by the end of the incubations, although the supersaturated treatment remained strongly supersaturated (Table 2, Supplementary Figure S1).

Dissolved oxygen (DO) was not measured at the end of the incubations due to sample volume requirements for DIC and A_T analyses, but the increases in DIC were likely associated with the cumulative O₂ demand by pteropod respiration during the incubations and, as such, would have

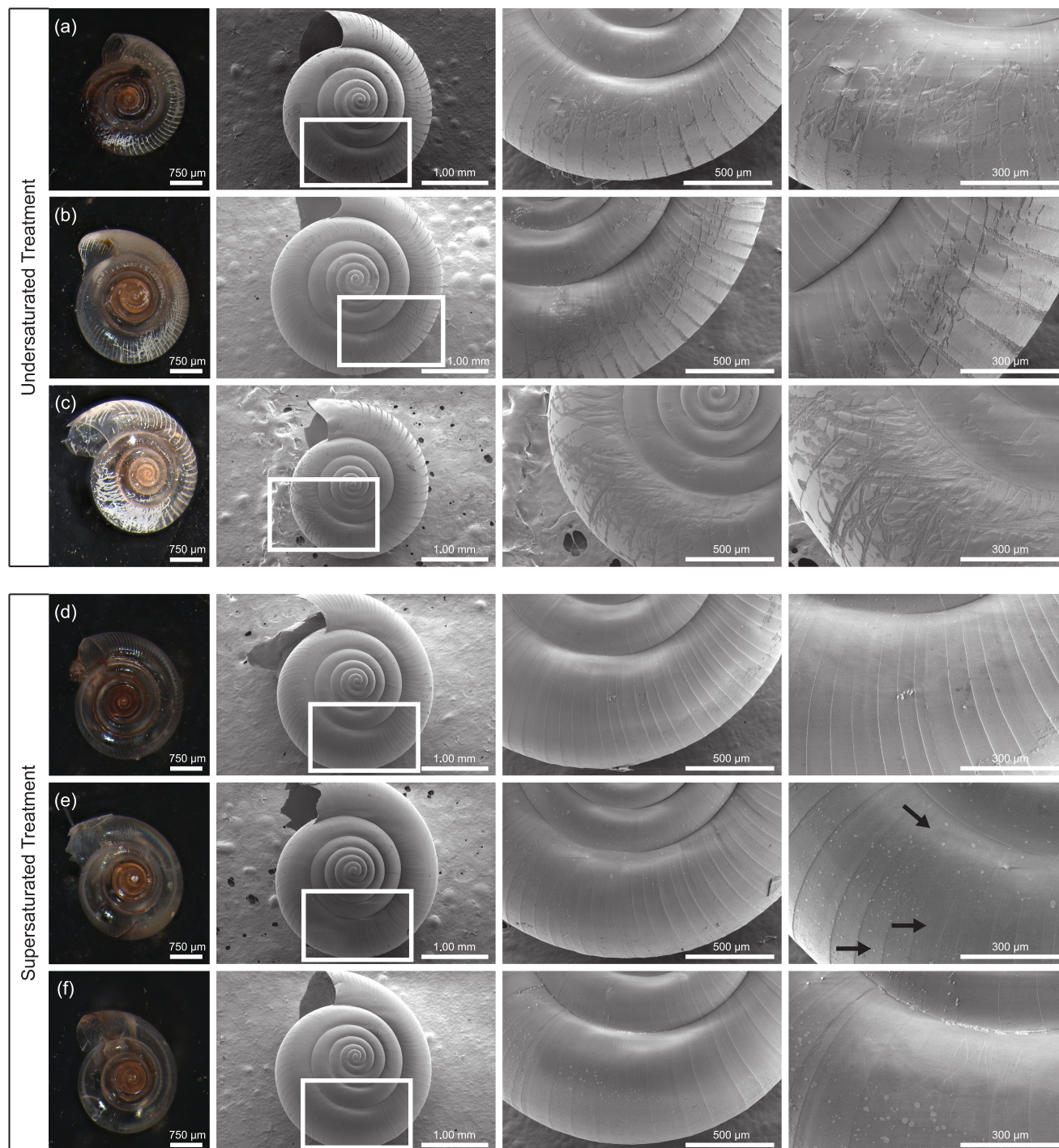


Figure 2. Light microscope (left column) and SEM (three rightmost columns) images of six *L. helicina* specimens (rows a–f) whose periostraca were subjected to manually induced damage (on the outermost one or two whorls, opposite the aperture) prior to incubation in natural seawater for 8 d. Individuals (a–c) were incubated in conditions undersaturated with respect to aragonite, while individuals (d–f) were incubated in conditions supersaturated with respect to aragonite. Each specimen was chosen to represent the observed ranges in degree of shell dissolution along scratches (increasing from a to c) and visibility of manually induced abrasion (d = scratches not visible, e = scratches almost undetectable (black arrows), f = scratches visible) observed amongst the total pool of specimens.

resulted in similar decreases in DO concentrations in both treatments (Table 2). Given the replenishment of O_2 from the bottle headspace when the bottles were opened, DO concentrations (Table 2) remained well above the average threshold for hypoxia used in the literature [$70.4 \pm 3.0 \mu\text{mol kg}^{-1}$ (Vaquer-Sunyer and Duarte, 2008)] and were more than sufficient for pteropods (Supplementary Methods, Supplementary

Table S1, and Supplementary Figure S2). Although experimental conditions (Ω_{Ar} , pH, and DO concentrations) changed during the incubations (except for temperature, which was held constant), all remained within the natural seasonal ranges typically experienced by *L. helicina* over its vertical habitat [0–200 (Lalli and Gilmer, 1989) m] in the Salish Sea (Figure 1).

