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Research Article

Parasites affect hemocyte functionality in the hemolymph of the invasive Atlantic blue crab *Callinectes sapidus* from a coastal habitat of the Salento Peninsula (SE Italy)

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Abstract

The blue crab *Callinectes sapidus* is an invasive alien species in the Mediterranean Sea. In native habitats, it suffers mortalities determined by different pathogens, including viruses, dinoflagellates, and bacteria. In invaded habitats, scant information is available on the occurrence of parasites in blue crabs, and no comparisons have been made with native brachyuran species. Here, a preliminary screening of the hemolymph of *C. sapidus* hemolymph as well as of three indigenous crabs (*Eriphia verrucosa*, *Carcinus aestuarii* and *Pachygrapsus marmoratus*), captured in the Acquatina Lagoon (Salento Peninsula, SE Italy), evidenced the presence of unidentified protozoans in more than 70% of the analyzed specimens, with a low number of suspected infections due to dinoflagellates of the genus *Hematodinium*. In *C. sapidus,* the occurrence of parasites was accompanied by a decrease of hemocyte functionality, interfering with the role of the cells in innate immunity (i.e. phagocytosis or DOPA production). More studies are necessary in order to verify the impact of this alien brachyuran on invaded ecosystems as mediated by the spread of its parasites and other pathogens.

Keywords: *Callinectes sapidus*, parasites, invasive alien species, crab hemocytes, phagocytosis.

Introduction

The Atlantic blue crab *Callinectes sapidus* Rathbun, 1896 (Decapoda, Brachyura, Portunidae) is native to the north-western Atlantic Ocean coasts; the first record from European waters was in 1901 along the Atlantic coasts of France (Nehring, 2011*)*; subsequently, it extended its presence throughout the Mediterranean Sea (Mancinelli *et al.,* 2017a). In particular, the blue crab has been recorded in the Adriatic (Dulčić *et al.,* 2011; Mancinelli *et al.,* 2016; Cilenti *et al.,* 2015) and Ionian Seas (Mancinelli *et al.,* 2013a, 2017b; Carrozzo *et al.,* 2014), where a number of established, fully reproductive populations have been identified. Currently, *C. sapidus* is considered an invasive alien species (IAS) in the Mediterranean Sea (Katsanevakis *et al.,* 2014).

The introduction of alien species is at present of serious concern worldwide, as it has been acknowledged to threaten the biodiversity and functioning of invaded ecosystems (Katsanevakis *et al.,* 2014). In addition, growing attention has been recently given to the possibility that species may be introduced together with their parasites and pathogens, releasing them into the new environment, infecting native species, altering pre-existing infectious disease dynamics ("pathogen pollution": Daszak *et al.,* 2000), and ultimately increasing their ecological impact (Goedknegt *et al.,* 2016). Many studies have documented how pathogens introduced with alien species can affect native communities, especially when introduced hosts act as reservoirs from which infection can "spill over" to native species (Prenter *et al.,* 2004; Colla *et al.,* 2006; Lawson Handley *et al.,* 2011 and literature cited). Furthermore, the presence of parasites as well as other infectious pathogens are considered important ecological factors affecting individuals, populations, and ecosystems, involved in global-scale declines of a wide range of marine and terrestrial species (Harvell *et al.,* 2002; Granek *et al.,* 2005; Panek, 2005; Lips *et al.,* 2006).

In native habitats, *C. sapidus* is subjected to a wide spectrum of parasitic pathogens, including viral, bacterial, and micro algal agents (Flowers *et al.,* 2016; Shields, 2015 and literature cited). In invaded Mediterranean habitats, the occurrence of parasites in the species is to date virtually unexplored, even though it is an appreciat-

