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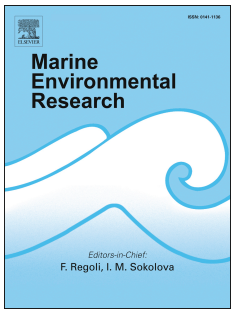

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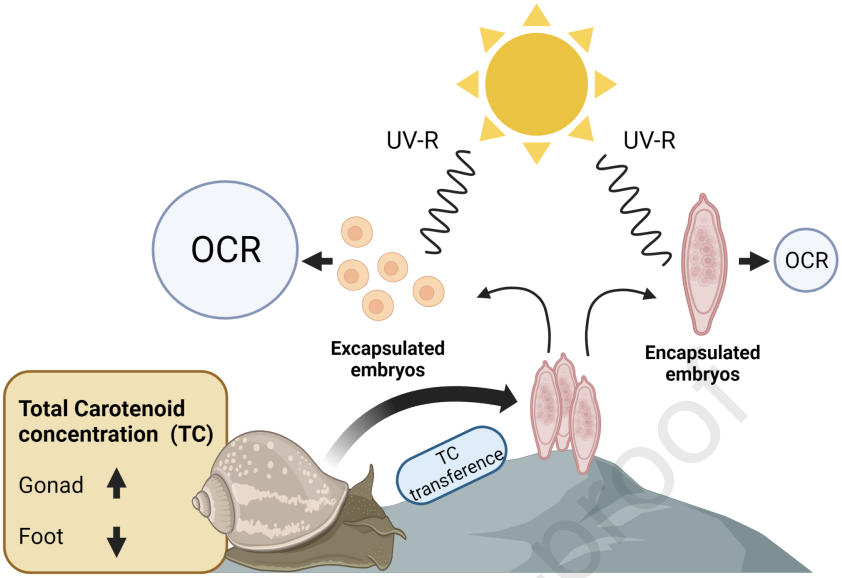

UV-R mitigation strategies in encapsulated embryos of the intertidal gastropod *Acanthina monodon*: a way to compensate for lack of parental care

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Key words: Embryos, Capsules, UV radiation, Carotenoids, Intertidal gastropod, Oxygen consumption rate.

Abstract

Intracapsular embryonic development in the intertidal zone exposes embryos to various stress sources characteristic of this environment, including UV-R. They require defensive mechanisms to mitigate its adverse effects. The presence of total carotenoids (TC), and mycosporine-like amino acids (MAAs) was studied in adults, in encapsulated embryos, and in the egg capsule walls of the intertidal gastropod *Acanthina monodon*. Oxygen consumption rates (OCR) were determined in encapsulated and excapsulated embryos exposed to photosynthetically active radiation (PAR) and PAR+UV-A+UV-B to understand if the capsule wall is a protective structure for encapsulated embryos. The results showed the presence of TC in adult pedal and gonad tissues, and in all encapsulated stages. MAAs were not detected. The physical structure of the capsule wall retained most wavelengths, being particularly efficient in the UV-B range. Excapsulated embryos exposed to PAR+UV-A+UV-B radiation increased its OCR compared to encapsulated embryos, indicating the protective character of the capsule wall.

1. Introduction

The intertidal habitat is an area of high physiological stress for species that inhabit there, because they are periodically exposed to stressful environmental conditions due to tidal changes (Womersley and Edmonds, 1952; 1958; Taylor and Butler, 1978; Little and Kitching, 1996). The exposure of intertidal organisms to different stressors during low tide can generate lethal and sublethal effects, affecting their distribution and abundance (Fierro et al., 2017). Tidal cyclicity leads to strong variation in temperature, desiccation, salinity, oxygen and food, as well as to elevated levels of ultraviolet radiation (UV-R / >280 – 400 nm) (Rawlings, 1996; 1999; Przeslawski et al., 2004; Przeslawski, 2005; Gosselin and Jones, 2010). Ultraviolet-B radiation (UV-B, >280 – 320 nm) is a highly damaging abiotic factor for the cell, because its direct absorption generates changes in the DNA structure (Vincent and Neale, 2000), which affects the processes of reading and replication of the genetic material, generates mutations in its structure and induces morphological and functional anomalies (Häeder et al., 1998; Day and Neale, 2002; Nigel and Gwynn-Jones, 2003; Lamare et al., 2007). This is common in early developmental stages of marine organisms because they are highly vulnerable to changes in the surrounding environment (Lamare et al., 2007; Przeslawski et al., 2015). Although the effect of UV-B radiation has been of major concern during the last decades due to the significant increase in the generation of cell damage in marine organisms, it is important to point out that most of the UV-R reaching the earth's surface corresponds to UV-A radiation (320 - 400 nm) (Whitehead et at., 2004). Its absorption can also generate photo-oxidative damage in the cell (Rijstenbil, 2001), however the frequency of this type of cytotoxic compounds are lower considering the reduced energy it possesses compared to UV-B (Vincent and Neale,

2000; Whitehead et at., 2004).In invertebrates with external fertilization or mixed development (*sensu* Pechenik, 1979), planktonic larvae with a high degree of transparency and without physical protection (e.g. shells or body plates) are highly vulnerable to UV-R (Lister et al., 2010). However, larval lability during this developmental phase could be compensated by the presence of photoprotective compounds, chromatophore expansion capacity or vertical migration strategies in the water column that minimize cell damage from UV-B exposure (Hylander and Hansson, 2010; Bashevkin et al., 2020). For intertidal species with internal fertilization (e.g. gastropods), direct embryonic development and no maternal protection, the viability of eggs laid in benthic gelatinous masses or capsule structures could be mediated by the role of the enveloping wall acting as a physical protective structure. Added to the presence of photoprotective compounds, this may ameliorate the effects of UV-B radiation during intracapsular development (Karentz et al., 1991; Adams and Shick, 1996; Rawlings, 1999; Yanar et al., 2004; Paredes-Molina et al., 2016). Previous studies indicate that capsule walls can act as a barrier between the external environment and the interior of the capsule, impacting for example oxygen diffusion to the intracapsular fluid (Segura et al., 2010; Chaparro et al., 2020), providing protection against desiccation and potentially reflecting part of the UV-R (see review in Rawlings, 1999); embryos without such capsule protection can suffer direct impacts upon exposure to solar radiation (e.g. mortality, Rawlings, 1996). The use of photoprotective compounds is a strategy widely used by marine invertebrates against UV-R; they can absorb different wavelengths, dissipating the energy without generating reactive oxygen species (ROS, Adams and Shick, 1996). Mycosporine like amino-acids (MAAs) are secondary metabolites that can absorb radiation between 310 and 362 nm (Takano et al., 1978); their presence has been recorded in different gastropod species (e.g. several gastropod species, Przeslawski,

