SCIENTIA MARINA 79(2)
June 2015, 257-262, Barcelona (Spain)
ISSN-L: 0214-8358
doi: http://dx.doi.org/10.3989/scimar.04172.21B

Physiological adaptation to Mediterranean habitats of the native crab *Pachygrapsus marmoratus* and the invasive *Percnon gibbesi* (Crustacea: Decapoda)

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Summary: Markers of oxidative stress in the hepatopancreas and carotenoid and vitamin E content in the carapace of the invasive crab *Percnon gibbesi* and the native crab *Pachygrapsus marmoratus* were compared as indicators of their physiological condition and environmental adaptation. Crabs similar in size were collected from coastal waters of Portals Vells (Mallorca Island, Spain; 39°28'18.79"N, 2°28'19.63"E) at <1 m depth. Vitamin E levels were significantly higher in *P. marmoratus* than in *P. gibbesi*, whereas all carotenoid concentrations, except astaxanthin, were significantly higher in *P. gibbesi*. Catalase, glutathione peroxidase and glutathione reductase activities were significantly greater in *P. marmoratus*. Malondialdehyde concentration, a marker of lipid peroxidation, was higher in the hepatopancreas of *P. gibbesi*. The differences in carotenoid and vitamin E contents and in the antioxidant enzyme activities are a reflection of the different physiological activity of the two species and may suggest that *P. gibbesi* is not a potential competitor for the native crab *P. marmoratus*.

Keywords: antioxidants; carotenoids; alien species; Mediterranean Sea; oxidative stress; vitamin E.

Adaptación fisiológica a los hábitats del Mediterráneo del cangrejo nativo *Pachygrapsus marmoratus* y el invasivo *Percnon gibbesi* (Crustacea: Decapoda)

Resumen: Se evaluaron marcadores de estrés oxidativo en el hepatopáncreas, y el contenido de carotenoides y de vitamina E en el caparazón del cangrejo invasivo *Percnon gibbesi* y del cangrejo nativo *Pachygrapsus marmoratus* como indicadores de su estado fisiológico y su adaptación al ambiente. Cangrejos de tamaño similar se capturaron en las aguas costeras de Portals Vells (Mallorca, España; 39°28'18.79"N, 2°28'19.63"E) a <1 metro de profundidad. Los niveles de vitamina E fueron significativamente mayores en *P. marmoratus* que en *P. gibbesi*, mientras que todas las concentraciones de carotenoides, excepto la astaxantina, fueron significativamente mayores en *P. gibbesi*. Las actividades enzimáticas de la catalasa, glutatión peroxidasa y glutatión reductasa fueron significativamente mayores en *P. marmoratus*. La concentración de malondialdehído, como marcador de peroxidación lipídica, fue más elevada en el hepatopáncreas de *P. gibbesi*. Las diferencias observadas en los niveles de carotenoides y vitamina E y en las actividades de las enzimas antioxidantes son un reflejo de la diferente actividad fisiológica de ambas especies, lo que puede sugerir que *P. gibbesi* no sea un competidor potencial para el cangrejo autóctono *P. marmoratus*.

Palabras clave: antioxidantes; carotenoides; especies invasoras; mar Mediterráneo; estrés oxidativo; vitamina E.

Citation/Como citar este artículo: Tejada S., Deudero S., Box A., Sureda A. 2015. Physiological adaptation to Mediterranean habitats of the native crab *Pachygrapsus marmoratus* (Crustacea: Decapoda) and the invasive *Percnon gibbesi* (Crustacea: Decapoda). Sci. Mar. 79(2): 257-262. doi: http://dx.doi.org/10.3989/scimar.04172.21B

Editor: Ch. Zeng.

Received: October 31, 2014. **Accepted**: February 13, 2015. **Published**: March 24, 2015.