ed shellfish product, in particular in Greece and Turkey (Mancinelli *et al*. 2017c). The only exception is represented by an unconfirmed claim of infection in blue crabs from the Salento Peninsula (SE Italy) by syndinid dinoflagellates of the genus *Hematodinium* (Mancinelli *et al.,* 2013b). Species belonging to the genus (e.g. *H. perezi*) are known to determine high mortality in native habitats (Messick, 1994; Messick & Shields, 2000; Shields *et al.,* 2015). Besides *C. sapidus*, they have been reported in several crustacean species in North American as well as in European waters, including *Cancer pagurus* Linnaeus, 1758 (Stentiford *et al.,* 2003), *Chionoecetes opilio* (O. Fabricius, 1788) (Shields *et al.,* 2005), and *Nephrops norvegicus* (Linnaeus, 1758) (Stentiford & Neil, 2011; Beevers *et al.,* 2012). Indeed, *Hematodinium* dinoflagellates are currently recognized as significant pathogens for commercially important crustaceans worldwide (Stentiford, 2012). These parasites form "silent" blooms in the water column with the whole crustacean assemblages that are under the threat of disease. Although the possible influence of environmental factors related to e.g. climate change and ocean acidification on both host and protista pathogen need yet to be clarified (Stentiford, 2012; Adlard *et al.,* 2014), areas newly colonized by alien host species can offer to pathogens favorable conditions for their survival and spread. Nevertheless, the presence of parasites in crab species introduced in the Mediterranean Sea as well as the susceptibility of native brachyurans to parasitic infections has been scarcely investigated so far.

In this study, we analyzed *Callinectes sapidus* specimens from a lagoon located in the Salento Peninsula (SE Italy). Their hemolymph was screened to detect the presence of parasites, including *Hematodinium* spp. A comparison was carried out with the native crab species *Eriphia verrucosa* (Forskål, 1775), *Carcinus aestuarii* (Nardo, 1847), and *Pachigrapsus marmoratus* (Fabricius, 1787), which were sampled contemporaneously. Furthermore, an effort was made to perform a previously unattempted morph-functional characterization of hemocytes of blue crabs from non-native habitats, considering the possible effects of parasite presence on the cells functionality.

Accordingly, here we performed a preliminary morphological investigation on hemocytes under light microscope and an analysis of two important properties of these cells, i.e. phagocytosis and DOPA production.

Materials and Methods

Animals

Crabs were captured with modified commercial crab traps (60×60×60 cm) of the type described in Carrozzo *et al.* (2014) in the Acquatina Lagoon (40°27'22" N, 18°12'24" E, Adriatic Sea, SE Italy; Fig. 1). Two traps were deployed in May 2016 at a depth of 70 - 100 cm, baited with crushed mussels. Trap deployment was carried out between 6:00 and 7:00 p.m. and retrieved the day after between 9:00 and 10:00 a.m. Sampling operations were repeated adopting an identical procedure from June to September 2016 at a monthly frequency. After retrieval, crabs were harvested from the traps and transferred in refrigerated containers to the laboratory, where they were identified to the species level, enumerated, and sexed af-