2004; Przeslawski et al., 2005; *Siphonaria denticulata*, Przeslawski, 2005; *Crepipatela dilatata* and *C. peruviana*, Paredes-Molina et al., 2016). Carotenoids are yellow-orange pigments that absorb wavelengths from 380 – 510 nm (Flores et al., 2005); their presence has been identified in early stages of development (Heras et al., 2007; Borodina et al., 2013; Paredes-Molina et al., 2016). Both photoprotective compounds are synthesized *de novo* by bacteria, algae and fungi and transferred to higher trophic levels (Liaaen-Jensen, 1978; Bedoux et al., 2020). Once assimilated by consumers, and stored in specific organs (e.g., pedal muscle, gonadic tissues; Bandaranayake and Des Rocher, 1999; Lamare & Hoffman, 2004; Paredes-Molina et al., 2016), they can be transferred by the adults to their offspring through the maternal line as has been recorded in eggs and larval stages of different invertebrate species (e.g. crustaceans, Hernández Moresino and Helbling, 2010; polychaetes, Marsh et al., 1990; echinoderms, Adams and Shick, 1996, McClintock and Karentz, 1997; Lamare and Hoffman, 2004; molluscs, Przeslawki 2004; Paredes-Molina et al., 2016).

Acanthina monodon (Pallas, 1774) is a carnivorous muricid gastropod widely distributed in the central and southern coast of Chile (Poblete et al., 1987; Büchner-Miranda et al., 2021; Salas-Yanquin et al., 2022). It is a dioecious species with internal fertilization, whose embryos and nurse eggs are packed inside small (5.0 to 12.8 mm in length; Gallardo,1979) capsular structures which are attached by the female to the rocky substrate of intertidal and subtidal zones. It had been suggested that *A. monodon* capsules laid in the intertidal are mainly attached inside rock crevices or under shaded sites (Gallardo, 1979). This species presents direct development without physical parental care;

105 juveniles from 0.82 to 1.3 mm in length hatch from the capsules (Gallardo, 1979; Chaparro
106 et al., 2018) after 50 to 80 days of intracapsular development (Gallardo, 1979).

107 Considering the stress generated by UV-B radiation in the intertidal zone,
108 reproductive strategies seem to be fundamental to ensure the viability of the early
109 developmental stages of intertidal species (Biermann et al., 1992; Rawlings, 1996). Protected
110 oviposition sites, as well as the transfer of photoprotective compounds from adults to
111 embryos, nurse eggs, and/or capsule walls could be determinant when facing exposure to
112 UV-B radiation in the intertidal zone. This is especially relevant when low tide periods
113 coincide with the maximum levels of solar radiation (Rawlings, 1999; Cubillos et al., 2018),
114 which may exacerbate cell damage, inducing abnormalities during early development
115 (Lamare et al., 2007) and triggering physiological changes (e.g. oxygen consumption rate,
116 Chaparro et al., 2020).

117 The objectives of this study were to evaluate the photoprotection mechanisms of the
118 encapsulated embryos of *A. monodon* and to estimate the capacity of the capsule wall to act
119 as a protective tool against UV-R exposure, thus avoiding changes in embryonic
120 metabolism. It is postulated that encapsulated embryos of *A. monodon* contain
121 photoprotective compounds that reduce the absorption of UV-R radiation, and that the
122 structural conformation of the capsule wall provides physical protection to the embryos,
123 acting as a dissipation filter against direct UV-R exposure to which they are exposed during
124 low daytime tides.

2. Materials and methods

2.1. Sampling and collection site

Adult *Acanthina monodon* snails were collected from the mid-low rocky intertidal zone of Calfuco beach (39° 46′ 50″ S, 73° 23′ 34″ W), Valdivia, Chile. Those egg capsules (length of the capsules excluding stalk: 6-10 mm) of this species containing embryos at different stages of development were detached from egg masses laid on the rocky intertidal substrate using a scalpel and deposited in a Falcon tube with filtered seawater (1 µm). The adult individuals and capsules were transferred to the laboratory and kept in an aquarium with circulating seawater (35 psu, 12–13 °C temperature and constant aeration) for a maximum of 3 days before use.

2.2. Environmental radiation

During the 2022 Southern Hemisphere summer, a daily cycle of ambient radiation was recorded during a clear day for Calfuco beach between 8:00 and 18:30 h using a portable PMA-2100 radiometer (Solarlight, Pennsylvania, U.S.A.) fitted with UV-B (PMA 2106) and PAR (PMA 2132) sensors. UV-B and PAR irradiance values were expressed in W m^{-2} and µmol m^{-2} sec^{-1}, respectively.

2.3. Sample processing

2.3.1. Pedal muscle and gonad tissues

Adult *A. monodon* specimens were dissected using surgical scissors to extract pedal muscle and gonad fragments (n=13). Tissue was deposited in 1.5 ml Eppendorf centrifuge tubes and frozen at -20 °C until biochemical determination of photoprotective compounds.

146 *2.3.2. Capsule wall*

147 The capsules were delicately cleaned using a brush to remove epibionts. The
148 capsule walls, from which embryos at different stages of development were extracted, were
149 deposited in 1.5 ml Eppendorf centrifuge tubes and frozen at -20 °C until analysis of
150 photoprotective compounds. The capsule walls used in the MAAs and TC analyses were in
151 the same developmental stages indicated for the embryos.

152 *2.3.3. Capsule wall transmittance*

153 Capsules containing embryos at different stages of development (initial stage,
154 veliger larvae and pre-hatching juveniles) were opened from the side using dissection
155 scissors. After removing the contents, the capsule wall was extended and deposited inside a
156 well of a microplate (96 wells, Corning). Then a transmittance scan of the capsular wall
157 was performed for a range of 295-700 nm using a plate reader (SpectroStar, BMG).
158 Transmittance values were estimated for the UV-B (295 - 320 nm), UV-A (320-400 nm)
159 and PAR (400-700 nm) radiation bands. Empty wells of the same microplate were used as
160 controls.

