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SHORT COMMUNICATION

Direct and indirect acquisition of photoprotective compounds in crab larvae of coastal Patagonia (Argentina)

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First larval stage (Zoea I) of the crab *Cyrtograpsus altimanus* can obtain photoprotective compounds (PPCs) from their mother (indirect acquisition) and also via feeding on PPC-producers such as phytoplankton (direct acquisition). The bioaccumulation of PPC resulted in higher survival of larvae exposed to ultraviolet radiation (UVR), when comparing larvae with high and low content of PPC. Thus, both ways of acquiring PPC may contribute to maximize survival of Zoea I when they are exposed to natural UVR.

KEYWORDS: bioaccumulation; crab larvae; *Cyrtograpsus altimanus*; patagonia; planktonic larvae; photoprotective compounds; UVR tolerance

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INTRODUCTION

Solar ultraviolet radiation (UVR, 280–400 nm) is known to cause potential negative effects to zooplankton in the upper water column (Speekmann *et al.*, 2000; Browman, 2003). For example, early studies determined mortality in several species of crab larvae owing to exposure to UVB (280–315 nm) radiation (Damkaer *et al.*, 1980; Morgan and Christy, 1996). However, one of the strategies of zooplankton to cope with UVR is the acquisition and bioaccumulation of photoprotective compounds (PPCs) which include pigments like carotenoids and UV-absorbing compounds (UACs) such as mycosporine-like aminoacids (MAAs). MAAs have maximum absorbances in the UVR region (typically 310–360 nm; Karentz *et al.*, 1997; Sommaruga and Garcia-Pichel, 1999), whereas carotenoids absorb within the range of the visible radiation (400–700 nm) and often provide protection as antioxidants (Edge *et al.*, 1997). PPC with maximum absorbance within the UVR range (UV-absorbing compounds) can act as natural UVR sunscreens. Since PPC are not synthesized by metazoans, they can be acquired from dietary items that may include phytoplankton and macroalgae (Adams and Shick, 1996; Helbling *et al.*, 2002; Moeller *et al.*, 2005).

Adults of the crab *Cyrtograpsus altimanus* (Rathbun, 1914) are dominant in tidal pools of coastal Patagonia and are an important component of the aquatic food web (Galván *et al.*, 2009). In the natural environment of this species, macro- and microalgae are plentiful and it has been shown that adults can bioaccumulate PCC in their tissues (Hernández Moresino and Helbling, 2010). These authors suggested that newly hatched larvae (Zoea I) had PPC which was "inherited" from their mother, thus providing initial protection to the larvae. However, after hatching, larvae also feed on other organisms such as phytoplankton (Anger, 2001), and thus, they not only might be initially protected by PPC acquired from the progenitor (indirect trophic acquisition), but also might be able to acquire PPC by feeding on phytoplankton (direct trophic acquisition).

The main objective of this work was to experimentally assess the importance of indirect and direct acquisition and further bioaccumulation of PPC, on larvae of the coastal crab *C. altimanus*. We also tested the role of PPC in conferring protection to the larvae against UVR.

Organism collection and maintenance

The larvae used in our experiments were obtained from (a) females previously fed on different diets in the laboratory (indirect acquisition) and (b) ovigerous females collected in the field (direct acquisition).