INTRODUCTION

The rapidly accelerating human activities over the past century (trade, transport and tourism) have dramatically enhanced the spread of alien species (Streftaris et al. 2005). Invasive species often represent a potential risk to native organisms because they compete intensely with resident species, thus altering the population dynamics (Galil 2012). The Mediterranean Sea is prone to biological invasion, with around 600 introduced species (Zenetos et al. 2005). *Percnon gibbesi* (H. Milne Edwards, 1853) is a small grapsid crab with a subtropical distribution. The native distribution of the crab extends from Chile to California and from Florida to Brazil in America and from the Gulf of Guinea to the Azores in Africa (Manning and Holthuis 1981, Galil et al. 2002). The first Mediterranean observations of *P. gibbesi* were from the Island of Linosa, Italy, in 1999 (Relini et al. 2000) and it can now be found in many Mediterranean countries, including Spain, France, Greece, Turkey, Libya and Malta (Deudero et al. 2005, Thessalou-Legaki et al. 2006, Elkrwe et al. 2008). In the Balearic Islands, the first observations were in 1999 in Mallorca and Minorca (Garcia and Reviriego 2000), where this species had established populations. In a decade, this species colonized most rocky shores throughout the Mediterranean basin (Katsanevakis et al. 2011). The wide distribution of these crabs may reflect the general characteristics of invasive species: high abundance in their native range, wide diet, short generation times and ability to survive in a wide range of physical conditions (Ehrlich 1989).

Individuals of *Percnon gibbesi*, originally from the Atlantic Ocean, entered the Mediterranean Sea through the Strait of Gibraltar (Pipitone et al. 2001). This species lives in shallow rocky bottoms with boulders and has a very limited bathymetric distribution, mainly restricted to the infralittoral zone from 0 to 4 m depth, with maximum abundances at 1 m depth and with a maximum of activity during the day (Deudero et al. 2005, Yokes and Galil 2006). *Pachygrapsus marmoratus* is native to the Mediterranean Sea, inhabiting supralittoral crevices (Augusto and Flores 2001), and is known to exploit most of the trophic sources of the intertidal zone during its adult life (Cannicci et al. 2007).

The function of antioxidant enzymes in protecting cells and organisms against the deleterious effects of reactive oxygen species (ROS) and their role as biomarkers of physiological stress have received much interest in recent years in many organisms, including crustaceans. (MacFarlane et al. 2006, Vijayavel and Balasubramanian 2006, Martin-Diaz et al. 2008, Pereira et al. 2009). Organisms have a complete network of antioxidant defence systems consisting of enzymes and numerous non-enzymatic antioxidants (Rahman 2007). Antioxidant enzymes are subject to variations due to intrinsic biological cycles, physicochemical environment changes and pollutants (Sheehan and Power 1999). Carotenoids and vitamin E are widely distributed in nature and present in many plants, algae, micro-organisms and animals. Carotenoids are synthesized de novo only in higher plants and protists, so animals have to obtain them from the food, although crustaceans are also considered an important source of natural carotenoids (Sachindra et al. 2006). It has been pointed out that carotenoids are effective quenchers of ROS and can prevent lipid peroxidation in marine animals, including crustaceans (Miki et al. 1994, Liñán-Cabello et al. 2002). Vitamin E is a lipid-soluble antioxidant which protects cell membranes from peroxidative damage (Rigotti 2007).

P. gibbesi is an opportunistic feeder, feeding primarily on algae, and its distribution is on the shallow infralittoral rocky shores. These characteristics suggest that the ecological niche of *P. gibbesi* does not interfere with that of other benthic organisms such as *P. marmoratus* (Deudero et al. 2005). Moreover, previous studies have found no competitive interactions between *P. gibbesi* and *P. marmoratus* that could be a consequence of their physiological status and this finding is also supported through biochemical analysis (Deudero et al. 2005, Sciberras and Schembri 2008). We hypothesize that aerial exposure could result in increased antioxidant enzymes and vitamin E content as a response to UV radiation and atmospheric oxygen. In addition, differences in carapace carotenoids and in their colouration could also be related to their habitat preferences. The aim of the present work was to compare markers of oxidative stress in the hepatopancreas of the native *P. marmoratus* with those of the invasive *P. gibbesi* and to assess the carotenoid and vitamin E content in the crabs' carapace as indicators of their physiological behaviour, habitat use and distribution.