161 *2.4. Identification and collection of encapsulated embryos*

162 During the identification of embryonic stages used in the analysis of
163 photoprotective compounds, each capsule was cut in the basal area with surgical scissors,
164 allowing the embryos to be removed. The embryonic content of each *A. monodon* capsule
165 was deposited in a Petri dish with filtered seawater (30 psu).

166 Embryos were photographed with a digital camera (Model Q-imaging) mounted on
167 a stereo microscope (Olympus, model SZ61) for identification of developmental stages and

size estimation. The developmental stages used in the investigation were early embryos (shell-less, egg-trochophore stages), veliger larvae (500 – 800 µm), and pre-hatching juveniles (without velum ≥ 850 µm). The sizes of the shelled stages (initial veliger larvae (n=10) and pre-hatching juveniles (n=10)) were obtained from photographs that were analyzed using the image processor (ImageJ). All embryos from each measured capsule were deposited in Eppendorf tubes and kept at -20 °C until the determination of photoprotective compounds.

2.5. Photoprotective compounds

2.5.1. Extraction and determination of mycosporine like-amino acids (MAAs)

Tissue samples of *A. monodon* (foot and gonad of adults, capsule walls and early embryos, veliger larvae and pre-hatching juveniles) were dried at 45 °C for 48 hours in an oven (Memmert 854). Then the samples were ground using a porcelain mortar until a fine powder was obtained. The extraction of MAAs was carried out on 10 mg of dried tissue that was homogenized with 1 ml methanol (HPLC grade), following the methodology described by Cubillos et al., (2015). The solution was kept in the dark at 4 °C for 24 hours. Subsequently, the samples were centrifuged at 5000 RPM for 10 min and the supernatant was filtered through a nylon filter (0.22 µm). Finally, 200 µl of the filtrate was injected into an HPLC (Agilent / HP 1050) using an autosampler whose liquid phase was composed of a solution of deionized water: methanol: formic acid (90:8:2%) using a Synergi-Fusion-RP column (250 x 3.00mm / 4 µm - 80Å - Phenomenex). Samples were analyzed isocratically at a flow rate of 1 ml min^{-1} (220 PSI) for 15 minutes using a diode array detector (DAD) sensor. To extract and quantify MAAs in capsule walls and embryos, we pooled between 10 to 15 capsules from the same developmental stage for each analysis.

2.5.2. Extraction and determination of total carotenoids (TC)

The protocol of Zheng et al. (2010) was used with some modifications for TC extraction. Dried tissue (45 °C for 48 h) from the foot (male and female), gonad (male and female), embryos (initial, veliger larvae and pre-hatching juveniles) and capsule wall (initial stages, veliger larvae and pre-hatching juveniles) was pulverized using a mortar and pestle until a fine powder was obtained. To quantify TC, 20 to 30 capsules of each embryonic developmental stage were pooled. Subsequently, 25 mg of dried tissue (foot, gonad, capsule walls and embryos) was homogenized with 1 ml of 90% (w/v) acetone and incubated during 2 h at 4 °C. The tubes were transferred to an ultrasonic bath where they remained at 4 °C (40hz) for a period of 2 h in dark conditions. Then the samples were centrifuged at 12,000 RPM for 10 min, generating a pellet. Finally, 200 µl of supernatant were deposited in a well of the microplate (96 wells) and their absorbance was determined at 480 nm, 510 nm and 750 nm. For the "blanks", 200 µl of 90% (w/v) acetone was added directly to the microplate wells. The TC concentration was determined using the following formula: $TC = 7.6 *(a_{480}-a_{750}) – (1.49*(a_{510}-a_{750}))$ and expressed in µg ml^{-1}.

2.6. Respiratory physiology

In order to evaluate the impact of UV-R on respiratory physiology, 48 capsules with early embryos (initial stage) were collected from the mid-lower intertidal of Calfuco beach. The yellow coloration of the capsules was considered for preliminary identification of the initial developmental stage (Chaparro et al., 2018). The capsules were immediately transferred to the laboratory, where they were kept in an aquarium with a continuous flow of seawater (30 psu) taken directly from the collection site. The external surface of the capsules was gently cleaned of epibionts using a brush. The capsules were used for OCR

measurements the next day. For OCR quantifications, 24 individual capsules were placed in 8 ml of filtered seawater (30 psu) inside an 8 ml respiration quartz chamber and was then sealed hermetically. Simultaneously, other 24 capsules were opened from the apical section with a surgical scissors. All the embryos excapsulated from each capsule were placed inside their respective respiratory chambers. This experiment allowed to identify the impact of radiation on both encapsulated and excapsulated embryos, to determine how sensitive they are to experimental radiation. All respiration chambers were made of quartz, allowing the penetration of all wavelengths used in the experimental radiation treatments.

Respiration chambers with encapsulated or excapsulted embryos were exposed under different combinations of fluorescent tubes ((PAR: Phillips Daylight 40 W / 100 µmol m^{-2} s^{-1}), UV-A (Phillips TL40W / 03 40 W / 62 W m^{-2}), and UV-B (20 W / 2.4 W m^{-2} Phillips UV-B TL2012)) placed 30 cm above the experimental aquaria. The radiation treatments PAR (> 400 – 700 nm), and PAR+UV-A+UV-B (> 280 – 700 nm) were achieved using Clear-220 PAR (Johnson, UK) and cellulose acetate (CDA; Graphix / USA) cut-off filters, respectively over the oxygen chambers (ESM 1). Experimental radiation was controlled using a portable radiometer (Solarlight, PMA-2100) fitted with a UV-A, UV-B and PAR sensors. Dissolved oxygen concentration inside each respiration chamber was monitored using a non-invasive Fibox 3 oxygen-sensing system (PreSens, gmbH). Measurements for each chamber were made at the beginning of the experiment and after 4 h. This is the maximum aerial exposition period for capsules of *A. monodon* laid in the lower intertidal level (Chaparro et al., 2020). Two chambers without capsules served as controls for each set of measurements (Chaparro et al., 2018). After each measurement, the specific development level of each embryo was identified. Capsule OCRs were expressed

as mg oxygen consumed h^{-1} $capsule^{-1}$. Sea water used in the respiration chambers had been filtered (0.5 µm) and sterilized with UV light, maintained at 13 °C and salinity of 30 ± 1 psu. To avoid the influence of UV and PAR lamps on the experimental temperature, respiration chambers were kept under a double controlled temperature system (water chiller and temperature-controlled room, both set up at 13 °C).