(a) *Indirect acquisition of PPC*: Adult males and pre-ovigerous females of *C. altimanus* were collected from tidal pools in Puerto Madryn (42°47′S, 65°00′W) on the Atlantic coast of Patagonia during low tide in mid-July 2009 (austral Winter). They were transported in thermally insulated containers to Estación de Fotobiología Playa Unión (EFPU) and split in two aquaria (60 × 40 × 20 cm; length × width × depth). Each aquarium contained 30 pre-ovigerous females, 15 males, and ca. 25 L of seawater, that were fed with one of two different diets: (a) PPC diet, which consisted of a mixture of *Porphyra columbina* (intertidal red macroalgae with high UAC content and moderate carotenoid content) and muscle tissue of the Argentinean hake *Merluccius hubbsi* (which lacks PPC), (b) no-UAC diet, which consisted only of *M. hubbsi*. Feeding of adults was *ad libitum* for ca. 3 months. During this period, larvae from the first spawning were discarded (to avoid potential interference from the feeding history of the adults) and only larvae from the subsequent spawning were used for experimentation. Before the second spawning began, three females from each diet were randomly selected and the PPC contents in their gonads, digestive glands and embryos were analyzed (see below). The contents of PPC in newly hatched larvae (batches of 200–300 individuals) originated from three females fed on each diet were also analyzed. The aquaria were kept inside an aerated culture chamber at 15°C, with a 12:12 h photoperiod (120 W m^{-2} visible radiation or PAR), and seawater was renewed weekly.

(b) *Direct acquisition of PPC*: Ovigerous female *C. altimanus* were collected from tidal pools in Puerto Madryn during the austral Spring and Summer (hatching season) of 2009–2010. They were transported to EFPU and individually placed in 2-L aquaria with *in situ* collected seawater until their embryos hatched (1–7 days later). The newly hatched larvae from a single field-collected female were divided into two feeding groups of 300 individuals incubated in two aquaria (17 × 17 × 4 cm, length × width × depth) with 500 mL of autoclaved seawater. Larvae were fed on two diets consisting of cultures of the dinoflagellate *Prorocentrum micans* with high and low contents of UAC, and moderate content of carotenoids in both cases (see online Supplementary data, Appendix S1 for details on these cultures). The initial concentration of *P. micans* in each diet was 5000 cells mL^{-1}, and the water was partially renewed every 48 h. Larvae were kept in these conditions for 6 days, and groups of 20–30 larvae were collected every 2 days for PPC analysis. This experiment (repeated measurements, without replicates) was performed twice, so each aquarium from each experiment was considered a replicate.

Survival of larvae

Newly hatched larvae from the indirect acquisition experiment with PPC and no-PPC diets were placed in

groups of 20 individuals inside 180-mL glass beakers with autoclaved seawater (without food) and exposed under a solar simulator (SOL 1200W, Dr Hönle AG, Gräfelfing/München, Germany) at 15°C. Two radiation treatments were done using cutoff filters on top of the beakers: P treatment, larvae received PAR (400–700 nm); and PAB treatment, larvae received the full spectrum of radiation (PAR, UVA and UVB, 280–700 nm). The beakers were placed at 85 cm from the solar simulator so the samples received irradiances of 1.22, 49.1 and 125.8 W m^{-2} for UVB, UVA and PAR, respectively. The radiation level was chosen based on UVB irradiance commonly measured in the study area at summer noon, when UVB reaches ca. 1.8 W m^{-2} at the sea surface (Villafañe *et al.*, 2004b). Radiation treatments were performed in triplicate, therefore a total of six beakers were used for each exposure and diet. Dead larvae were recorded at different time intervals (20–120 min) until all larvae in the PAB treatment were dead. These experiments were carried out twice.

Analysis and measurements

The contents of PPC in macroalgae, dinoflagellates, fish tissue, adult and larval crabs were estimated from methanolic extracts of samples using a UV–VIS spectrophotometer (Hewlett Packard 8453E). The UAC contents were estimated by peak analysis of the spectrophotometric scans at 310–360 nm (Helbling *et al.*, 1996). The total carotenoid contents were also estimated from the spectrophotometric scans using the equation of Wellburn (1994). UAC contents are expressed as optical density (OD), while carotenoid contents are expressed as weight of total carotenoids. Both PPC contents are normalized by dry weight or by cell numbers in the case of *P. micans*.

Further details about analysis and measurements can be found in Supplementary data, Appendix S2.