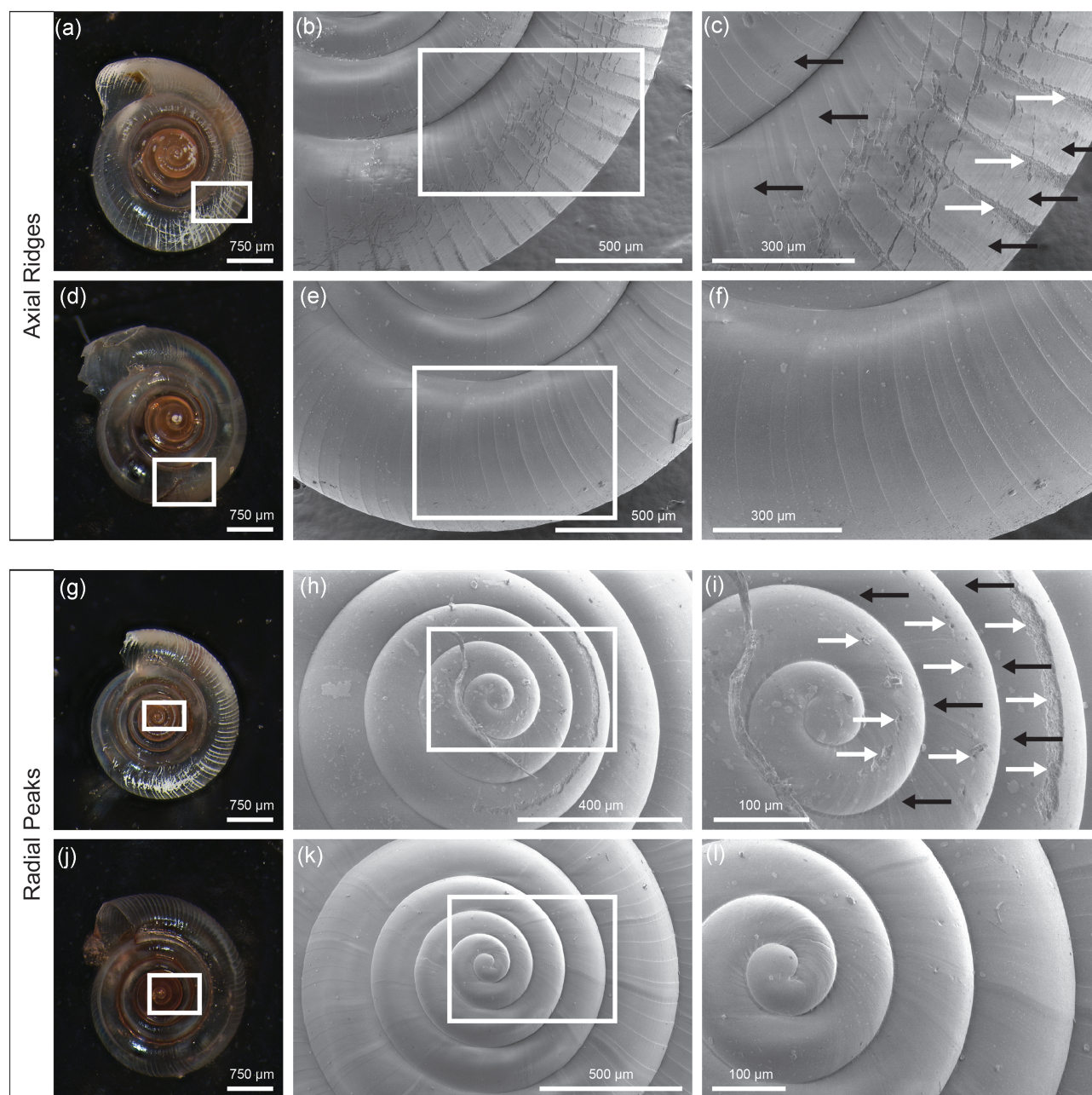


Figure 3. Light microscope (left column) and SEM (two rightmost columns) images contrasting the conditions of the axial ridges and radial peaks on the shells of *L. helicina* incubated for 8 d in natural seawater that was either undersaturated (a–c, g–i) or supersaturated (d–f, j–l) with respect to aragonite. White arrows in (c) indicate dissolution observed along the axial ridges, and in (i) indicate the dissolution along the radial peaks of the inner whorls. Black arrows indicate areas between the axial ridges and radial peaks that remained pristine on individuals from the undersaturated treatment. No shell damage was detectable along the axial ridges (d–f) or radial peaks (j–l) on individuals from the supersaturated treatment. Some salts/minerals appear to have precipitated on some shells during preservation [e.g. in panels (f) and (l)], but these precipitates can be distinguished from dissolution damage because they appear to sit on top of the shell rather than appearing as a cavity in the shell. A fibre is seen on the surface of the shell in panels (h) and (i), which likely fell onto the shell prior to being placed in the SEM.

Discussion

This study represents the first experiment to physically manipulate the integrity of a pteropod periostracum in order to test its ability to protect the shell from dissolution in undersaturated water. We intentionally damaged a small, specific area of the periostracum on live *L. helicina* before incubation in seawater that was either undersaturated or supersaturated with respect to aragonite. Resulting light microscope and SEM images reveal that dissolution only occurred where the ptero-

pod periostracum had been damaged, both intentionally from manual scratching and incidentally from contacting the glass walls of the incubation bottles during the experiment, and the shell was exposed to aragonite undersaturated water (Figure 2a–c, Figure 3a–c, g–i, Supplementary Figure S4). In contrast, areas where the periostracum remained intact did not dissolve despite being exposed to the same undersaturated conditions (Figure 3c, i, Supplementary Figure S4). These results, which occurred in 100% of the individuals from the undersaturated