2.7. Capsule wall histology

Capsules containing early embryos (n=3) and advanced embryos (n=3) were selected for histology analysis. Empty capsules were fixed with 7% formalin, then dehydrated using a battery of increasing of alcohol solutions, cleared and embedded in paraffin wax. Subsequently, the blocks were sectioned at 7 µm. Finally, the histological sections were stained with hematoxylin-eosin using the protocol of Montuenga et al. (2009) and photographed using a microscope (Olympus BX-41) fitted with a digital camera. The images were processed using Image J software.

2.8. Statistical analysis

The assumptions of normality and homoscedasticity of the data were identified using Kolmogorov-Smirnov test and Levene's test, respectively. Data that did not pass these criteria were analyzed by nonparametric tests. Comparison of carotenoid concentrations between the foot and gonads of adult *A. monodon* specimens was performed by Mann-Whitney tests, while TC content in capsule walls and embryos of different developmental stages (initial, veliger larvae and pre-hatching juveniles) were analyzed with a Kruskal-Wallis test for each case.

Transmittance data of the capsule walls were analyzed by independent ANOVAs for each type of radiation (UV-B, UV-A and PAR), the common categorical variable was

the developmental stage (initial, veliger larvae and pre-hatching juveniles). The wavelength of the radiation was used as the covariate. Data transformation was performed using Ln (x) to achieve the ANOVA assumptions for both PAR and UV-A treatments.

The embryos respiratory response (OCR) of the encapsulated and excapsulated treatments exposed to PAR and PAR+UV-A+UV-B radiation were analyzed by t-tests. A significance level of $p = 0.05$ was applied in all statistical analyses. Statistical procedures were carried out using SIGMAPLOT (V.11) and STATISTICA (V.7.0) software.

3. Results

3.1. Environmental UV-R parameters at Calfuco beach

During a clear summer day in the Southern Hemisphere, the maximum UV-B and PAR irradiance levels measured for Calfuco beach were 2.5 W m^{-2} and 2070 µmol m^{-2} sec^{-1}, respectively. These were recorded at 14:17 pm. The daily UV-B and PAR doses received were 54.95 kJ m^{-2} and 13.890 kJ m^{-2}, respectively (Fig. 1).

3.2. Photoprotective compounds

3.2.1. MAA determination

Isocratic HPLC analyses indicated that no mycosporine-like amino acids were present in any of the tissues analyzed (foot/gonad of adult specimens, capsular wall, early embryos, veliger larvae and pre-hatching juveniles).

3.2.2. Total carotenoid (TC) determination

TC levels were significantly higher (Mann-Whitney test: P = 0.001; N = 13, Fig. 2) in gonads (2.577 ± 1.183 µg ml^{-1}, mean ± SD) than in the pedal tissue (0.325 ± 0.326 µg ml^{-1}, mean ± SD).

The capsule wall of *A. monodon* also showed the presence of TC. A significant difference in TC level was identified between capsular walls containing embryos at different levels of development (Kruskal-Wallis test: $H_{(2, 37)} = 26.21$ $P < 0.001$, N = 38; Fig. 3). The lowest values were in the walls containing early embryos (multiple comparisons, $P < 0.05$), whereas TC levels were higher in the walls of capsules containing

287 veliger and pre-hatching juvenile stages of development, and did not differ significantly
288 (multiple comparisons, P > 0.05).

289 The presence of TC was found in encapsulated embryos at all developmental stages.
290 The concentration of these photoprotective molecules did not show a significant difference
291 between encapsulated embryos at different developmental stages (Kruskal-Wallis test: $H_{(2,}$
292 $_{31)}$ 4.067, P = 0.1309, N= 31, Fig. 4). The concentration range varied between 1.0845 µg ml
293 $^{-1}$ (pre-hatching) and 1.3577 µg ml $^{-1}$ (veliger larvae).

294 *3.3. Capsule wall transmittance*

295 The capsule wall of *A. monodon* acted as a filter over the entire wavelength range
296 tested (220-700 nm) for all stages of embryonic development. A significant correlation was
297 identified between the transmittance of the capsule wall across the range of the respective
298 radiation bands (UV-B, UV-A and PAR type) and transmittance through the capsule walls
299 (ANCOVA: $F_{(1,146)}$ 367.6; P < 0.001, Fig. 5A; ANCOVA: $F_{(1,116)}$ 2340.5; P < 0.001, Fig.
300 5B; ANCOVA: $F_{(1,449)}$ 7581.5; P < 0.001, Fig. 5C).

301 No significant differences in transmittance levels were identified between capsule
302 walls containing different stages of embryonic development (ANCOVA: $F_{(2,146)}$ 0.0367; P
303 = 0.963, Fig. 5A) subjected to UV-B radiation. In contrast, for capsular walls subjected to
304 UV-A radiation (ANCOVA: $F_{(2,116)}$ 88.973; P < 0.001, Fig. 5B) and PAR (ANCOVA:
305 $F_{(2,449)}$ 1124.3; P < 0.001, Fig. 5C), significant differences were identified between the
306 embryonic developmental stages. A post hoc Tukey test showed a significant difference (P
307 < 0.05) for both PAR and UV-A treatments between initial stages, veliger larvae and pre-
308 hatching juveniles.

3.4. Capsule wall as a barrier for embryo protection against UV radiation: OCR responses.

Early embryos, encapsulated and excapsulated, showed no significant difference in OCR when were exposed to PAR (T test: $P = 0.345$, Fig. 6A); while a significant difference was found for OCR between embryo conditions when they were exposed to PAR+UV-A+UV-B treatment (t test: $P = 0.040$, Fig. 6B).

3.5. Capsule wall structure

The capsule wall of *A. monodon* is composed of 3 layers, inner, middle and outer. The mean total thickness ranged from 38.3 µm (± 0.46) to 46.5 µm (± 0.70). The inner layer is thin and homogeneous in appearance. The intermediate layer is the widest and has abundant vacuoles in its middle section. The outer layer has numerous dendriform lateral projections with mean lengths of 28.2 µm (± 1.8) and 13.3 µm (± 2.4), for the early and advanced stages, respectively (Fig. 7).