Statistical analysis

A two-way analysis of variance (ANOVA) test was used to evaluate differences in PPC contents between diets and

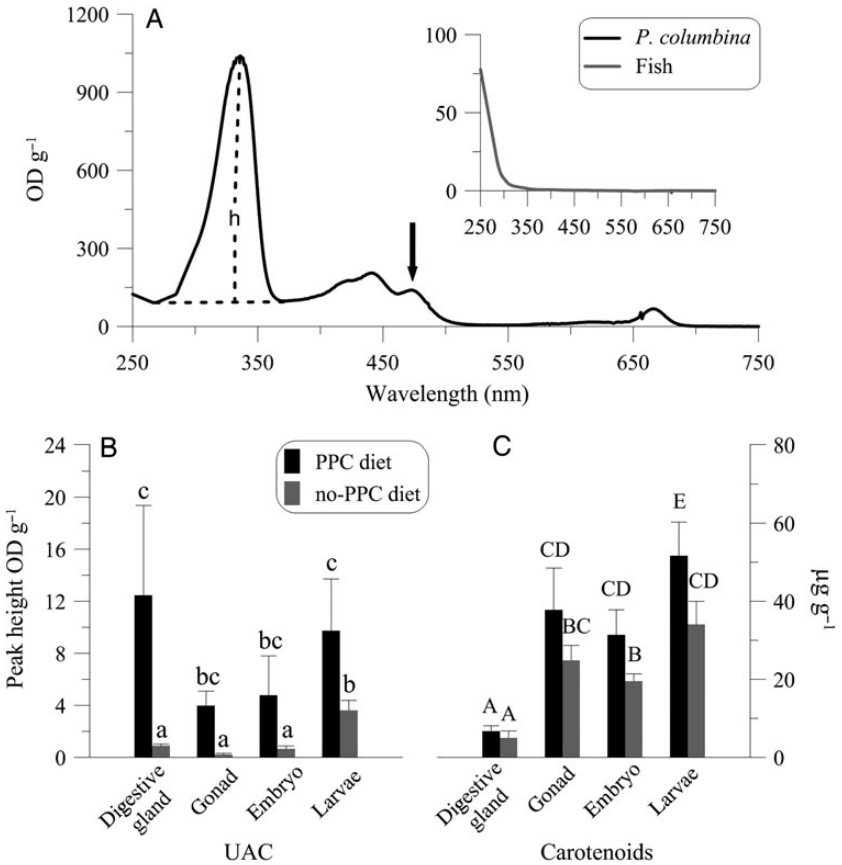

Fig. 1. (**A**) Absorption spectra for the red macroalgae *P. columbina* (black line) and for the muscle tissue of fish (gray line, insert), where *h* indicates the height of the peak of absorbance at 337 nm and the arrow indicates the peak of absorbance at 470 nm. (**B**) Content of UAC in different tissues of adult females of *C. altimanus* (fed with PPC and no-PPC diets) and their ZI larvae. (**C**) The same as (B) but indicating carotenoid contents. Data in (A) and (B) are expressed as OD normalized by dry weight of sample, while data in (C) are expressed as weight of total carotenoids normalized by dry weight of sample. Bar height represents average contents and error bars indicate one standard deviation ($n = 3$). Lower case and capital letters on top of the bars indicate significant differences between diets and samples, for (B) and (C), respectively.

samples/tissues in the indirect acquisition experiment. A one-way ANOVA was used to evaluate differences in UVB-induced mortality of larvae from mothers subject to the two diets. Also a one-way repeated measurements ANOVA was used to evaluate differences between PPC contents in larvae during the direct acquisition incubations. When applicable, *post hoc* pairwise comparisons were performed using the Tukey HSD test.