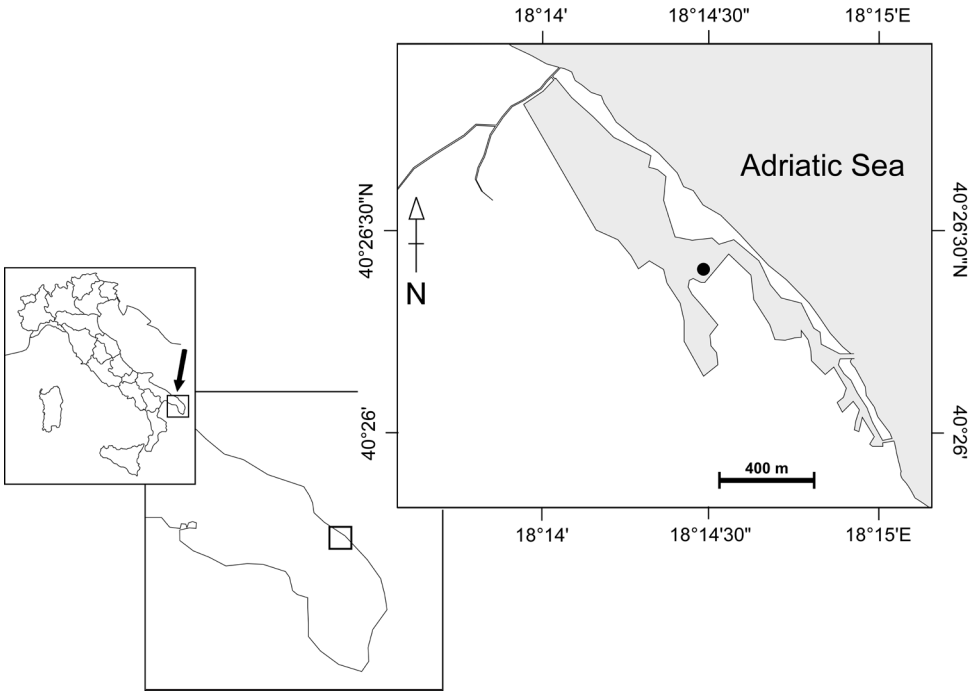

Fig. 1: The Acquatina lagoon. The sampling station used during the study period is indicated by a full circle.

ter examination of the shape of the abdomen apron. For each specimen the total wet weight (WW) was determined with an electronic balance to the nearest 0.01 g. Specimens characterized by a WW < 60g [corresponding to an approximate carapace width of 94 mm according to Ju *et al.* (2001)] were classified as juveniles.

Hemolymph sampling and analysis for parasites

The hemolymph of introduced and indigenous crab species was analyzed to verify the presence of parasites. From each individual, a hemolymph sample was collected from the arthrodial membrane covering the articulated base of the 5th walking leg with a plastic syringe containing an anti-coagulant (0.3 MNaCl, 0.1M glucose, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA, pH 5.4) to avoid hemocyte agglutination.

Parasites were detected by examining individual hemolymph smears. One drop of hemolymph was mixed (1:1) with one drop 0.25% Neutral Red (in filtered seawater, FSW), placed on a hemocytometer and observed under a microscope (Nikon Eclipse 50i). The presence of atypical cells was evaluated in both sides of the hemocytometer (i.e. 4.5 ml of undiluted hemolymph were analyzed). Parasite cells take up the neutral red dye while host hemocytes do not, therefore vacuoles in parasites result bright red, while vacuoles in hemocytes are only rarely stained. When red stained cells were observed, we considered the individual as parasitized.

Culture chambers and basic morphology of hemocytes

Pools of hemocytes obtained from at least 4 specimens were used in each experiment. The hemolymph, collected in Eppendorf cuvettes, was centrifuged at 780*g* for 10 min. The supernatant was discarded while pellets (hemocytes) were suspended in FSW to a final concentration of 10^6 cells mL^{-1}. Sixty µL of hemocyte suspension were placed in the center of a coverslip coated with poly-L-lysine. To allow hemocyte adhesion, the cells were placed in a moist chamber for 60 min, at room temperature. For hemocyte examination by light microscopy (LM), slides were rinsed in phosphate-buffered saline (PBS: NaCl 8 g L^{-1}, KCl 0.20 gL^{-1}, KH_2PO_4 0.20 gL^{-1}, Na_2HPO_4 1.14 gL^{-1}, pH 7.3), fixed for 10 min with neutral-buffered formalin (NBF) fixative followed by routine staining with 10% Giemsa. Coverslips were subsequently mounted on microscope slides with 80 % glycerol. Two hundred hemocytes in each of 10 optic fields (1250X magnification) were observed and counted.

Phenoloxidase (PO) activity in hemocytes

A cytoenzymatic assay was performed to evaluate the phenoloxidase activity on blue crab cells. Hemocytes were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 min and then incubated for 60 min in a saturated solution of L-DOPA. Cells were rinsed, examined by light microscope and counted (at least 150 cells in each of 10 optic fields at 1250X magnification). Positive cells converted L-DOPA to dopachrome and appeared dark brown. Samples from 5 parasitized and 5 non-parasitized specimens were analyzed.