4. Discussion

The conditions of the intertidal environment are highly stressful for organisms that develop there (Przeslawski et al., 2004; Salas-Yanquin et al., 2022). This stress condition can be accentuated when periods of low tide occur during the maximum daily peak of ambient radiation (Cubillos et al. 2018). In a nearby area at Calfuco beach, in a midday summer the daily doses of UV-B are high enough to produce stress to intertidal organisms (e.g. intertidal algae, Huovinen et al., 2006). This can produce damage, especially to early stages of development, impacting the normal development of individuals (Lamare et al., 2007) or even causing death (Hernández Moresino and Helbling, 2010), due to the high embryonic susceptibility to environmental radiation. Successful development of encapsulated embryos in the intertidal environment will depend on the existence of mechanisms that are able to mitigate physiological stress (e.g. transfer of photoprotective compounds through the yolk), and also through the generation of embryo-enveloping structures (e.g. capsular walls or gelatinous matrices), which ameliorate the noxious UV-B effect (Rawlings, 1996). The results of the present study show that *A. monodon* possesses embryonic photoprotection strategies against UV-R that involve the presence of photoprotective compounds, as well as egg capsules with a capsule wall that act as a radiation filter, protecting the embryos during their intracapsular development.

Presence of photoprotective compounds

The presence of total carotenoids (TC) recorded in both adult tissues and all encapsulated embryonic stages of *A. monodon* has also been identified in numerous species, forming part of photoprotection strategies (e.g. *Actinia equinosa*, *A. tenebrosa*, Tauber, et al. 1980; several animal species, Matsumo 1989; *Holothuria atra*, Bandaranayake and Des

345 Rocher, 1999; several marine animals, Maoka, 2011; *Crepipatella dilatata, C. fecunda,*
346 Paredes-Molina et al., 2016). High levels of TC in the gonad tissue of *A. monodon*
347 reinforces the idea that gametes are the vector through which these photoprotective
348 molecules are transferred from parents to embryos, as has been identified in invertebrate
349 species like the mussel *Mytilus californianus* (Petes et al., 2008), the urchin
350 *Strongylocentrotus* sp. (Lamare and Hoffman, 2004) and the starfish *Anasterias antarctica*
351 (Pérez et al., 2015).

352 TC concentrations in *A. monodon* did not show variation during embryonic
353 development, suggesting a constant and continuous photoprotective role during
354 encapsulated development. In species such as the urchin *Strongylocentrotus*
355 *droebachiensis*, no variation in TC was identified when eggs were compared with pluteus
356 larvae (Griffiths, 1966). It appears that these photoprotective compounds transferred from
357 the female to the eggs are stable during embryonic development.

358 Potential increases in the content of TC in encapsulated embryos would involve an
359 exogenous route of entry, a difficult situation for encapsulated embryos of *A. monodon*,
360 taking into consideration that the capsule wall generates its isolation from the external
361 environment. Notwithstanding the above, the study by Paredes-Molina et al., (2016) found
362 that TC in encapsulated embryos of the intertidal populations of *Crepipatella dilatata* and
363 *C. peruviana* varied during embryo development. An increase in TC content was observed
364 as embryonic development progressed in *C. dilatata*, while it decreased in *C. peruviana*.
365 Interestingly, the increase in TC in the former species is due to nurse egg consumption
366 (Andrade-Villagran et al., 2018; Chaparro et al., 2019); they contain this type of
367 photoprotective compound and energetically support the direct development of embryos

368 inside the capsules (Paredes-Molina et al., 2016). This situation does not occur in *C.*
369 *peruviana*, whose encapsulated development is indirect, hatching as a veliger larva that is
370 fueled during the encapsulated phase only by the egg yolk provided by the female (Cubillos
371 et al., 2007; Chaparro et al., 2012). The reduction of TC content during the early
372 developmental stages of *C. peruviana*, has been associated with a potential conversion of
373 carotenes to other photoprotective compounds (Paredes-Molina et al., 2016).

374 This study identified the presence of carotenoids in the capsule walls of *A.*
375 *monodon*. The origin of these molecules could be explained by maternal transfer, as has
376 been suggested for hatchling calyptraeid gastropods (Paredes-Molina et al., 2016). There
377 may also be the possibility that part of the TC originate from the nurse eggs that the mother
378 makes available inside capsules for feeding by the embryos (Paredes-Molina et al., 2016).
379 The ingestion of nurse eggs by *A. monodon* occurs once the embryos reach the trochophore
380 stage (Gallardo, 1979). Although ingestion occurs by engulfment of the eggs as a unit
381 (Gallardo, 1979), part of the nurse eggs may disintegrate in the intracapsular fluid and their
382 remains become integrated into the internal wall of the capsules, but there is the possibility
383 that biofilm growing on the capsule wall could apport TC (Cancino et al., 2000). This could
384 explain the rise in TC as the embryonic development advances. Future research is needed to
385 identify the origin of the carotenes present in the capsular walls of *A. monodon*.

386 On the other hand, MAAs were not identified in *A. monodon*, neither in adult tissues
387 nor in encapsulated embryonic stages. It should be noted that this species is carnivorous,
388 and basically preys on mollusks and cirripedes (Soto et al., 2004; Büchner-Miranda et al.,
389 2021). Considering that MAA are molecules generated by autotrophic organisms, the way
390 in which they could be transferred to *A. monodon* adults is indirectly through the prey

391 consumed, particularly from herbivorous species as has been described in several groups of
392 marine invertebrates (e.g. echinoderms, Lamare and Hoffman, 2004; McClintock and
393 Karentz, 1997; crabs, Hernández Moresino et al., 2014). However, it seems that the MAA
394 photoprotective pathway is not a principal strategy of carnivorous species such as *A.*
395 *monodon*, and in this case the photoprotective role would be played by other
396 photoprotective compounds such as carotenoids (Sanchez et al., 1999).

397 *Capsule wall*

398 Capsule walls in some gastropod species can act as a protective barrier against the
399 effects of UV-B radiation for embryos (e.g. *Nucella* spp., Rawling, 1996). The capsule wall
400 of *A. monodon* is able to retain almost all radiation at different wavelengths. Considering
401 that UV-B radiation is highly damaging, the ability of the capsule wall of *A. monodon* to
402 reduce its penetration into the capsule by > 99.5% favors the development of early stages in
403 the intertidal environment. This radiation barrier capacity is particularly high in *A.*
404 *monodon*, even higher than that recorded in capsule walls of the gastropod *Nucella*
405 *emarginata*, where the retention of incident UV-B was also very high with values of about
406 95% (Rawling, 1996). Considering the direct intracapsular development of our model
407 species (e.g. hatching as a crawling juvenile), the capsule wall offers permanent protection
408 for the embryos until hatching. This reproductive strategy favors species that reproduce in
409 intertidal environments despite not having the parental care of the parents and remain for
410 months in that environment prior to hatching (Gallardo, 1979).