Indirect acquisition of PPC (maternal transfer)

The macroalga *P. columbina* exhibited a characteristic absorption peak in the UVR range at 337 nm (UAC compounds) and at 470 nm (carotenoids), while the fish tissue did not absorb significantly in any range (Fig. 1A). Peak analysis of PPC in different tissues of females of *C. altimanus* and newly hatched larvae (Zoea I) from these females showed significant differences between diets, both in UAC and carotenoid contents ($F_{(3,16)} = 3.9$, $P < 0.03$ and $F_{(3,16)} = 5.6$, $P < 0.01$, Fig. 1B and C, respectively). *Post hoc* tests indicated differences between diets in all samples in the case of UAC ($P < 0.05$), and differences in embryos and larvae ($P < 0.01$) in the case of carotenoids, with samples from the PPC diet having higher values than those from no-PPC diet. Larvae fed on a PPC diet had double the UAC content and ca. 50% more carotenoid contents than those fed on the no-PPC one.

Direct acquisition of PPC (bioaccumulation by larvae)

The absorption spectra of *P. micans* (Fig. 2A) showed no significant differences in the carotenoid content between diets ($F_{(1,4)} = 4.68$, $P = 0.1$), so we refer to these diets as UAC-rich and UAC-poor diets. Peak analysis of larvae from the different diets throughout the time of feeding (Fig. 2B) showed significant differences in UAC ($F_{(3,6)} = 6.33$, $P = 0.03$). This indicated that larvae fed on UAC-rich diet of *P. micans* accumulated higher contents of UAC than the ones fed on the UAC-poor diet. However, carotenoid contents (Fig. 2C) did not show differences between diets through the time of feeding ($F_{(3,6)} = 0.6$, $P = 0.65$).