Phagocytosis

After adhesion, blue crab hemocytes were incubated at RT, in a moist chamber, with 50 µL of a yeast (*Saccharomyces cerevisiae*) suspension in FSW (yeast/hemocyte ratio = 10:1) for 60 min; the yeast was consequently removed by repeatedly dipping each coverslip in excess FSW. Cell monolayers were fixed and stained as described above (Giemsa) before their observation. Hemocytes from 5 parasitized and 5 non-parasitized specimens were analyzed by counting 150 cells in each of 10 optic fields (1250X magnification).

Statistical analysis

All data are reported as means ± SD. All the assays were repeated at least three times. Differences were considered statistically significant at the level of $P<0.05$. Preliminary X-square tests were performed for each crab species to test for sex-related differences in the proportion of parasitized specimens; since no significant differences were generally detected (P always > 0.05), data for both sexes were cumulated in further analyses. Comparisons of L-DOPA oxidizing abilities were performed using Student's t-tests.

Results

Eighty-two specimens of *Callinectes sapidus* were captured in the Acquatina lagoon. The average wet weight of blue crabs was 214.7 g (± 82.5 SD); weights varied considerably, ranging from 44.5 to 388.4 g. Specifically, two juveniles weighing < 60 g were captured; the remaining specimens were adults, with a prevalence of males (overall sex ratio of 3:1). Two ovigerous females were also sampled. In addition, a total of 24 adult brachyurans belonging to three native species were analyzed, i.e. the eriphiid *Eriphia verrucosa* Forskål, 1775 (n = 10, 143.3 ± 103 g average wet weight, 5:1 sex ratio males:females), the grapsid *Pachygrapsus marmoratus* Fabricius, 1787 (n = 8, 16.9± 4.1 g, all males), and the portunid *Carcinus aestuarii* Nardo, 1847 (n = 6, 24.9 ± 11.3 g, all males).

Parasites presence in hemolymph smears of crabs

Given the low number of juvenile blue crabs collected (2), they were not further analyzed. The examination of hemolymph smears from the remaining adult specimens (80) revealed that most of them (i.e., 71%) were parasitized. Regarding the native crab species, we found 70% parasitized specimens in *E. verrucosa* and 50% in *C. aestuarii*, while in *P. marmoratus* only 25% of the inspected individ-

uals were parasitized (Fig. 2). The smears were generally dominated by the host hemocytes (Fig. 3A), but in few cases (5%) in both *C. sapidus* (Fig. 3B) and *E. verrucosa* parasite cells were more than 80% of the total hemolymph cells.

After the Neutral red assay, based on microscopical observations of parasite morphology, we suspected the occurrence of *Hematodinium* spp. and of other parasites (Fig. 3C). We found that in 6% and in 3% of *C. sapidus* and *E. verrucosa* individuals, respectively, *Hematodinium*-like cells occurred (Figs. 3D and 3E respectively); however, no external (e.g., lethargy, weakness) or internal (e.g., milky hemolymph) signs of infection were observed. Furthermore, in these individuals the number of suspected *Hematodinium* cells was very low (2.88 ± 1.53% of total cells).

Cytochemical properties of C. sapidus hemocytes and their response to parasite presence

After Giemsa staining of *C. sapidus* hemolymph, the presence of cells with or without granules was highlighted. The granular and semi-granular cells were 9-20 μm in size while the hyalinocytes had a size varying between 12-22 μm. After the cell adhesion on the glass slides, the frequency of each cell type became easily detectable (Fig. 4): hyalinocytes were the most abundant cells (46.9 ± 2.4% of total cells), while granular and semi-granular cells were equally present (26.5 ± 8.4% and 26.6 ± 8.7%, respectively).

