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Are embryonic developing modes determinant in the acquisition and levels of photoprotective compounds in slipper limpets of the *Crepipatella* genus?

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ABSTRACT

The type of embryonic development (mixed and direct) and its influence on the accumulation and translocation of photoprotective compounds from the mother to the encapsulated embryo was studied in the intertidal gastropods *Crepipatella peruviana* and *Crepipatella dilatata* during their reproductive peak. HPLC/MS was used to determine type and levels of sunscreen compounds (total carotenoids; TC/and mycosporine-like amino acid; MAA) in brooding females, capsule walls and early and pre-hatching embryos of both species. Photoprotective compounds were only quantified in nurse eggs of *C. dilatata*. Our results indicate that females of both species can accumulate TC and MAA at different levels, and they are able to transfer them selectively to capsule walls, embryos and nurse eggs. Palythine-serine (MW = 244 Da; λ_{max} = 320 nm) and MAA-330 (MW = 234 Da; λ_{max} = 330 nm) constitute total MAA pool in brooding females, whereas brooded embryos incorporate palythine (MW = 244 Da; λ_{max} = 320 nm) to the MAA pool. Although TC was transferred from the mother to the embryo through the yolk in both species, MAA trespass showed differences. Females of *C. peruviana* transfer MAA to their embryos through the embryonic yolk; *C. dilatata* can transfer MAA only through their nurse eggs, which are consumed by embryos during the terminal stages of intracapsular development. Differences between mixed and direct embryonic development, as well as environmental UV-R levels, which the recently hatched larvae and juveniles of *C. peruviana* and *C. dilatata* are exposed to, would determine levels of sunscreen compounds in each species. Higher TC and MAA levels in pre-hatching larvae of *C. peruviana* compared to *C. dilatata*, indicate a necessity of *C. peruviana* for protection against UV-R radiation during approximately 15 days when their veliger larvae remain in the water column before metamorphosis is complete. Conversely, low photoprotective levels in pre-hatching juveniles of *C. dilatata* could be related to low UV-R exposure levels due to the direct incorporation to the benthos and the presence of a protective shell.

1. Introduction

Acclimation potential and adaptation are determinant in the capacity that organisms have to tolerate different levels of environmental stress [1,2]. Such capacities are mainly related to the environment in which they develop [3], the intensity and time of stressor exposure [4–6] and the developmental stage of the exposed organism [7]. In general, early developmental stages of marine invertebrates are highly susceptible to fluctuations in their environmental conditions [8–10]. Ultraviolet radiation (UV-R; 280–400 nm) may vary throughout the year and may affect marine invertebrate larvae [3]. The highest UV-B radiation (280–400 nm) levels of the year are observed during summer periods [11], being coincident with reproduction of some marine and estuarine species of mid-latitude areas [12–14]. The above can seriously compromise the equilibrium between protection and cellular damage in their early developmental stages [3,8]. Prolonged UV-B exposure in marine invertebrates can cause fertilization problems in free spawning organisms [15], increasing the frequency of abnormalities [3,8,16], reducing larval growth rates [17,18], to finally increase mortality rate [16,19–22]. These factors can impact the fitness of those species, which can be identified through reductions in larval settlement [23] and recruitment of juveniles [24].

UV-B can generate macromolecular damage, especially in those species that have swimming larvae and that remain exposed to their levels in the water column [3,25]. For example, direct UV-B exposures to sea urchin larvae (*Sterechinus neumayeri*) can generate increased levels of DNA mutations, through the generation of cyclobutane pyrimidine dimers (CPD) [26]. Indirect UV-R exposure can produce oxidative damage to macromolecules (e.g. lipids, proteins and DNA) in early developmental stages of marine invertebrates, as was observed in sea urchin larvae

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(*Tripneustes gratilla*) [8]. Macromolecular UV-R induced damage in marine organisms has been mitigated by their displacement to less exposed areas [27]. Acquisition and accumulation of UV-R screening compounds has also been reported as an efficient mechanism to reduce cellular damage in several taxa of marine organisms (e.g. Porifera, Cnidaria, Annelida, Mollusks, Platyhelminthes, Arthropoda, Bryozoa, Echinodermata and Chordata), [28–33]. Particularly, UV-R screening compounds has been described in several groups of mollusks from intertidal to subtidal habitats from polar to tropical latitudes [33–39].

Mycosporine-like amino acids (MAA) are efficient UV-R blocking compounds and have been able to reduce cellular damage in several marine invertebrate taxa [22,32,33,40–44]. MAA are colorless molecules with low molecular weight (244–1050 Da) that can absorb wavelengths between 310 and 364 nm [28,35,45–50], and dissipate their energy without reactive oxygen species (ROS) generation [51]. Carotenoids are larger molecules than MAA and they are formed by chains of 40 carbon atoms [52] characterized by a yellow-orange coloring, which have been attributed to photoprotective functions in marine organisms [53, 54]. Because mollusks lack the biochemical pathway to synthesize MAA and CT, they must acquire these compounds through food [40, 43,55], maternal trespass [5] or symbiotic associations [56]. In this context, herbivorous diet of intertidal gastropods has been considered decisive in the accumulation of photoprotective compounds [34]. Once photoprotective compounds are assimilated in adult organisms, they can be transferred to gonadal tissue and eggs [7]; offering protection against environmental radiation to the initial developmental stages [5,19]. A previous study indicated that gastropods that lay their eggs in intertidal zone require higher levels of protection for their early developmental stages, to reduce the damage caused by extended periods of UV-R exposure [34]. Although the presence of high levels of sunscreen compounds in early developmental stages appears to be determinant in protection levels against UV-B, exposure time in the water column may be determined by the development mode of each species.

