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Response to comment

Vulnerability of pteropod (*Limacina helicina*) to ocean acidification: shell dissolution occurs despite an intact organic layer
By Bednarsek et al

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We thank Bednarsek et al. for their detailed consideration of our paper. We welcome this opportunity to clarify some misconceptions of our work and expand further on how our methods differ from previous studies.

We will address the three issues raised by Bednarsek et al. in turn.

1.1. *Dissolution in the pteropod* Limacina helicina *is only happening if the periostracum is damaged. The dissolution is readily evident under the light microscope and without removal of the periostracum. (p. 10, 11 in Peck et al.).*

In response to *Dissolution in the pteropod* Limacina helicina *is only happening if the periostracum is damaged*.

Bednarsek et al. (2014) allude to the thin periostracum of pteropods offering insufficient protection against shell dissolution in understaturated waters. We maintain that the periostracum of *Limacina helicina*, when fully intact, fulfils its purpose of protecting the aragonite beneath and that for dissolution of the outer surface of the shell (of a healthy living specimen) to occur understaturated water must make direct contact with the aragonite. To achieve this contact, water must penetrate the protective periostracal layer on the shells outer surface through a breach or perforation. We (Peck et al., 2016) considered methods of periostracal breaching that were evident to us at the magnification we were working to, however we did not consider the occurrence of micro to nanno scale perforations and thank V. Garilli (pers. comm.) for bringing our attention to the possibility of periostracal breaches on a smaller scale than we have currently identified (Garilli et al., 2015). In addition, lateral penetration of waters between the aragonite and periostracum away from the location of the breach will cause dissolution to occur beneath an area of intact periostracum (as presented in Peck et al., 2016; Fig. 7). Hence, while a periostracum may appear to be intact in SEM images, if there is dissolution of the aragonite layer there must be a perforation or breach in the periostracum at some scale somewhere on the shell.

To address the claim made that Bednarsek et al. that our findings differ substantially from the previous findings of her group and also Busch et al. (2014) and Lischka et al. (2011) we consider two explanations for the fact that we (Peck et al., 2016) do not report *uniform patterns of dissolution affecting large areas of the shell beneath the periostracum* observed in previous studies. In fact Peck

et al. (2016) do report uniform shell dissolution, please refer to figure 9 (d and e) and text on page 11 under sub-heading *4.5 Dead animal shell dissolution.* We state that internal shell dissolution, manifested by uniformly opaque shells beneath a pristine periostracum (caption to Fig. 9) is a post-mortem feature. What we failed to mention in Peck et al. (2016) is that internal dissolution of the shell can also be observed in specimens that are living, but in poor health. We also failed to be explicit in our determination of animal health, and suitability for further shell condition examination, at the end of the incubation. In the incubations presented in Peck et al. (2016), all specimens that entered the incubation (actively swimming and appearing to be in good physical health), were recovered in the same state. However, in concurrent incubations not presented in Peck et al. (2016) we observed some fatalities and deteriorations in animal health that we would like to discuss here.

Figure 1 shows specimens recovered following an 8 day incubation within waters manipulated to Ω_{Ar}=0.85 (not presented in Peck et al., 2016, but from the same cruise). Of the thirteen specimens shown, eleven specimens were actively swimming and met the criteria for activity Stage I proscribed by Lischka et al. (2011) to be 'animal expanded, actively moving, soft tissue appears clear and in good condition'. The specimen circled in red was no longer living, or activity Stage V according to Lischka et al. (2011), that is 'animal retracted, soft tissue appears strongly decomposed, individual clearly dead'. The condition of the shell was compromised from internal dissolution (in response to tissue decay and pH imbalance inside the shell) as evidenced by the shell having become uniformly opaque. The specimen circled in orange is of particular interest since it is retracted inside its shell but, as the body could be seen to be actively moving inside and the soft tissue appeared clear and in good condition, this specimen fitted the criteria for activity Stage II (Lischka et al., 2011). Although this animal was technically alive, it was clearly in a poor state of health and the semi-opaque nature of the shell indicates that the shell condition may already have been compromised, i.e. the animal had bigger issues to deal with than maintaining an internal pH balance. We did not use this specimen to assess shell condition in response to exposure to undersaturated waters. However, according to the methods of Lischka et al. (2011) and Busch et al. (2014), all animals that were alive at the end of the incubation (meeting the criteria for activity stages I through to III [Lischka et al., 2011]), regardless of their state of health, were assessed for shell condition. We therefore propose that the differing results between Peck et al. (2016) and previous studies which note large-scale uniform dissolution across shells may have included shells of animals that were not in good health.