411 The ability of the capsule wall to filter different wavelengths is related to the
412 composition, morphology and thickness of the wall, as has been identified in different
413 species and populations of *Nucella* (Rawling, 1996). This ability to attenuate radiation

414 would be associated with the acellular structure of the wall, and also with the presence of
415 carotenoids identified in the capsule wall of *A. monodon*. The capsule wall of this species is
416 composed of three layers (outer, middle and inner) with a total thickness of approx. 45 µm,
417 which is concordant with previous studies by Büchner-Miranda et al., (2018) for the same
418 species. An interesting aspect of the capsule morphology is the presence of a highly
419 vacuolated intermediate layer, which could serve for storage of liquids and/or gases,
420 functioning as a reinforcement to the barrier capacity against solar radiation, particularly
421 during the aerial exposure period. The presence of lateral dendriform projections distributed
422 throughout the outer wall of the capsule (Büchner-Miranda et al., 2018) could also provide
423 a "double skin" function against solar radiation, interfering with the direct action of
424 radiation on the outer layer of the egg capsule. Future studies should investigate the role of
425 capsule wall vacuoles and dendriform projections as potential UV-R attenuators.

426 *OCR as a proxy for capsule wall function as a barrier for embryo protection against UV radiation.*

427 Aerial exposure of intertidal egg capsules during low tide impacts encapsulated
428 embryos, both in the long term and immediately during exposition (Pechenik, 1986; 2018;
429 Rawlings, 1999; Moran, 1999; Przeslawski, 2005; Chaparro et al., 2018; 2020; Salas-
430 Yanquin et al., 2022). In the latter case, metabolic responses serve as a proxy to identify
431 how stressful that environment may become for embryos. Our results indicate that exposure
432 to PAR radiation did not generate alterations in embryo OCR, either in those that received
433 this stimulus protected by the capsular wall or in those that were not, due to being
434 excapsulated. This result makes it possible to deduce that the excapsulated condition of
435 embryos *per se* (change from intracapsular fluid to seawater), did not generate impacts on
436 the respiratory rates in early embryos when exposed to PAR radiation. However,

437 excapsulated embryos exposed to PAR+UV-A+UV-B showed differences in OCR
438 compared to encapsulated embryos. The excapsulated embryos significantly increased their
439 oxygen consumption levels, which makes the stressful condition that UV-B radiation
440 generates evident. The noxious effect of UV-B in early developmental stages of marine
441 organisms is widely known, which can trigger cellular (Lesser, 2006), anatomical (Lister *et*
442 *al*., 2010) and physiological (Singh et al., 2015) problems, finally causing death in extreme
443 cases (Lamare et al., 2007).

444 The above-mentioned results reinforce the protective role that the capsule wall has
445 for the encapsulated embryos of *A. monodon* against radiation. This protective role matches
446 the results on the radiation absorbance capacities of capsular wall which prevent the UV-R
447 penetration towards the interior of the capsules where embryos develop. This result agrees
448 with those reported for *Nucella emarginata*, whose capsular walls have a high absorption
449 level of UV-B radiation (Rawlings, 1996). UV-B radiation is highly damaging because
450 direct absorption can induce the formation of photo-products such as CPDs and 6-4 PP in
451 DNA or generate high levels of photo-oxidative damage to macromolecules through
452 indirect absorption (Lesser, 2006). Therefore, the protection of encapsulated *A. monodon*
453 embryos is imperative to ensure the viability of offspring in an intertidal environment with
454 abundant environmental stressors (Pechenik, 1986; 2018; Przeslawski, 2005).

455 *Oviposition behavior*

456 Oviposition site can be also an important issue in reproductive strategy to protect
457 offspring from physical stressors including UV-R, particularly in intertidal environments
458 (Rawlings, 1999). *A. monodon* females tend to lay their egg capsules in places protected
459 from radiation, such as in rock crevices or shaded intertidal sites (Gallardo, 1979). Thus,

460 the selection of oviposition site by females may be key to embryo survival during
461 encapsulated development (Spight,1977). However, in some cases females laid its egg mass
462 on UV-R exposed sites (Salas-Yanquin, pers. obs.). A non-protected laying site can result
463 in the death of many of the embryonic masses (e.g. *Thais lamellosa*, Spight, 1977). The
464 arrangement of the egg capsules within the capsular mass is another aspect that could
465 reduce the impact of solar radiation. Egg capsules of *A. monodon* are laid on the substrate
466 side by side and with little space between them, forming a relatively compact capsular mass
467 (Gallardo, 1979). Thus, the height of one capsule allows shading of the neighboring
468 capsule, generating a sort of protection from incident environmental radiation.

469 Offspring developmental stage at hatching from capsules (e.g. crawling juveniles in
470 species with direct development or veliger larvae in species with mixed development, see
471 Pechenik, 1979) confers different levels of protection against solar radiation, depending on
472 the presence of photoprotective compounds and/or the development of hard protective
473 structures (Paredes-Molina et al., 2016). Before hatching, *A. monodon* crawling juveniles
474 (Chaparro et al., 2018; Salas-Yanquin et al., 2021) had been protected inside capsules so
475 their first direct exposure to UV-R occurs when juveniles leave the capsule, when the UV-
476 B stress could generate cellular and physiological damage (Steeger et al., 2001; Lister et al.,
477 2010). However, the presence of a well-developed and calcified shell in hatchlings (Salas-
478 Yanquin et al., 2021; 2022) would provide an important protective role against UV-R
479 exposure (Paredes-Molina et al., 2016). The relationship between photoprotection level and
480 embryonic development has been studied in gastropods of the genus *Crepipatella*. In the
481 limpet *C. dilatata*, with direct intracapsular development, lower values of photoprotective
482 pigments (carotenes and MAA) were identified compared to the congeneric and sympatric

483 species *C. fecunda* (currently named *Crepipatella peruviana*) (Paredes-Molina et al., 2016).
484 In the latter species, hatched veliger larvae remain in the water column for about 15 days
485 before settling (Chaparro et al., 2002; Rivera-Figueroa et al., 2021), so photoprotective
486 compounds would be of high importance for larvae in that habitat, unlike *C. dilatata*, where
487 newly hatched crawling juveniles possess a well-developed protective shell (Chaparro et
488 al., 2005; 2019).