MATERIALS AND METHODS

Sampling area and experimental design

Specimens of *Percnon gibbesi* and *Pachygrapsus marmoratus* (n=14 for each species) were collected by apnoea diving in Portals Vells (Mallorca, Balearic Islands, western Mediterranean; 39°28'18.79"N, 2°28'19.63"E) in July 2009 at <1 m depth. Both crab species were collected in the same area and in a similar way and carapace length was recorded using a calliper (accurate to ±0.1 mm). The work was carried out in accordance with the EU Directive 2010/63/EU for experiments with animals.

Crabs were immediately dissected and tissues (hepatopancreas and carapace) were frozen in liquid nitrogen and maintained at –70ºC until processing. The hepatopancreas was homogenized with a Potter-Elvehjem glass/Teflon homogenizer in five volumes (w/v) of 100 mM Tris-HCl buffer pH 7.5. Each homogenate was briefly sonicated (2-3 s) using an ultrasonic processor and centrifuged at 9000 g at 4°C for 15 min. After centrifugation, supernatants were collected and immediately used for biochemical assays.

All assays were performed in duplicate and results were normalized to the total protein content of the samples (Biorad Protein Assay) using bovine serum albumin as standard.

Vitamin E and carotenoid pigment determination

Samples of carapace from *P. gibbesi* and *P. marmoratus* were defrosted and dried until all surface water had evaporated. Samples were freeze-dried and weighed before being homogenized. Vitamin and pigments were then extracted using n-hexane after deproteinization with ethanol containing 0.2% butylated hydroxytoluene. The extract was filtered and the residue re-extracted with fresh solvent until a colourless solution was obtained. Vitamin E (α-tocopherol) and carotenoid concentration were determined by high performance liquid chromatography (HPLC) in the n-hexane extract of carapace homogenates after drying in a N_2 current and dissolving in methanol. The mobile phase consisted of 550:370:80 acetonitrile:tetrahydro furan:H_2O. The HPLC was a Shimadzu with a diode array detector and the column was a Nova Pak, C18, 3.9×150 mm^2. Vitamin E was determined at 290 nm, cryptoxanthin, β-carotene and lycopene were determined at 460 nm; and lutein/zeaxanthin and astaxanthin were determined at 450 nm. The flow rate was fixed at 1 mL min^{-1}. Identification of peaks was based on the comparison of their retention times and diode array spectra, taken during analysis, with the corresponding data obtained by analysing standard compounds.

Antioxidant enzyme activities

All activities were determined with a Shimadzu UV-2100 spectrophotometer at 20°C with methods previously described (Sureda et al. 2011). Catalase activity was measured in the samples by monitoring the decrease in absorbance of hydrogen peroxide (H_2O_2) at 240 nm. Superoxide dismutase activity was determined with the xanthine oxidase-cytochrome C method. The cytochrome C reduction by superoxide anions generated by xanthine oxidase/hypoxanthine reaction was detected at 550 nm. Glutathione peroxidase activity was measured using hydrogen peroxide H_2O_2 and glutathione as substrates and glutathione reductase and nicotinamide adenine dinucleotide phosphate (NADPH) as enzyme and non-enzymatic indicators, respectively. Utilization of NADPH by the glutathione system was recorded at 340 nm. Glutathione reductase activity was determined by measuring the rate of conversion of glutathione disulfide to glutathione by monitoring oxidation of NADPH in the assay system at 340 nm.

Lipid peroxidation assay

Malondialdehyde (MDA), as a marker of lipid peroxidation, was analysed in hepatopancreas homogenates by a colorimetric assay kit (Calbiochem, SanDiego, CA, USA). Samples were placed in glass tubes containing nmethyl-2-phenylindole in acetonitrile:methanol (3:1). Hydrochloric acid (HCl) (12 N) was added and samples were incubated for 1 h at 45°C. The absorbance was measured at 586 nm and the MDA concentration was calculated using a standard curve of known concentration.