A second consideration is the post-collection storage of specimens. To eliminate any possibility of post-collection alteration of the shell, due to pH change within a solution, we rinsed our specimens in buffered deionised water before air-drying and storing in individual specimen slide cells. Cross-reference to photographs of individual shells taken and catalogued at the time of collection (at sea) confirms that no post-collection alteration occurred to specimens presented in Peck et al. (2016).

With regards to the account that *dissolution in the pteropod* Limacina helicina *is only happening if the periostracum is damaged* we qualify our statement as follows. The dissolution of the shell of a *healthy*, living specimen is only susceptible to dissolution on the *outer* surface when the periostracum has been breached and the underlying aragonite is exposed to undersaturated waters (either directly or through the lateral propagation of water between the aragonite and periostracal layers). As shown in Peck et al. (2016), uniform dissolution across large areas or the entirety of an individual's shell is consistent with internal dissolution of the shell (Peck et al. 2016; Fig. 9d and e) and representative of the animal's inability to maintain a suitable internal pH, rather than external corrosion of the shell due to exposure to undersaturated waters.

To ensure that future studies are directly comparable we suggest that, similar to Peck et al. (2016), any specimens not meeting the criteria of activity stage I of Lischka et al. (2011) should not be pooled with those that do meet the criteria. Given the rapid deterioration of pteropod shells from the inside when the animal is in a poor state of health, or dead, we urge careful examination of activity of specimens upon collection wherever possible. Every effort should be made to ensure that dead or dying specimens are not used for shell condition analysis to assess the impact of ocean acidification since they will bias the data set.

In response to *dissolution is readily evident under the light microscope and without removal of the periostracum.*

Our approach to analysing the condition of the shell incorporated both light microscope and SEM imagery. We are pleased to see Bednarsek et al. adopt our approach of not removing the periostracum before SEM imaging in their figure 1, however we are disappointed that Bednarsek et al. do not provide light microscope images for comparison. We find that the images provided by Bednarsek et al. (Fig. 1) entirely support our claim that the periostracum does not need to be, and should not be, removed to in order to inspect exterior shell condition. We note that the specimen shown in their figure 1b has survived numerous fracture and repair events and has suffered associated dissolution in this area as we also observe. The specimen in their figure 1d has numerous linear scars on the inner whorl which, as we suggest, may have resulted from predatory scratches or microbial attack of the periostracum. As highlighted by Bednarsek et al., shell dissolution is clearly apparent beneath the periostracum, supporting our case that removing the periostracum before imaging is unnecessary. While the periostracum remains on the shell, it is not intact as claimed by Bednarsek et al. Perforation to the periostracum are clearly visible within the field of view their images figures 1c, 1e and 1g. From these perforations, water penetrating beneath the periostracum will begin to dissolve the shell and further undermine the periostracum (Peck et al., 2016) increasing the area vulnerable to dissolution, even if the periostracum directly above the site is intact. Furthermore, the occurrence of micro to nanno perforations cannot be excluded at this magnification (V. Garilli, pers. comm.).