The lysosomes were not clearly visible after the Neutral red staining because of the low permeability of the

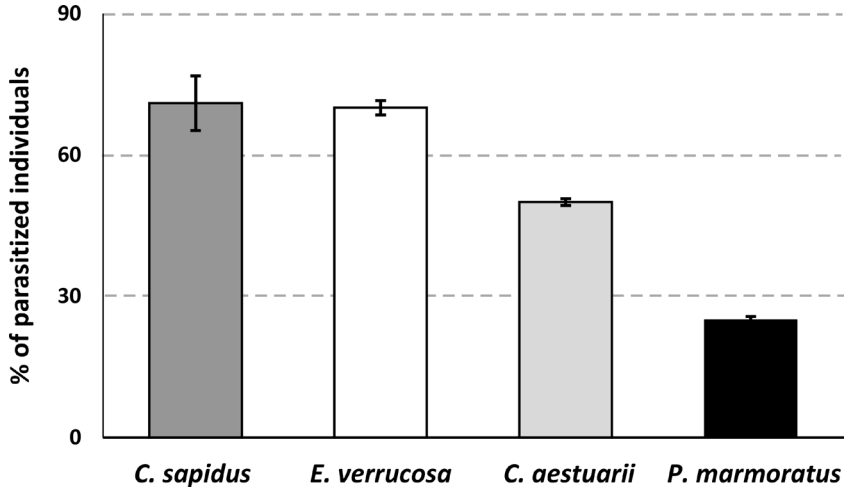

Fig. 2: Percentage of parasitized crabs. Data represent mean ± SD.

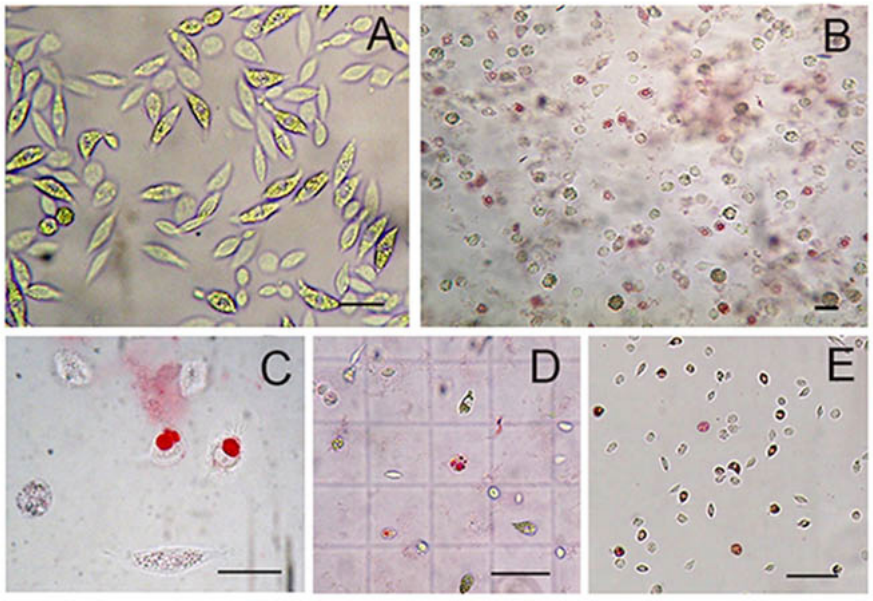

Fig. 3: Light micrographs of hemolymph smears from *C. sapidus* (A, B, D) and *E. verrucosa* (C, E). Scale bars are 20 μm (A, B, C) and 50 μm (D, E).