Statistical analysis

All statistical analyses were carried with SPSS® (Statistical Package for the Social Sciences) v. 16.0. A Shapiro-Wilk W-test was applied to assess the normal distribution of the data. The statistical significance of the data was assessed by one-way ANOVA. Results are expressed as mean±SEM and $p<0.05$ was considered statistically significant.

RESULTS

No statistical differences were reported in the mean size of crabs (2.05±0.18 cm for *Percnon gibbesi* and 2.12±0.17 cm for *Pachygrapsus marmoratus*).

Vitamin E and carotenoid concentration determined for the carapace of *P. gibbesi* and *P. marmoratus* are presented in Table 1. Vitamin E levels were significantly higher in *P. marmoratus* than in *P. gibbesi*. All carotenoid concentrations, except for astaxanthin, were significantly higher in *P. gibbessi* (lutein/zeaxanthin,

Table 1. – Vitamin E and carotenoid concentrations (mg/100 g) determined in carapaces of *Percnon gibbesi* (n=14) and *Pachygrapsus marmoratus* (n=14) captured in Portals Vells (Mallorca, Balearic Islands, western Mediterranean by apnoea divers. Significant differences were analysed with one-way ANOVA. Values are expressed as mean±SEM and $p<0.05$ was considered statistically significant.

	Percnon gibbesi	*Pachygrapsus marmoratus*	p value
Vitamin E, mg/100 g	21.8±1.3	28.1±1.9	p=0.009
Lutein/Zeaxantin, mg/100 g	0.99±0.10	0.37±0.04	p<0.000
Criptoxanthin, mg/100 g	5.60±0.58	3.19±0.21	p<0.000
Lycopene, mg/100 g	1.01±0.08	0.74±0.06	p=0.006
β-carotene, mg/100 g	1.25±0.14	0.59±0.12	p=0.001
Astaxanthin, mg/100 g	3.74±0.36	4.64±0.31	p=0.061

Table 2. – Antioxidant enzyme activities and malondialdehyde (MDA) concentration determined in hepatopancreas of *Percnon gibbesi* (n=14) and *Pachygrapsus marmoratus* (n=14) captured in Portals Vells (Mallorca, Balearic Islands, western Mediterranean by apnoea divers. Significant differences were analysed with one-way ANOVA. Values are expressed as mean±SEM and $p<0.05$ was considered statistically significant.

	Percnon gibbesi	*Pachygrapsus marmoratus*	p value
Catalase, K (s^{-1})/min/mg	13.8±1.7	20.6±2.6	p=0.034
Superoxide dismutase, pmol/min/mg	1.21±0.16	1.18±0.05	p=0.882
Glutathione peroxidase, nmol/min/mg	3.02±0.25	3.97±0.39	p=0.041
Glutathione reductase, nmol/min/mg	3.70±0.31	10.1±2.9	p=0.030
MDA, nmol/mg	2.37±0.13	1.72±0.12	p=0.001

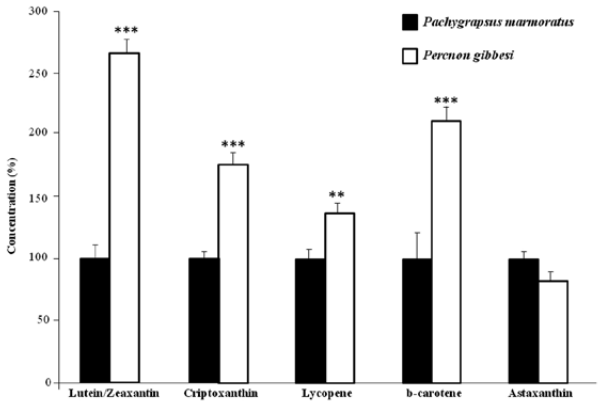

Fig. 1. – Carotenoid concentrations (% in comparison with *Pachygrapsus marmoratus*) determined in carapaces of the native and the invasive *Percnon gibbesi* in Portals Vells (Mallorca, Balearic Islands, western Mediterranean in July 2009 at <1 m depth. ** p<0.01 and *** p<0.001 (one-way ANOVA).