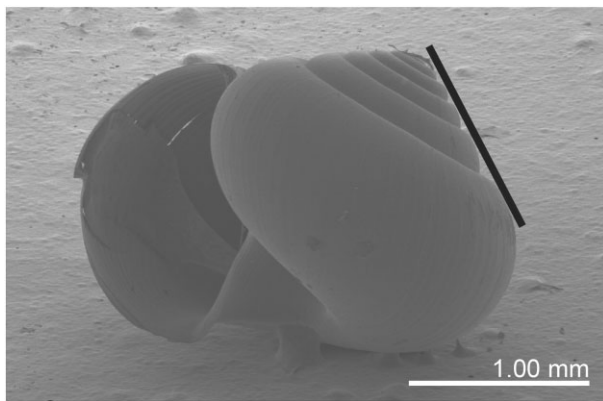


Figure 4. Side-profile of a *L. helicina* shell. The black line is a cross-sectional representation of a flat surface (such as the walls of the incubation bottle), and indicates that only the radial peaks (most elevated points of the whorls) are affected when such a surface contacts anywhere on the shell spire (e.g. when the animal bumps into the sides or bottom of the incubation bottle while swimming).

treatment, provide strong evidence that the *L. helicina* periostracum protects the shell from dissolution in undersaturated water.

Incidental damage to the periostracum (and associated dissolution) occurred during the incubations along the axial ridges of the outer whorl and the radial peaks of the inner whorls that was not anticipated (Figure 3). This damage confirms and enhances our findings by adding a consistent level of periostracum damage that was not manually induced by an experimenter. It was observed that the pteropods repeatedly contacted the walls of the incubation bottles as they swam around or periodically remained on the bottom (Supplementary Videos). Since the axial ridges and radial peaks are the highest points of the shell (Figure 4), they would have made contact with the glass walls and prevented the lower-elevation points between them from making contact. Dissolution was observed along the axial ridges of the outer whorl and the radial peaks of the inner whorls on 100% of the shells from the undersaturated treatment, even though these areas were not manually scratched (Figure 3a–c, g–i, Supplementary Figure S4). These same regions were pristine on 100% of the shells from the supersaturated treatment, indicating that the aragonite below the periostracum was not directly damaged by the process of contacting the glass walls (Figure 2d–f, Figure 3d–f, j–l, Supplementary Figure S3, Table 1). We therefore infer that the observed dissolution on the axial ridges and radial peaks of the shells from the undersaturated treatment was caused by a breach in the periostracum that resulted from contacting the glass walls, which allowed undersaturated water to come into direct contact with the aragonite shell. Any natural damage to the periostracum that may have occurred prior to collection (and thus resulted in dissolution during incubation) should be randomly dispersed on the shell since pteropods do not typically encounter hard flat surfaces in their natural environment. Busch *et al.* (2014) also observed similar axial ridge dissolution on *L. helicina* (also collected from the Salish Sea) after incubating them in undersaturated water and suggested that these ridges are simply more prone to dissolution than the rest of the shell. We contend that it is more likely that this dissolution was caused by the periostracum being worn away

from repeated contact with the surfaces of their incubation containers as in our study.

Except for the small patches of dissolution observed upon collection on the centre one or two whorls, the remaining unscratched internal control areas of the shells between the axial ridges and radial peaks remained pristine on 100% of the shells from the undersaturated treatment (Figure 3c, i, Supplementary Figure S4). This stark contrast in shell condition in the undersaturated treatment between areas that sustained damage to the periostracum (i.e. the manually scratched areas and the axial ridges and radial peaks), where dissolution was observed, and the remaining internal control areas of the shell that did not sustain periostracum damage and did not dissolve demonstrates the protective properties of the pteropod periostracum. We conclude that the external shell surface of a healthy, living adult *L. helicina* is protected from dissolution in waters undersaturated with respect to aragonite if the periostracum remains intact, while a breach in the periostracum allows dissolution to occur.