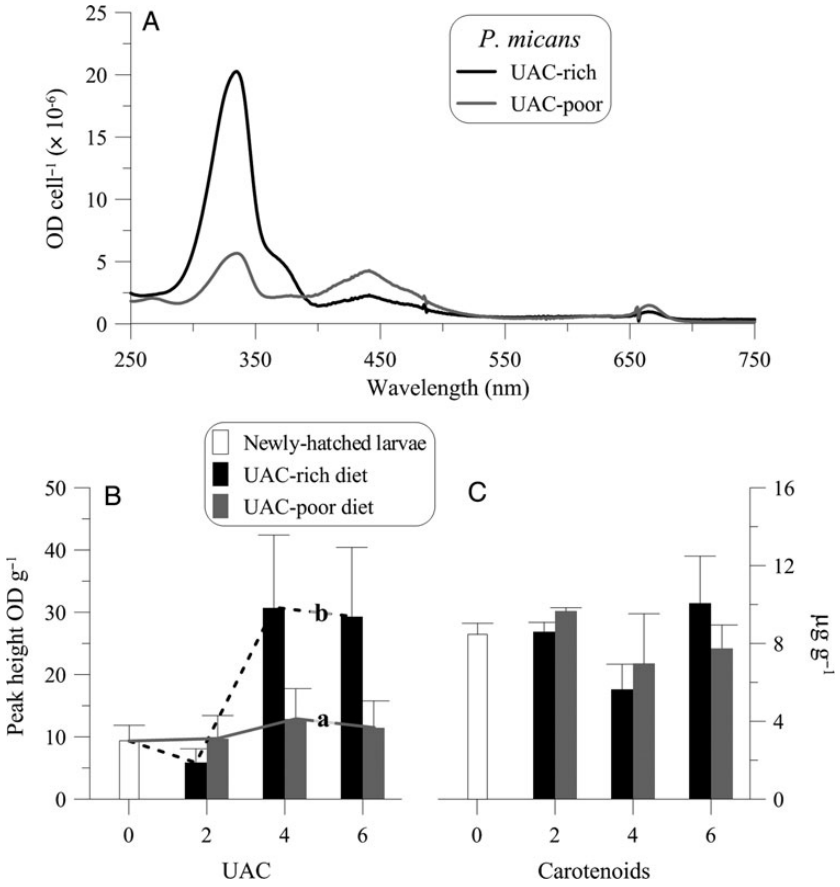

Fig. 2. (**A**) Absorption spectra of *P micans* after 6 days of exposure to full radiation (PAB treatment, black line) and PAR only (P treatment, gray line). (**B**) UAC contents at different time of feeding of *C. altimanus* larvae from UAC-high and UAC-poor diets. (**C**) The same as (B) but indicating carotenoid contents. Data in (A) and (B) are expressed as OD normalized by dry weight of sample, while data in (C) are expressed as weight of total carotenoids normalized by dry weight of sample. Bar height represents average contents and error bars indicate half-mean range ($n = 2$). Lines connecting bars and lower case letters in (B) indicate significant differences between diets through the time of feeding.

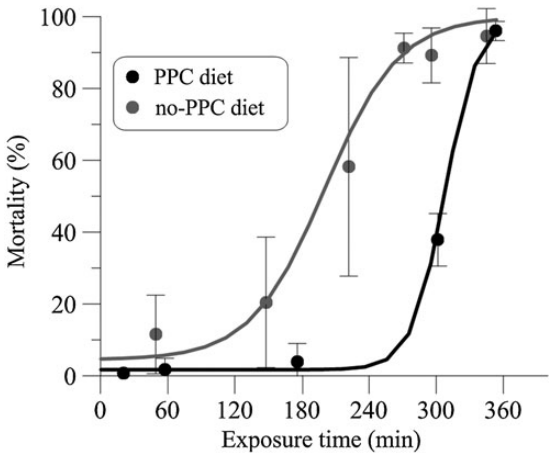

Fig. 3. Mortality (%) as a function of exposure time to UVR. ZI larvae of *C. altimanus* from females fed with PPC and no-PPC diets (black and gray symbols, respectively). Error bars indicate one standard deviation from two experiments with triplicates ($n = 6$). Lines represent the sigmoidal function fitted to data.

Survival of larvae with different PPC content

Larvae from the indirect acquisition experiments experienced no mortality when exposed to PAR only (control), so, we show (Fig. 3) the mortality due to UVR as a function of exposure time, and for diets with and without PPC. The mortality was best fitted ($R^2 = 0.98$ and 0.77 for PPC and no-PPC diets, respectively) with sigmoidal curves. Significant differences ($F_{(1,4)} = 6.83$, $P < 0.01$) were found in threshold time (Th_t) with larvae starting to die after 254 and 139 min of exposure to UVR for the PPC and no-PPC diets, respectively. Also larvae from the PPC diet were more resistant than those from no-PPC diet in terms of lethal time of exposure for 50% mortality (LT_{50}) ($F_{(1,4)} = 6.83$, $P < 0.01$), with values of 304 and 196 min, respectively.

We demonstrate that newly hatched larvae in the laboratory had an initial content of PPC that was related to the items eaten by their progenitor. These "inherited" PPC may provide larvae a first level of protection to cope with potentially damaging UVR exposure. Our results agree with previous studies which also found indirect transfer of UAC in crustacean zooplankton, e.g. *Idothea baltica* (Helbling *et al.*, 2002), or *Cyclops abyssorum tatricus* (Orfeo *et al.*, 2011). Moreover, indirect transfer of carotenoids has also been documented in other crustacean zooplankton (Hairston, 1979).

Larvae were also able to bioaccumulate UAC when feeding on a dinoflagellate which is a potential prey and which is highly abundant on the Atlantic coasts of Patagonia during late Spring and Summer (Villafañe *et al.*, 2004b), coincident with the hatching season of *C. altimanus*. This suggests that *C. altimanus* larvae may use a second level of protection against UVB by directly feeding on their planktonic prey and thus presumably obtain protection for an extended period through development.

Our data also suggest that PPC from mother's diet conferred on larvae a higher UVB tolerance of ca. 80% (in terms of Th_t), comparing larvae from crabs reared with PPC and no-PPC diets. This increased tolerance would have been due not only to UAC, but also to carotenoids. Further experiments are needed to explore the relative contribution to the UVB tolerance of each of these two types of PPCs.