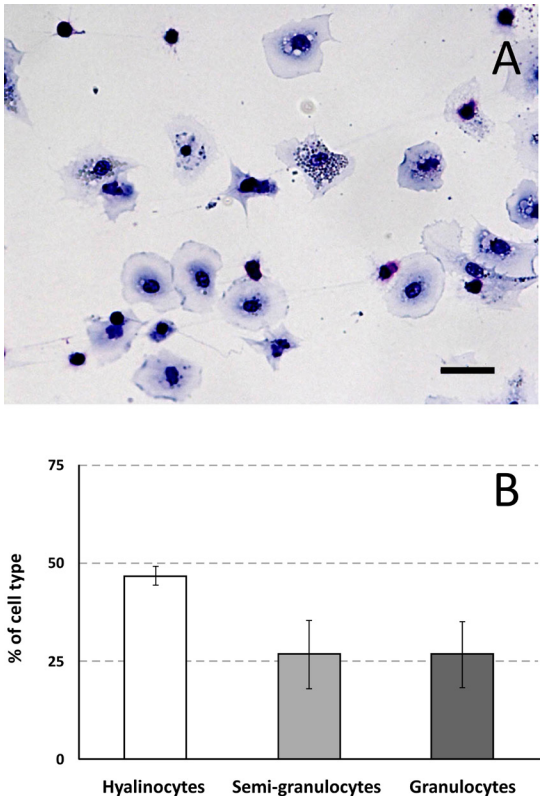

Fig. 4: Light micrographs of *C. sapidus* hemocytes stained with Giemsa; A) hemocytes after adhesion on glass slide; scale bar is 20 µm; B) frequency of cell types; data represent the mean ± SD.

membrane to this dye. Lipids were present as individual droplets in different cell types, but they were more frequent in hyalinocytes (87.5 ± 2.1%) than in granulocytes (13 ± 3.5).

Differences in cell functionality were investigated by comparing non-parasitized and parasitized blue crabs. Specifically, the hemocyte cytoplasm was able to oxidize polyphenol substrates such as L-DOPA (Fig.5A). As shown in Fig. 5C, a decrease in the number of positive cell was observed in parasitized individuals: 2.2 ± 0.5 % of dopachrome positive cells if the presence of parasite was detectable, while in non-parasitized animals 8.7 ± 0.6% of cells were dopachrome (t-test, $P<0.05$). In culture chambers, both granulocytes and hyalinocytes actively phagocytose yeast cells with an increased phagocytosing activity observed for hyalinocytes (Fig. 5B). The number of phagocytosing hemocytes peaked after 2h of incubation in the presence of yeast at the optimal temperature range of 20–23°C. The frequency of phagocytosing cells was 21 ± 2.3% in non-parasitized animals. The phagocytosis rate (Fig. 5C) resulted lower in parasitized crabs in which 7.9 ± 0.96% of phagocytosing cells were found.

Discussion

The results of the present investigation indicated a high frequency of parasitized individuals in both *C. sapidus* and in the native *Eriphia verrucosa*, while for other indigenous crabs (i.e., *C. aestuarii* and *P. marmoratus*)