167% higher; cryptoxanthin, 76% higher; lycopene, 36% higher; and b-carotene, 112% higher) (Fig. 1).

Antioxidant enzyme activities and MDA concentration are shown in Table 2. The activities of catalase, glutathione peroxidase and glutathione reductase were significantly greater in *P. marmoratus* than in *P. gibbesi* (49, 31 and 173%, respectively). No significant differences were found for superoxide dismutase activity. MDA concentration was higher in the hepatopancreas of *P. gibbesi* than in that of *P. marmoratus* (38% higher).

DISCUSSION

The grapsid crab *Percnon gibbesi* (H. Milne Edwards, 1853) is one of the most invasive decapod species to enter the Mediterranean (Streftaris et al. 2005). It can form successful populations in a short time everywhere (Deudero et al. 2005, Azzurro et al. 2011, Katsanevakis et al. 2011). The high capability of dispersion of *P. gibbesi* in the Mediterranean Sea suggests that this species could be a potential competitor for the native crab *Pachygrapsus marmoratus*. However, previous studies have reported that *P. gibbesi* does not overlap with *P. marmoratus* (Deudero et al. 2005). In fact, *P. marmoratus* mainly inhabits supralittoral crevices (Augusto and Flores 2001, Deudero et al. 2005), whereas *P. gibbesi* shows no tendency towards a semi-terrestrial lifestyle, with limited bathymetric distribution restricted to the infralittoral zone from 0 to 4 m depth. Laboratory experiments indicated that when competing for space, *P. marmoratus* dominates the interactions with *P. gibbesi* (Sciberras and Schembri 2008). The present results provide new biochemical evidence suggesting that *P. gibbesi* and *P. marmoratus* do not compete for space and there is no spatial overlap, so *P. marmoratus* is not excluded from its natural habitat by the alien species.

In the present work, different carotenoid contents and vitamin E levels were found in the two crab species. This could be directly related to their patterns of habitat use and different lifestyle, since most crustaceans acquire carotenoids from their diet and their coloration can change with the diet (Méndez Casariego et al. 2011). The higher carotenoid content in the invasive *P. gibbesi* can be related to the yellowish and reddish colouration that this crab exhibits helping this species to mimic in their habitat. This aposematic mimetic adaptation could be a consequence of the presence of red algal species such as *Peyssonellia* sp. or *Corallina mediterranea* inhabiting the sciaphile rocky bottoms. The crab feeds on these algae, so their carotenoids be incorporated into the carapace, resulting in a similar chromatic pattern. By contrast, *P. marmoratus* showed lower values of carotenoids with brownish colouration, because this crab lives in the supralittoral zone, where this brown colour is dominant. *P. marmoratus* exhibits colouration that resembles the colour and pattern of the surrounding environment in order to avoid detection by predators. In fact, antipredator adaptation is considered the most important factor influencing evolution of colouration (Hinton 1976). That is why *P. marmoratus* exhibits a colouration similar to the brown algae in which this crab lives, as a strategy to avoid predators. By contrast, the invader *P. gibbesi* lives completely submerged amongst boulders or in sciaphilic rocky bottoms covered by encrusting red coralline algae and, consequently, its carapaces have a similar chromatic pattern to the sea bottom. Nevertheless, in this study, no statistical differences were found in the astaxanthin pigment, which is used in aquaculture and in fish farming for the pigmentation of animals (Carter et al. 1994). This indicated that astaxanthin did not contribute to the differences between the two crabs studied. Therefore, in the case of *P. gibessi*, increased carotenoid content in comparison with *P. marmoratus* might be linked to the foraging activity together with the habitat preferences towards crevices and rocky shores.