Protection offered by the periostracum helps explain how *L. helicina* manages to deal with natural exposures to aragonite-undersaturated water, for example, throughout their evolutionary history during high CO₂ periods such as the Paleocene–Eocene Thermal Maximum (Hönisch *et al.*, 2012; Janssen *et al.*, 2016; Peijnenburg *et al.*, 2019), or currently through their distributions in regions with naturally acidic water such as the Salish Sea (Mackas *et al.*, 2013; Keil *et al.*, 2021), the upwelling region of the California Current System (Mekkes *et al.*, 2021b), and the Canada Basin of the Arctic Ocean (Comeau *et al.*, 2012b). One study has assessed *in situ* *L. helicina* shell condition in the Salish Sea (Bednaršek *et al.*, 2021), and although they observed shell dissolution associated with low Ω_{Ar} , the authors also noted that this species persists in these chemically unfavourable conditions, possibly due to life history and physiological adaptations. Busch *et al.* (2014) also noted that the persistence of this species in the corrosive conditions of the Salish Sea warranted further investigation. Some pteropod species also appear to perform diel vertical migrations (Wormuth, 1981) which may expose them daily to undersaturated waters in regions with shallow aragonite saturation horizons such as the Salish Sea (Ianson *et al.*, 2016; Evans *et al.*, 2019; Jarníková *et al.*, 2022) or the California Current System (Mekkes *et al.*, 2021b).

Retaining a thin periostracum as a defence against seawater that is corrosive to CaCO₃ would be a useful evolutionary strategy for a holoplanktonic calcifying species since this layer is lightweight and, as it consists of non-living tissue, requires no energy expenditure once formed. Except in polar oceans where sea ice is present, pteropods are unlikely to regularly encounter solid objects or particles that would cause abrasive damage to the periostracum. Nonetheless, other factors may potentially damage the periostracum over the pteropod life cycle. Being comprised of organic material, the periostracum is subject to microbial degradation (Harper, 1997). Little is known about this process in pteropods, with only one study to date investigating the link between bacteria and shell dissolution, which found no significant effect, although they did not focus on the periostracum (Bausch *et al.*, 2018). Failed predation attempts by predators that do not ingest the whole shell (e.g. the predatory gymnosome pteropod *Clione limacina*) may cause scratch-like damage to the periostracum (Peck *et al.*, 2016a, 2018). The centre whorls are the most common area that dissolution is observed on the shells of *L.*

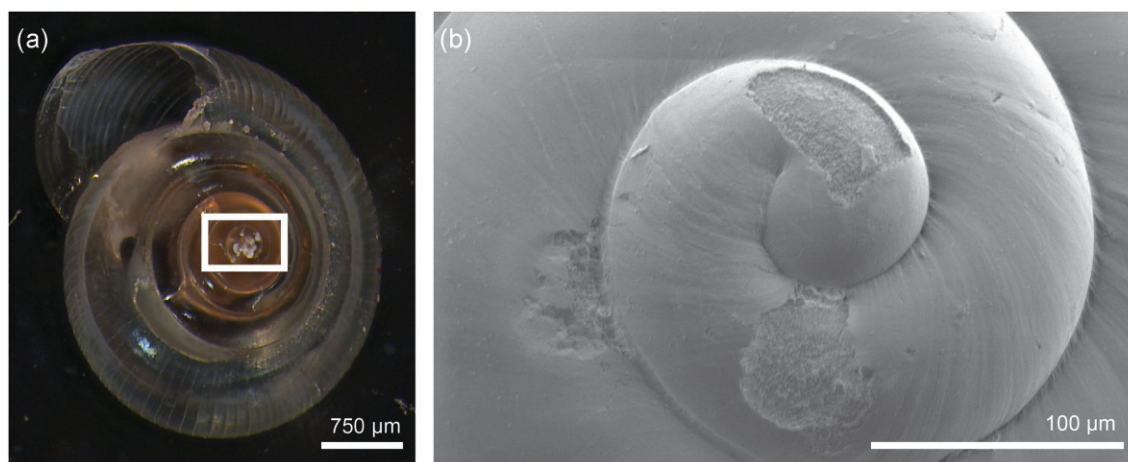


Figure 5. Light microscope (a) and SEM (b) images of shell dissolution on the centre two whorls of a *L. helicina* shell collected from the northern Salish Sea and incubated in natural seawater supersaturated with respect to aragonite for 8 d.