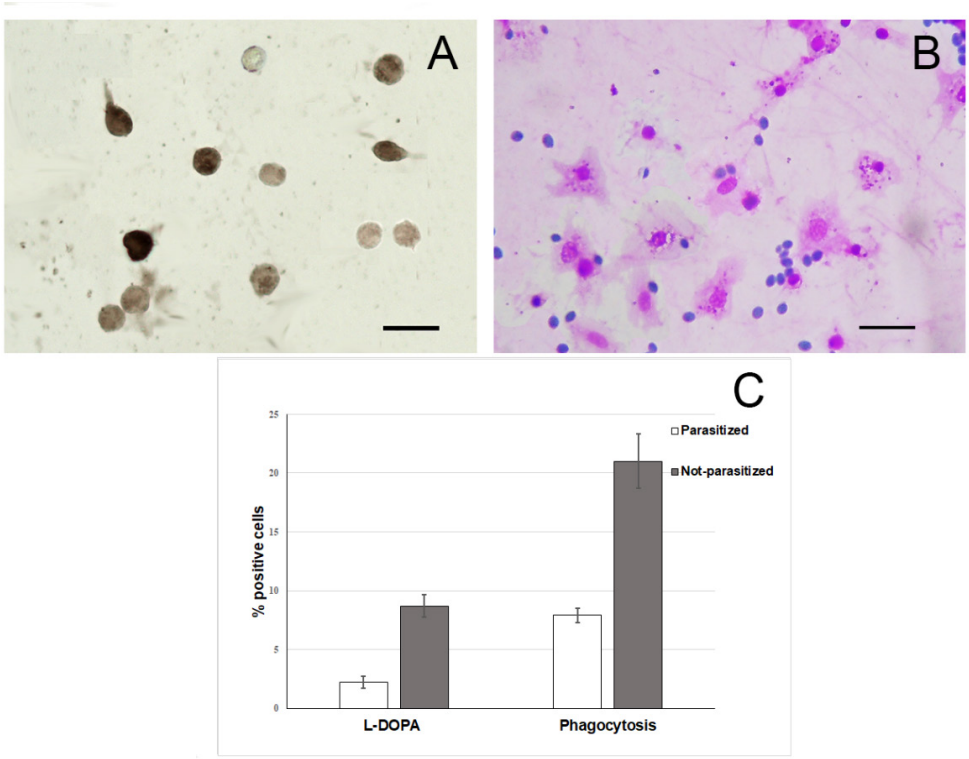

Fig. 5: Light micrographs of hemocytes from *C. sapidus* hemolymph; A) hemocytes treated with L-DOPA: stain for dopachrome production; B) phagocytes with ingested yeast cells. Scale bars are 20 µm; C) Percentage of dopachrome positive and phagocytosing cells in parasitized and unparasitized individual of blue crabs; data represent the mean ± SD.

the incidence of parasites was lower. In addition, an in-depth analysis of hemocytes functionality focused on *C. sapidus* highlighted that the occurrence of parasites was accompanied by a reduction in both the number of cells containing L-DOPA protein and in phagocytic activity.

This study analyzed three native crab species only in summer and, for some of them (i.e., *P. marmoratus* and *C. aestuarii*) a small number of specimens was inspected. While considerable efforts have been made to investigate the occurrence of endoparasites and other pathogens in Mediterranean fish species (e.g. Carreras-Aubets, 2011; Pérez-i-García *et al.,* 2017), the present investigation represents one of the first to focus on a crustacean multi-specific assemblage, and on brachyuran species. In addition, comparative information on introduced and native crab species is scant, and mostly focused on ectoparasites (Innocenti & Galil, 2007; Goedknegt *et al.,* 2017).

The hemolymph analysis evidenced the presence of parasitic protozoan cells in a high number of *C. sapidus* (71% of analyzed individuals) and *E. verrucosa* (70%) individuals. Noticeably, none of the crabs appeared lethargic, or showed clear signs of an on-going disease in terms of behavioral or exoskeleton anomalies. Conversely, both *C. aestuarii* and *P. marmoratus* showed a considerably lower number of parasitic cells in the hemolymph. Studies on *P. marmoratus* parasites are virtually non-existent with the exception of Kuris et al. (2004), focused on microphallid trematodes, where 33% of the individuals resulted infected. For *C. aestuarii* and the congeneric *C. maenas*, low infection frequencies are generally reported e.g., 7.1% for protozoan parasites (Stentiford, 2004), and 7.6% for haplosporidians (Stentiford, 2013), supporting the hypothesis that the species belonging to the genus may possess physiological properties that make them more tolerant than other decapod crustaceans to parasite infections (Leignel *et al.,* 2014 and literature cited). However, in the Volturno River estuary the species was reported as 100% infected by a shell disease syndrome (Mancuso *et al.,* 2013). Our data on *C. aestuarii* should be interpreted in with caution measure given the low number of analyzed specimens, and further investigations including a larger number of individuals are required. For *E. verrucosa*, data on parasite occurrence are scant (Vivares, 1971); conversely, for *C. sapidus* a considerable body of information is available (see references cited in the introduction), yet no data until now have been published on Mediterranean populations (but see Mancinelli *et al.,* 2013b for an exception). In addition, here an advanced screening of hemocytes morphology and functionality was performed. Hemocytes play a central role in the immune defenses of crustaceans (Söderhäll & Cerenius, 1998; Zhang *et al.,* 2006). Little information currently exists about the diversity, classification, morphology and function of *C. sapidus* hemocytes. To our knowledge, no study concerning morphological characterization of circulating hemocytes from the crab *C. sapidus* has previously been performed.