Vitamin E is the most important naturally occurring lipophilic antioxidant agent protecting cell membranes from free radicals generated from lipid peroxidation (Rigotti 2007). The concentration of vitamin E was higher in *P. marmoratus* than in *P. gibbesi* and this result may be related to the need for more protection against oxidation. The native *P. marmoratus* lives in shallow areas, with higher exposure to atmospheric oxygen and UV radiation, while *P. gibbesi* lives completely submerged. In fact, exposure to UV radiation and ROS can act synergistically to cause extensive DNA damage and lead to apoptosis (Lesser et al. 2001). Inadequate protection against UV radiation and ROS may result in damage to DNA, proteins and membrane lipids that will compromise the physiology and biochemistry of the organisms (Lesser et al. 2003). Consequently, the content of vitamin E in *P. marmoratus* should be higher to protect against oxidation and UV radiation as an adaptation to the supralittoral environment.

Stress has been defined as a condition in which the homeostasis of an organism is threatened or disturbed as a result of intrinsic and/or extrinsic stressors (Chrousos and Gold 1992). Stress leads to a cascade of molecular and physiological reactions that can be easily detected (Livingstone 1993), and environmental stress is a possible cause of an excess of free radical

production. Therefore, some antioxidant enzymes such as catalase, glutathione peroxidase, and glutathione reductase have been proposed as biological indicators of stress in aquatic organisms (Box et al. 2009, An and Choi 2010, Vidal-Linan et al. 2010, Sureda et al. 2011). In this study, the use of a battery of biomarkers has been useful for indicating different responses in the two crabs. *P. marmoratus* showed an elevated antioxidant system in comparison with *P. gibbesi*, which may be related to the habitat where *P. gibbesi* lives, on the shallow rocky bottoms (Galil et al. 2002) with maximum abundances at 1 m depth (Deudero et al. 2005), whereas *P. marmoratus* lives in supralittoral and mediolittoral crevices (Augusto and Flores 2001). This crab exhibits semi-terrestrial habits and it is actively searching for food in terrestrial zones (Cannicci et al. 1999). Consequently, the environmental changes to which *P. marmoratus* is subjected are wider and the antioxidant system of this crab is therefore working at a higher level to avoid cellular damage, evidenced by the MDA results as an indicator of lipid injury.

The cellular antioxidant defence system that keeps ROS levels lower is one of the important biochemical strategies that gives protection to cells against the deleterious effects of endogenous ROS. ROS are neutralized by an elaborated antioxidant defence system consisting of several enzymes and numerous non-enzymatic antioxidants such as vitamin E. However, the invasive crab *P. gibbesi* showed a less active antioxidant system, although MDA was higher, than *P. marmoratus*. These results could be related to the adaptation of the alien species to the new habitat and environmental conditions, since its antioxidant system was not completely responding to the stress caused by a new habitat, and finally increasing the oxidative damage in cellular components, mainly in lipids. The change to a new environment may occur with altered oxidative stress and antioxidant defence of the crab *P. gibbesi* and this higher lipid peroxidation has been considered as an index of the induction of oxidative stress in marine invertebrates (Monserrat et al. 2007).

CONCLUSIONS

In conclusion, this is the first study quantifying the antioxidant defences, vitamin E and carotenoid content in two coexisting species, the native *P. marmoratus* and the invasive *P. gibbesi,* in Balearic coastal waters. Differences in carotenoid content could be related to the algal coverage of the habitat of both species as a means to mimic the surrounding environment. The higher antioxidant defence levels in *P. marmoratus* are probably an adaptation to the capacity of this species to live outside the water in direct contact with UV radiation and atmospheric oxygen. In addition, the low antioxidant enzyme activities together with an increased MDA may indicate elevated stress in *P. gibbesi*, which may be induced by several factors, including the new environment or possible interactions with other species—perhaps not *P. marmoratus* but other crabs that occur in the same habitat, such as *Eriphia verrucosa*.

ACKNOWLEDGEMENTS

This work was funded by the Spanish Ministry of Health and Consumer Affairs CIBEROBN CB12/03/30038.

Conflict of interest

The authors state that they do not have any conflicts of interest.

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