489 In conclusion, the photoprotection of encapsulated embryos of *A. monodon* is
490 supported by the presence of TC, both in the embryos and in the capsule wall. In addition to
491 this mechanism, capsule walls play a pivotal role as a physical protection structure for the
492 encapsulated embryos, attenuating PAR and UV-R. Determining if females have the ability
493 to select the oviposition site, as a complementary mechanism to reduce exposure to direct
494 solar radiation, as well as testing if subtidal and intertidal capsules of *A. monodon* show
495 similar mitigation strategies against UV-R, are subjects that merits future research.

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Figure captions

Fig. 1. Irradiance level of A) UV-B (280–320 nm; W m^{-2}) and B) PAR (400–700 nm; µmol m^{-2} s^{-1}) during a daily cycle under a clear sky condition at Calfuco beach (39° 46′ 50″ S, 73° 23′ 34″W) during summer (January 2022).

Fig. 2. Total carotenoid content in the foot and gonad of adult *A. monodon* (N = 13 gonad, N = 13 foot). Each bar in the figures represent mean (SD).

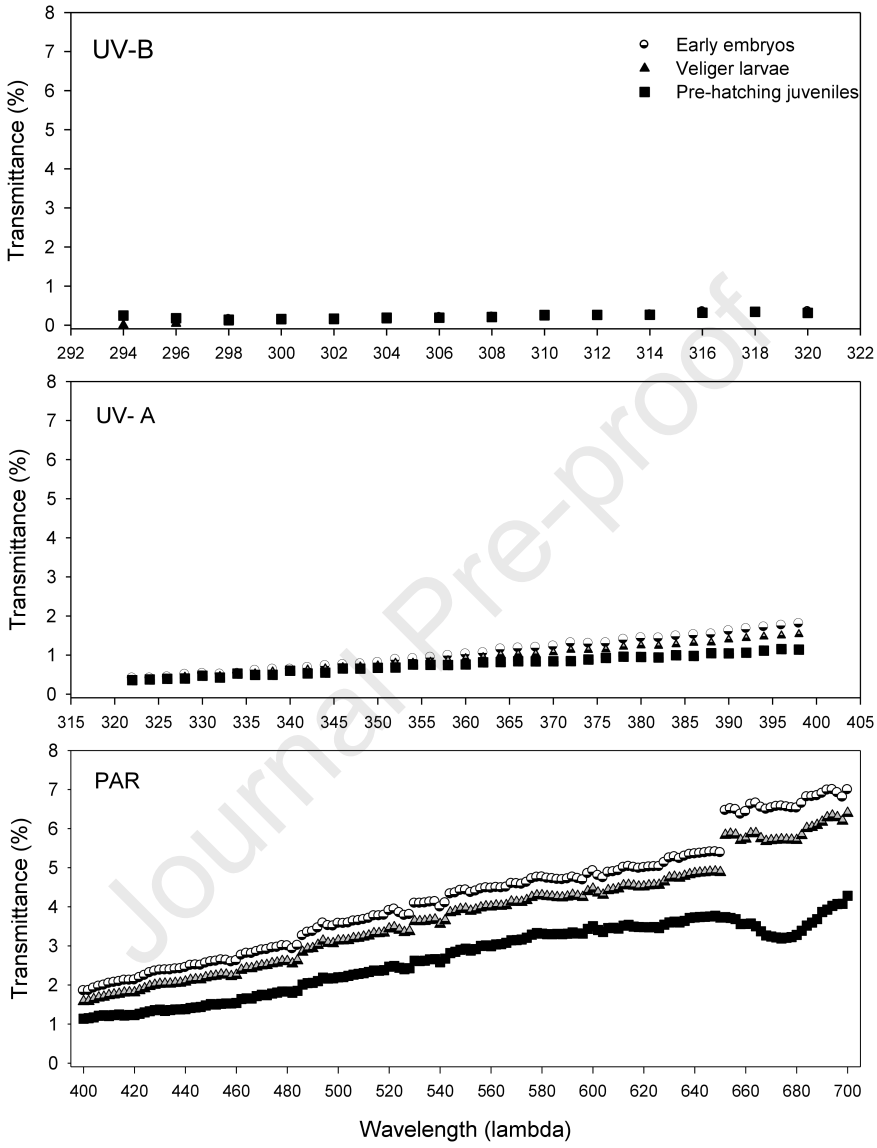
Fig. 3. Total carotenoid content in the capsular wall of *A. monodon* with different stages of embryo development (embryo N = 14, veliger N = 15, pre-hatching juvenile N = 9). Mean and SD. See Materials and methods section for definition of developmental stages.

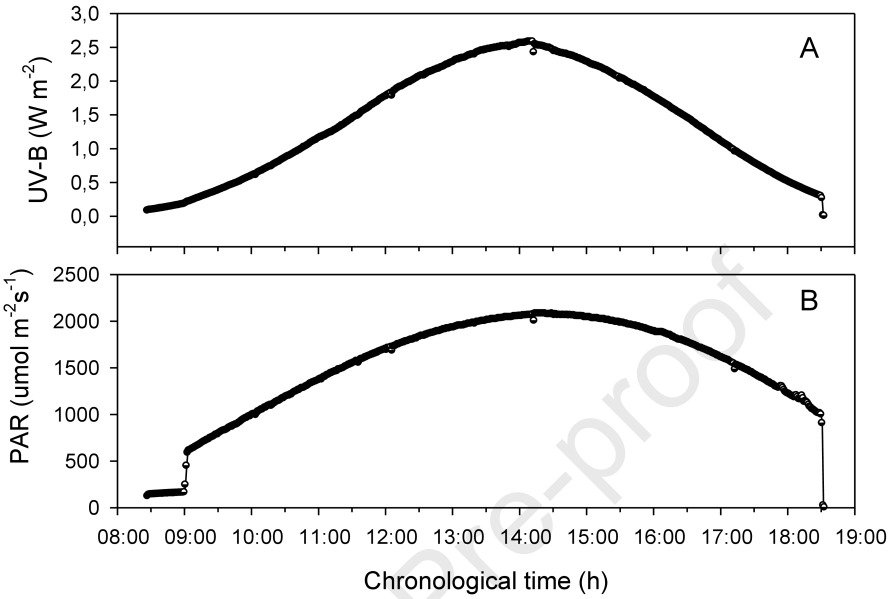
Fig. 4. Total carotenoid content in encapsulated embryos of *A. monodon* with different developmental stages (mean ± SD). Initial embryo (N = 7), veliger (N = 16), and pre-hatching juvenile (N = 8).