Indeed, the analyses confirmed the presence of the same cell types observed in other crabs; noticeably, they highlighted a relationship between the occurrence of parasites and the reduction in both the number of cells positive to L-DOPA and the phagocytic activity.

Identifying the cell types is a routinary and necessary step to study their respective functions; here we used cytochemical and functional methods to identify the blue crab hemocytes. The results indicated that the classification schemes of circulating cells reflects those of crustaceans described by Lin & Söderhäll (2011) and include granular, semi-granular, and hyaline cells. The latter, besides representing a lipid storage, resulted involved in phagocytosis, more than granular cells, generally implicated in encapsulation, early non-self recognition, melanization and coagulation (Lin & Söderhäll, 2011). The cytoenzymatic assay here performed to evaluate the PO activity through its catalytic conversion of L-DOPA (3,4-dihydroxy-L-phenylalanine, colorless) to dopachrome (red-brown color) confirmed, as in other crustaceans, the PO enzyme localization inside some hemocytes. Phenoloxidase (PO) is an important element of the marine invertebrates' innate immunity, being a primary component of the prophenoloxidase activating system (Söderhäll *et al.,* 1994; Söderhäll & Cerenius, 1998; Fan & Wang, 2002), able to convert polyphenols to quinones, which, in turn, polymerize to melanin (a toxic product).

The PO enzyme activity in hemocytes is often associated with physiological conditions of the crustaceans (Le Moullac & Haffner, 2000). Fluctuations of its activity can be explained in terms of stimulation by bacteria and bacterial products of processes involved in the formation of pro-PO (Hauton *et al.,* 1997). In this work, cells positive to PO activity in parasitized individuals resulted lower than in non-parasitized ones. Generally the PO activity increases after the crabs' treatment with β-1,3-glucan, LPS or inactivated *V. harvey* and *V. anguillarum* (Sritunyalucksana & Söderhäll, 2000). While, in the case of animals carrying reo-like virus infection (Chung *et al.,* 2015), slightly higher levels of PO gene transcripts have been evidenced in comparison to those that are uninfected. However, considering that also in the Chinese white shrimp *Fenneropenaeus chinensis* the levels of PO expression are down regulated (Li *et al.,* 2014) and that upon acute WSSV infection, these shrimps up regulate immune responsive genes, we can suppose a different response of hemocytes to the different extraneous organisms present in the hemolymph. Nevertheless, the reduction of PO positive cells here observed in parasitized crabs suggests considering the possible role of parasites in reducing crabs' defense ability.

Finally, here we evidenced, as in *Penaeus japonicus* (Bachère *et al.,* 1995) and in *Macrobrachium rosenbergii* (Itami *et al.,* 1998; Sung *et al.,* 2000) the ability of granular cells to phagocyte. The efficiency of cells to phagocytose foreign particles (i.e. yeast) in parasitized individuals was lower than in non-parasitized ones. Cells lacking granules were more active in carrying out this activity.

Conclusions

The results of the present study indicate that blue crabs in invaded Mediterranean habitats are highly parasitized, while in native brachyurans parasites occurrence varies considerably among the analyzed species. Furthermore, our results show that the presence of parasites affects the functionality of *C. sapidus* phagocytes, but no sign of disease has been evidenced. It is likely that the new environmental conditions help the blue crab to control parasites' survival and reproduction and to contrast the reduction of immune functions.

More advanced studies are needed to further verify this hypothesis, as well as other potential mechanisms of control on the spread of parasites. Furthermore, molecular investigations are necessary for a univocal identification and characterization of protozoa and other pathogens – including dinoflagellates of the genus *Hematodinium* - hosted by the crab hemolymph.

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