1.2. *Chemical and plasma etching treatment of the shell (as described in Bednaršek et al., 2012b) can induce dissolution damage (p. 2, 11 in Peck et al.).*

We wish to correct Bednarsek et al. on this point. We were very careful not to say that chemical or plasma etching treatment of the can *induce dissolution*. What we do say is

Wholesale removal of the periostracum inhibits recognition between dissolution which has occurred to the living specimen due to natural damage to the periostracum, as opposed to 'bleaching' of shells prior to analysis which can cause post-mortem damage to the crystalline fabric of the shells. The latter is particularly important as it is well known that shell microstructures contain both inter and intra crystalline organic matrix (Marin et al 1996), the selective removal of which may produce a corroded appearance (see Peck et al., 2015) which may be misinterpreted.

1.3. *Pteropods seem not to be so vulnerable to OA (p. 11 in Peck et al.).*

Again, we wish to correct Bednarsek et al. on this point. We were very careful not to say that pteropods seem not to be vulnerable to ocean acidification. What we do say is

While we propose that L. helicina helicina *are perhaps not as vulnerable to ocean acidification as previously claimed, at least not from direct shell dissolution, we have not assessed the energetic consequences of calcifying a shell in undersaturated waters and repairing and maintaining a damaged shell within waters of ΩAr≤1. Further investigation into the long term reproductive and somatic consequences of ocean acidification are needed.*

With regard to proposed method of removing the periostracum prior to SEM analysis by immersing specimens into 6% hydrogen peroxide, we would like to draw attention to the statement in Gaffey & Bronnimann (1993) [also cited by Bednarsek et al.] that *"even when buffered with NaOH, H_2O_2 caused dissolution and etching of carbonate".* It is unfortunate that Bednarsek et al. were unable to use the same specimens when comparing untreated (their Fig. 2 a, d, g) with treated individuals (their Fig. 2 b and c, e and f, h and i) since it is not possible to assess which features are inherent to the shell and which features are artefacts of removing the periostracum. One approach which may enable a true assessment of the impact that removal of the periostracum may have on the appearance of the underlying aragonite would be plasma etching or bleaching half of one individual shell. Light microscope images would also allow more thorough assessment.

We maintain that, for the purposes of analysing shell condition, our approach of rinsing in buffered, deionised water and air-drying prior to storage in individual specimen slide wells minimises the opportunity for shell condition to be altered in any way post-collection. We reiterate that an agreed, standardised approach to the preservation and analysis of pteropod shells that minimises opportunity for chemical or physical damage to the shell post-collection is a necessity.

To conclude, our findings, consistent with studies assessing the vulnerability of other mollusc shells to under saturated waters (e.g. Tunnicliffe et al., 2009; Garilli et al., 2015), indicate that the shells of healthy, living (actively swimming) pteropod *Limacina helicina* are only susceptible to dissolution on the external surface of the shell where both the periostracum has been breached and the aragonite beneath that breach is exposed to waters of $\Omega_{Ar} \leq 1$. As such, we maintain our conclusion that the extent of pteropod shell dissolution (of healthy, living individuals) is not a direct function of exposure to undersaturated waters, rather, it is dependent on the extent of periostracal damage combined with exposure to undersaturated waters.