Crepipatella peruviana and *Crepipatella dilatata* co-exist at mid-latitude areas of southern Chile. Although both species of Calyptraeid gastropods brood their encapsulated embryos in the pallial cavity of females [57,58] and share the same reproductive pattern, their embryonic development differs [57]. *C. peruviana* has a mixed embryo development. Their encapsulated embryos consume yolk as the main energy source until they reach the veliger stage, hatching from the capsule as swimming larvae that spend approximately 15 days in the water column before metamorphosis [12,57–59].

In contrast, *C. dilatata* has a direct embryo development where encapsulated embryos use yolk and nurse eggs as energy sources to continue developing, and start metamorphosis inside the capsule. Prehatching embryos emerge from their capsules as crawling juveniles that become incorporated directly into the benthos [57]. Therefore, newly hatched organisms of both species begin their independent life at different stages of development (veliger vs. juvenile) and UV-B exposure (planktonic vs. benthic).

Currently, there are no previous records indicating if embryonic development modes in marine gastropods determine photoprotective compounds trespass from females to their embryos; and how this situation can limit photoprotective compound levels in early developmental stages and pre-hatching embryos of *C. peruviana* and *C. dilatata*.

Species that have mixed embryonic development modes produce pelagic larvae spending several days in the surface of the water column, and can therefore potentially suffer critical levels of molecular damage if mitigation mechanisms are not efficient enough. In contrast, species with direct development have embryos that emerge from capsules as juveniles fitted with a carbonate shell. They would not need elevated levels of UV-R photo-protective compounds, because their shell may act as a physical barrier against UV-B radiation.

2. Materials and Methods

2.1. Sample Collection

Females of *C. peruviana* and *C. dilatata* were collected during the summer of 2014 from Pelluco (41°29′22″S–72°53′59″W–Puerto Montt) and Quempillén (41°52′14″S–73°46′04″W - Ancud), southern Chile. Clumped females were collected and transferred to Calfuco laboratory (Universidad Austral de Chile), together with the rocky substrate they were found on. Samples of the pedal tissues (10 mm^2) of taken from female of each species. Egg masses were also collected and encapsulated embryos categorized according to their development as follows: "early development" (230 μm for *C. peruviana;* and >300 μm for *C. dilatata*) and "pre-hatching development" (>329.5 μm for *C. peruviana;* and >800 μm for *C. dilatata*) [60,61]. Wall capsules of each egg mass that contained early and pre-hatching embryos were also collected separately. All materials were stored at −80 °C until further biochemical analysis. Prior to photoprotective compound extraction, tissue samples were dried using a laboratory stove (Memmert - Model U 40, USA) at 40 °C during 72 h, following the protocol used by Cubillos et al. [31]. The dried tissue was then converted into a fine powder using a mortar and pestle, and stored at room temperature in dark conditions until MAA and TC extraction. Approximately 4000 embryos of *C. peruviana* and *C. dilatata* in early developmental stages were collected. Approximately 3000 and 2500 embryos of *C. peruviana* and *C. dilatata* in pre-hatching stage were used respectively to extract photo-protective compounds.

2.2. Extraction and Analysis of Mycosporine-like Amino Acids (MAA)

MAA extraction was carried out using the protocol of Cubillos et al. [31]. Accordingly, 10 mg of fine powder was homogenized in 1 ml of 100% methanol (for HPLC analysis) and then sonicated for 5 min (Cole Palmer, model 8891, USA), and stored in dark conditions at 4 °C for 24 h. Then, samples were centrifuged at 12,000 rpm (Hettich, model Universal 16, USA) during 5 min and the supernatant was filtered using a syringe nylon filter of 0.22 μm. MAA levels were determined injecting 20 μl of methanolic extraction in a HPLC (Shimadzu, model Prominence UPLC, Japan), which was eluted by a mobile phase in an isocratic way during 10 min at 0.5 ml min^{-1} through an Xterra column (Waters, USA/4.6 mm by 250 mm). The mobile phase was composed of 6% MeOH, 6% ACN and 88% of a solution of diluted formic acid (99.8% MilliQ and 0.2% CH_2O_2). The HPLC system was provided by a DAD detector that was used to determine relative retention time (RRT) and levels of each MAA.

2.3. Mycosporine-like Amino Acids (MAA) Standards

MAA standards were generated through the collection of individual peaks obtained by the elution of methanolic extractions of embryonic tissue of *C. peruviana* in the HPLC system. Individual peaks were collected independently. Each isolated MAA was identified using an UHPLC/MS (Dionex-Ultimate 3000/Thermo TSQ vantage). For each isolated and identified MAA, a standard curve was created through serial dilutions, and their concentration were calculated through the peak area under the curve.

2.4. Extraction and Analysis of Total Carotenoids (TC)

Total carotenoid extractions were carried out using the methodology described by Zheng et al. [39]. Accordingly, 10 mg of tissue was homogenized in 1 ml of acetone (90%) in a centrifuge tube for 5 min. Tubes were sonicated using an ultrasonic bath (Cole Palmer, model 8891, USA) for 5 min and then centrifuged at 6000 rpm (Hettich, model Universal 16, USA) for 5 min at room temperature. Supernatant was collected and its absorbance was determined at 480 nm and 750 nm using a spectrophotometer (Shimadzu UV-Mini 1230). TC were re-extracted