helicina (Peck *et al.*, 2016a; Mekkes *et al.*, 2021b), including on roughly half of our shells, suggesting that natural damage to the periostracum on the centre whorls is common. These whorls are the oldest parts of the shell and have therefore had the most time for microbial degradation and failed predation attempts to occur (Peck *et al.*, 2016a). Additionally, the periostracum may be thinner on the centre whorls when the animal is first starting to secrete its shell, potentially limiting its protective capacity and making it more prone to damage. Dissolution has also been observed on other parts of the shell (e.g. Peck *et al.*, 2018), often being linked to lower aragonite saturation state. These observations indicate that natural damage to the periostracum is not limited to just the centre whorls. Understanding the conditions that promote natural periostracum damage, whether through bacterial degradation or mechanical damage, could allow us to better predict the risks posed to pteropods by OA-induced dissolution.

Superficial damage to the periostracum, such as the damage incurred in our experiment, or damage caused by microbial degradation or failed predation attempts in the wild, cannot be repaired if the shell has not been fully breached and the damage is located away from the growing edge, since the periostracum is only secreted at the mantle edge (Wagge, 1951; Beedham, 1965; Saleuddin and Petit, 1983; Harper, 1997). In some non-pteropod species of gastropods and some bivalves, a new periostracum-like organic layer is deposited during the process of shell repair after pieces of the shell are entirely removed (Watabe, 1983). In all documented cases of periostracum repair the mantle was able to access the outside of the shell, either through a full breach or at the growing edge (Andrews, 1934; Bubel, 1973; Pan and Watabe, 1989). Pteropods can repair full shell breaches by depositing new aragonite from the inside (Peck *et al.*, 2016a, 2018), but there is no evidence of periostracum replacement or repair in pteropods. Thus, a pteropod with a damaged periostracum must continue to thicken the shell from within as long as they are exposed to aragonite undersaturated waters, or the shell will eventually be breached (Peck *et al.*, 2016a). The few documented cases in the literature of pteropods thickening their shells in response to dissolution (Peck *et al.*, 2016a, 2018) suggest that the time scales of repair are either similar to, or shorter than, the time scales of dissolution. Otherwise, those individuals would probably not have survived, given that a shell breach

increases the risks of infection and predation and reduces the animal's ability to regulate buoyancy (Peck *et al.*, 2018). However, more research is needed to quantify rates and confirm whether pteropod shell repair can keep pace with continued dissolution.

The extent to which the periostracum may protect pteropods as OA increases in the future remains unclear. Although our results show that pteropods may currently be more resistant to OA-induced shell dissolution than previously thought, additional (and potentially multiplicative) stressors caused by OA and other aspects of climate change still pose threats independent of shell dissolution. For example, the progression of OA will increase energetic costs associated with calcification and other metabolic processes (Spalding *et al.*, 2017; Mekkes *et al.*, 2021a, b). Pteropods will therefore likely need to expend more energy to precipitate their shells (Spalding *et al.*, 2017), which requires sufficient food or energy stores to be available (Waldbusser *et al.*, 2013; Spalding *et al.*, 2017), and physiological limits in calcification rates may exist. The degree to which any increased energetic cost to precipitation might affect pteropod populations over time is unknown. Alternatively, producing thinner or less-dense shells (Mekkes *et al.*, 2021a, b) could circumvent the extra energetic cost, but may simultaneously compromise the animal's buoyancy or defence against predators. Other stressors associated with climate change, such as increased temperature, deoxygenation, or shifting trophic dynamics that result in reduced food availability or higher metabolic rates, will likely further increase these energetic costs. Larval and juvenile organisms are generally the most vulnerable to changing environmental conditions (Kroeker *et al.*, 2013), but due to the difficulty in maintaining pteropods in laboratory cultures, few studies (Comeau *et al.*, 2010; Thabet *et al.*, 2015; Manno *et al.*, 2016) have assessed the impacts of OA on these critical life stages. The role of the periostracum in protecting the larval shell during development also remains unknown.