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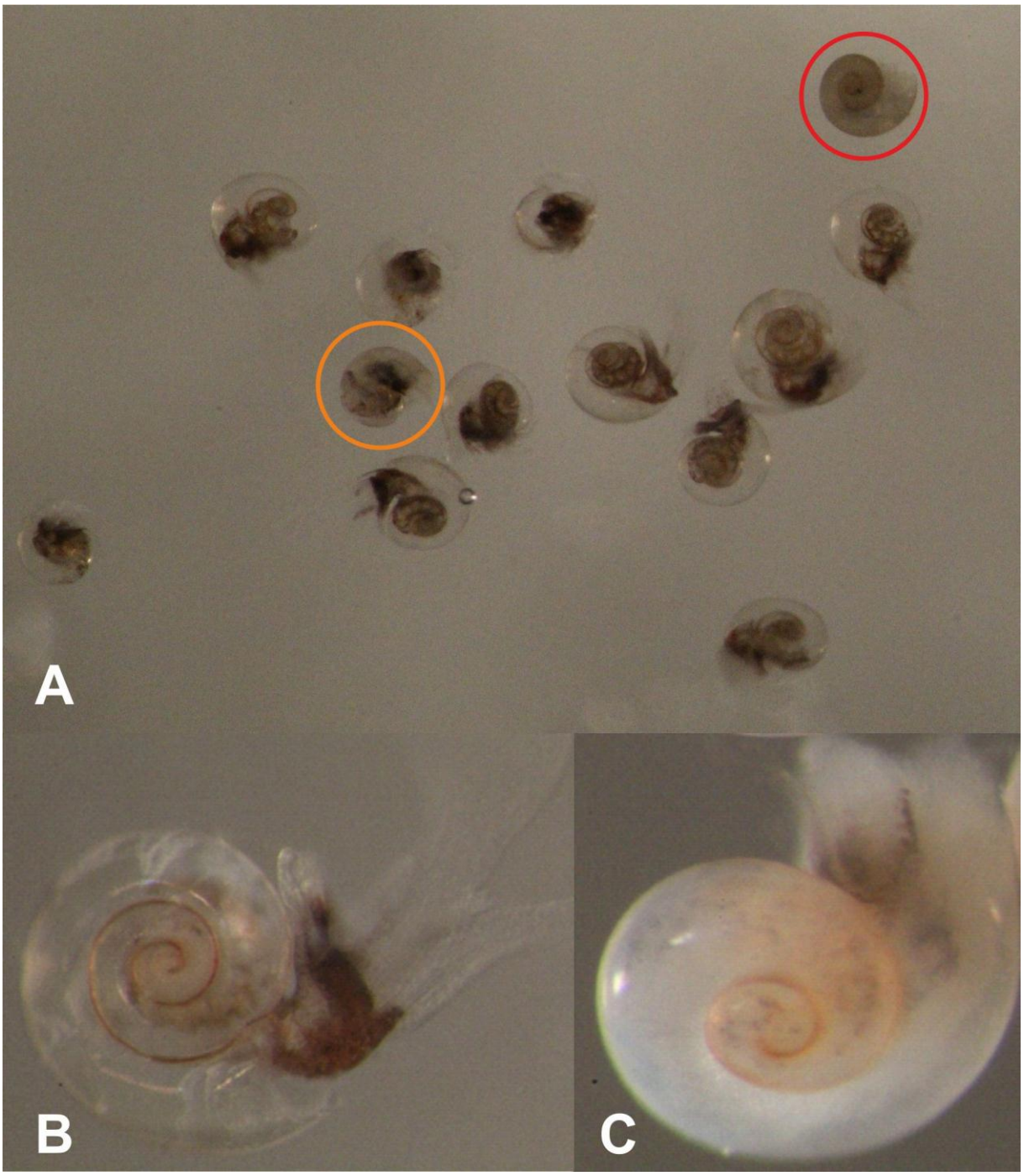

Figure 1. Light microscope image taken at sea on JR271 to illustrate the state of health of specimens recovered from an 8 day incubation experiment within waters of Ω_{Ar}=0.85. **A**. Of the 13 specimens recoverd, 11 were observed to be actively swimming. The specimen circled in red was no longer living and the uniformly opaque shell indicated that the shell condition has been compromised by internal dissolution. The specimen circled in orange remained retracted inside its shell throughout the examination period, but organs could be seen to be actively moving deeming the animal to be alive. However, the poor state of health of this animal relative to the other survivors was apparent. The semi-opaque appearance of the shell indicates that the shell condition may already have been compromised and we deem this specimen unsuitable for pooling with the actively swimming specimens. **B**. Actively swimming survivor which demonstrates linear opaque features on the shell indicative of dissolution along fracture and repair scars. **C**. A dead specimen presenting a uniformly opaque shell, indicative of internal dissolution of the shell.