Based on the UVB penetration in the study area (1% of surface irradiance at 12.4 m depth, i.e. $K_{UVB} = 0.37\ m^{-1}$; Helbling, unpublished data), ZI larvae of *C. altimanus* may be exposed to detrimental UVB levels if they swim in the first 3 m of the water column at noon in summer, when the maximum surface irradiance is ca. $1.8\ W\ m^{-2}$ (Villafañe *et al.*, 2004a). Zoea I larvae of the coastal crab *Cyrtograpsus* spp. may use a dispersal mechanism that involves temporal residence of larvae in the upper layers of the water column (Dellatorre, 2009). Therefore, indirect and direct acquisition of PPCs may act as redundant acclimation of larvae to enhance their resistance to ambient UVR while being transported offshore in upper layers of the water column.

SUPPLEMENTARY DATA

Supplementary data can be found online at http://plankt.oxfordjournals.org.

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Supplementary Appendix

S1: *Prorocentrum micans* cultures:

This dinoflagellate is a potential prey for *C. altimanus* larvae in the study area, and is known to increase its production of MAAs (mycosporine-like amino acids) when exposed to UVR (Marcoval *et al.*, 2007 and also corroborated in the present study). Briefly, an initial culture of *P. micans* was split in two UVR-transparent Plexiglas containers (1 L) and incubated at 15 ºC under artificial UVR + PAR. One of them was covered with a cut-off filter (Ultraphan UV Opak Digefra, Germany) which blocked the UVR and the other was left without filter. Both cultures were placed inside a culture chamber (Sanyo MLR-350) under a Q-Panel UVA 340 lamp and four Phillips daylight lamps, with 12:12 h photoperiod. Containers were placed at 25 cm from the lamps, thus receiving irradiances of 0.14, 3.06 and 26.4 W m^{-2} for UVB, UVA and PAR, respectively. This allowed us to have two different diets (UAC-high and UAC-poor) with the same species.

S2: Analysis and measurements

Estimation of photoprotective compounds (PPC): Fresh samples were placed in 15-mL centrifuge tubes with 5 mL of absolute methanol, sonicated for 20 minutes at 25 °C, and extracted for 1 hour. After that, the samples were centrifuged for 15 min at 1500 rpm and the absorption in the UV-visible range of the supernatant was measured with a Hewlett-Packard spectrophotometer. The amount of PPC compounds was estimated by using the optical density at 310-360 nm (for UV-absorbing compounds) and 470 nm (for total carotenoids) and normalized per gram of dry weight or per cell number in the case of *P. micans*. Dry weight was determined from sub-samples dried in the oven at 35 °C for 24 h (time needed to reach a constant weight). We are aware that UV-absorbing compounds are slightly underestimated by this procedure, as it was shown that 20% methanol is the best extraction solvent for these compounds (Tartarotti and Sommaruga, 2002). However, since no significant differences were found previously in our laboratory between the two extraction methods, we considered this procedure to be appropriate for the purposes of our investigation.

Radiation measurement: Irradiance levels both in the solar simulator and in the culture chamber were measured using a broadband filter radiometer ELDONET (Real Time Computers, Inc.) with channels for UVB, UVA and PAR.

Cut-off filters: Previous studies conducted with this species determined that neither PAR nor UVA had significant effects on larval mortality, whereas UVB caused significant mortality (Hernández Moresino and Helbling, 2010). Thus, two radiation treatments were used: P treatment (control), beaker covered with a cut-off filter (Ultraphan UV Opak Digefra, Germany) so the larvae received only visible radiation (PAR 400-700 nm); and PAB treatment, beaker covered with a 290 nm cut off filter (to screen out any potential UVC from the lamp of the solar simulator) so the larvae received the full spectrum of radiation (i.e., PAR, UVA and UVB).

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