Limitations

There was some variability in the strength of the manual scratching, an artefact of the extreme difficulty in gently scratching the delicately thin shells of these tiny organisms by hand while looking through a microscope. This variability

was revealed by the range in visibility of the scratches in SEM images of shells from the supersaturated treatment (where no dissolution occurred) (Figure 2d–f, Supplementary Figure S3). Some of the stronger scratches may have induced damage to the aragonite shell on some of the individuals rather than breaching the periostracum alone. However, scratches were entirely absent in SEM images of 4 of the 20 individuals from the supersaturated treatment and almost undetectable on another five (Figure 2d, Supplementary Figure S3). We assume that the scratches on individuals from the undersaturated treatment also followed a similar distribution of variability. Since dissolution was observed along the scratches in all individuals from the undersaturated treatment, removal of the periostracum was likely the main driver of the dissolution.

Pteropods are notoriously difficult organisms to maintain in laboratory incubations, typically exhibiting unnatural behaviours and high mortality rates (Howes *et al.*, 2014). Although pteropods in incubation containers have not been observed to feed using their typical mucous webs, they can direct food particles into the mouth using ciliated lobes (Howes *et al.*, 2014). No food was added to our incubation bottles, but *L. helicina* has been shown capable of surviving in captivity without food for up to 4 weeks (presumably on lipid and body reserves) (Lischka *et al.*, 2011). The pteropods used in this study were collected near the end of the spring/summer season of 2019 in the productive Salish Sea (March–September) (Simpson *et al.*, 2022), so they would likely have been relatively well-fed prior to collection, and yet we still saw strong dissolution in the areas of damaged periostracum. The effects of incubation conditions on pteropod behaviour or physiology should be considered during long-term experiments aimed to assess population-level impacts of OA. However, the aim of our study was to assess a short-term mechanistic response when a pteropod with a damaged periostracum is exposed to corrosive water.

Conclusions

Our findings demonstrate that the periostracum of *L. helicina* is an important first line of defence that protects the shell from exposure to corrosive water, and they strongly support the conclusion of Peck *et al.*, 2016a, b) that dissolution of the external shell surface on healthy, actively swimming adult *L. helicina* only occurs when the periostracum has been breached and the shell is exposed to undersaturated water ($\Omega_{Ar} < 1$). Since, pteropods currently play a large role in discussions about the global ecological implications of an acidifying ocean (Bednaršek *et al.*, 2016b; Manno *et al.*, 2017), recognizing this mode of defence is critical to accurately evaluating the broader risks posed by OA, especially when considering their use as bioindicators for water quality assessment. This work thus highlights the importance of understanding and considering the complex biology of species that are proposed as bioindicators to inform predictions about future impacts on ocean ecosystems.

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Data availability

Light and SEM images of all specimens along with water chemistry data for all incubation samples generated in this study are included in the supplementary material files as Supplementary Figures S3 and S4, and Supplementary Dataset 1, respectively. All other datasets analysed in this study are available on the National Oceanic and Atmospheric Administration's Ocean Carbon and Acidification Data System (OCADS) (<https://www.ncei.noaa.gov/products/ocean-carbon-acidification-data-system>): data used for DIC and A_T uncertainty analysis (Sastri *et al.*, 2022) (<https://doi.org/10.25921/5fza-nj73>), and data used to generate Figure 1 (Ianson *et al.*, 2018; Chandler *et al.*, 2021a, b) (<https://doi.org/10.7289/v5k64gcp>, <https://doi.org/10.25921/vr8n-x476>, <https://doi.org/10.25921/0cnc-x944>) (see Supplementary Methods for more detail).

Supplementary data

Supplementary material is available at the ICESJMS online version of the manuscript.

Author contributions

MRM conceptualized and carried out the experiment and wrote the manuscript. RO, PC, DI, and JD helped interpret results and reviewed and edited the manuscript. DI and JD obtained the funding.

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Conflict of interest

The authors have no conflicts of interest to declare.

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