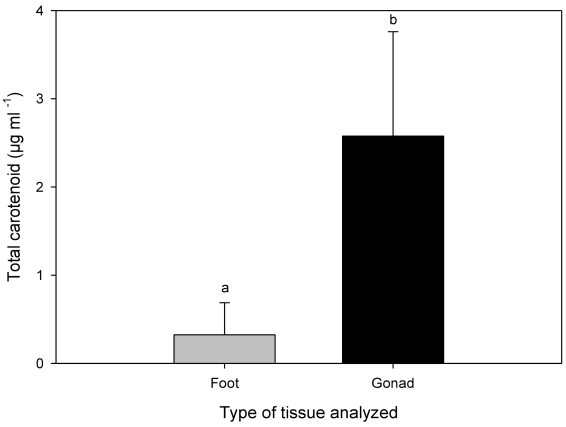
Fig. 5. Capsule wall transmittance of different wavelengths (UV-B, UV-A, and PAR). The capsules used had different levels of embryo development (initial embryo, veliger larvae and pre-hatching juvenile). Each point in the figures represent mean of the replicates (N = 48 for each development stage) measured according to the light spectrum and the corresponding stage of embryo development.

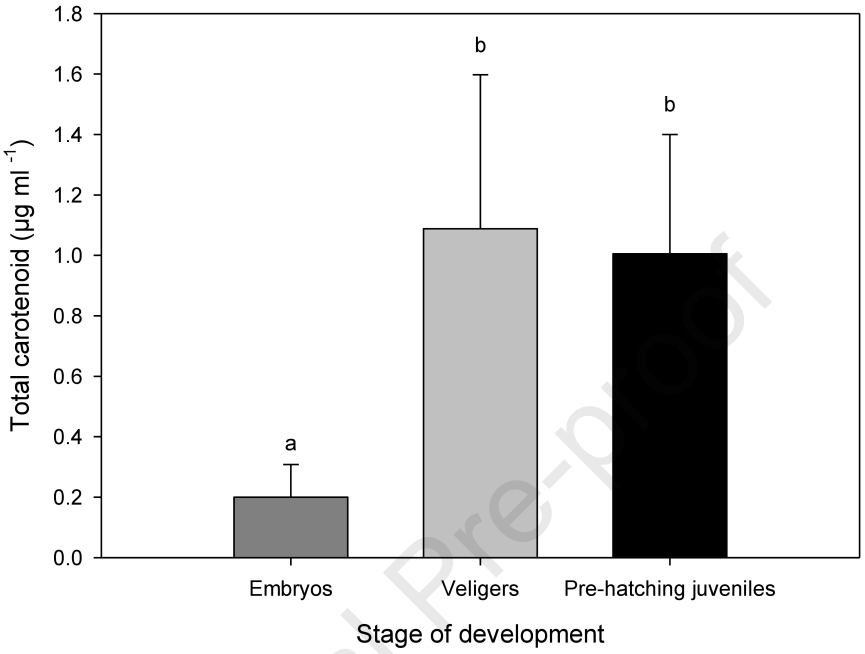
Fig. 6. Oxygen consumption rate (OCR) responses by encapsulated (N = 24) and artificially ex-capsulated (N = 24) early embryos when exposed to PAR and PAR+UV-A+UV-B radiation. Mean ± SD. Different letters indicate significant differences ($p < 0.05$).

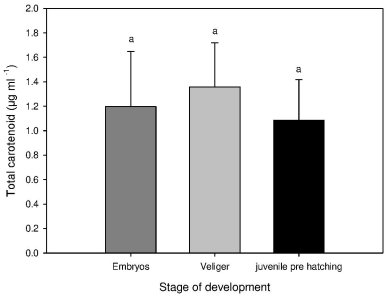
Fig. 7. Histological section of the capsular wall of *A. monodon* for stages A) early (embryos without shell) and B) advanced (veliger > 800 µm shell length). Empty arrow indicates lateral projections of the outer wall layer. Filled arrow indicates vacuolated middle layer. EL = external layer, ML = middle layer, IL = internal layer.

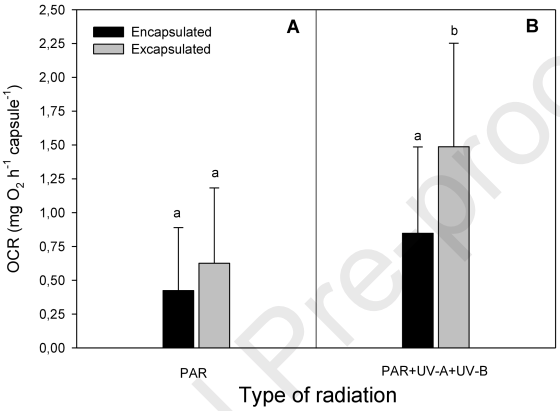


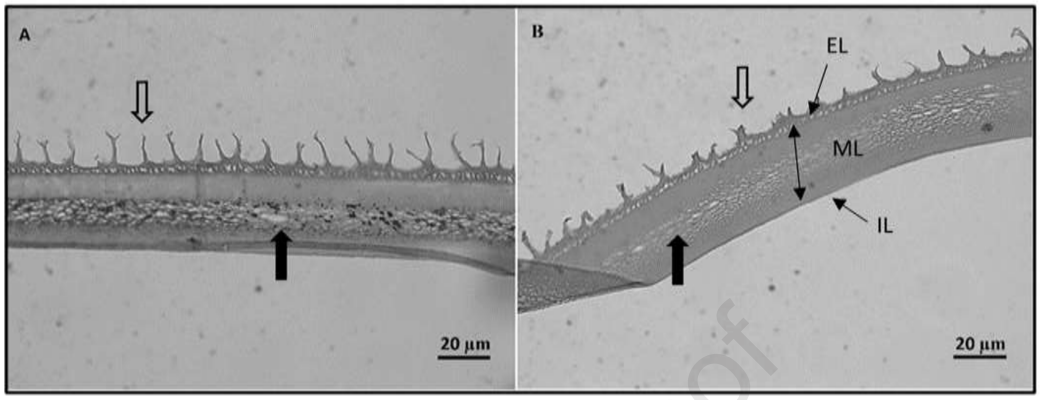












Highligths

- Total carotenoids (TC) were higher in gonad than in adult pedal tissues
- TC were recorded in all encapsulated embryo stages
- Capsule wall filtered over the entire wavelength range tested (220-700 nm)
- UV-R exposure increased oxygen consumption rate in excapsulated embryos

CRediT authorship contribution statement

V.M. Cubillos: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: