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Substrate preference and settlement behaviour of the megalopa of the invasive crab *Percnon gibbesi* (Decapoda, Percnidae) in the Mediterranean Sea

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Abstract
The transition from a planktonic to a benthic life is a critical phase in which sub-adults are particularly exposed to the risk of predation and dispersion into unsuitable habitats, and plays a crucial role in the distribution, structure and dynamics of marine populations. Settlement involves the selection of an adequate substrate that provides shelter and food during early life stages. *Percnon gibbesi* is an alien brachyuran crab that has invaded the Mediterranean, where it is preferentially associated to boulders covered with shallow algal turf. The mechanisms of substrate selection leading to the settlement of megalopae are still unknown in *P. gibbesi*, yet their knowledge may shed light on its high invasiveness. We examined the substrate preference and settlement behaviour of 36 megalopae of *P. gibbesi* using three natural substrates in an experimental mesocosm: gravel, cobbles and flat stones. Video recordings of 30-min trials were used to assess the substrate preference, measure the time to selection and observe the behaviour of the megalopae. Strong preference was given to hard and stable substrates i.e., cobbles and flat stones with interstices where to hide, which are also the most suitable as they provide shelter and food. Direct selection was the dominant behaviour followed by exploration and lastly by hesitation. The megalopae selected quickly the most suitable substrate to settle, likely enhancing their chances of survival. Our findings suggest that rapid settlement on a suitable substrate contributes to the success of the biological invasion of *P. gibbesi* along the Mediterranean coasts.

Keywords: Microhabitat, Larval stage, Brachyuran crabs, Light traps, Alien species

Background

Settlement and recruitment of marine organisms are complex processes affected by many biotic (larval morphology, pelagic phase duration, behaviour, etc.) and abiotic (oceanographic features, habitat structure, etc.) factors that occur at different temporal and spatial scales [1]. Many marine benthic organisms have a planktonic larval phase as part of their reproductive and dispersal strategy [2] that may disperse over large areas before settling and recruiting in a suitable habitat [3]. The transition from planktonic to benthic life (i.e., the settlement) is a critical step in the life cycle of benthic invertebrates that have a planktonic larva [4]. Two phases may be distinguished in this process: (1) a behavioural phase of search for a suitable substrate that provides shelter and food, and (2) a phase of permanent residence or attachment to the substrate, which triggers the metamorphosis [1]. The ability of recognizing a suitable substrate is therefore essential to enhance the survival chances of settlers and to guarantee the success of recruitment, and it ultimately determines distribution, structure and dynamics of animal populations.

Like most benthic marine organisms, brachyuran crabs have a pelagic larval phase followed by a benthic phase

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[5]. The first larval form—a zoea with several stages—is followed by a second form—a megalopa—that is able to actively swim and reach the habitat where settlement occurs. The morphology of the zoea stage is characterized by the presence of long carapace spines that have been shown to effectively deter mouth gape limited predators [6] and to reduce sinking rate [7]. Locomotion at this stage is usually limited to vertical movements and the active swimming capacity is reached once in the megalopa stage. A megalopa instead, shows most of the first crab characteristics: locomotive, feeding and sensory apparatus are developed and active swimming behaviour drives the animal to the benthic life.

Several different cues may generate settlement responses in the competent larvae of decapod crustaceans such as the presence of adult conspecifics [8, 9], light characteristics of crevices [10], structural characteristics of habitats [11], habitat-related chemical cues [4] or presence of an appropriate host in symbiotic species [12]. The absence of such cues when the larva is competent to settle and ready to metamorphose may cause a developmental delay of several hours–months [13] that may conclude with spontaneous metamorphosis or death, depending on the species [14]. Even if little is known about the substrate features that influence settlement, several studies carried out in the laboratory [15–18] as well as in the field [19, 20] have helped to describe the substrate preference in this delicate life phase.

Among the non-indigenous marine species occurring in the Mediterranean Sea, the crab *Percnon gibbesi* (H. Milne Edwards, 1853) is one of the most widespread and successful invaders [21–23]. Its native range is extended over the central eastern Pacific and central western and eastern Atlantic, including the Macaronesian archipelagos and Ascension Island [24]. It was first recorded in the Mediterranean in summer 1999 and in a dozen of years it has progressively colonized most coastal areas, starting from the central and western sectors [21, 22]. Its preferential habitat in the Mediterranean is made of boulders at 1–4 m depth covered with a shallow algal turf that offer plenty of safe shelters in the interstices [25]. The megalopa, like in other grapsoid crabs, is large-sized, well capable of swimming thanks to the presence of abundant natatory setae and with conspicuous teeth on the dactyli of the pereiopods [26].

While some aspects of the biology and ecology of adult *P. gibbesi* have been investigated [25–30], little is known on its larval stages. A major reason could be the low efficiency of traditional sampling gear (e.g., plankton nets) in collecting a sufficient number of living and healthy larvae. In fact, only descriptive studies on preserved larval material are available [31–33]. Information on the active substrate preference at settlement is unavailable for *P. gibbesi* in the Mediterranean as well as in its original distribution range, yet it may shed light on its successful invasion. A chance to study this topic and to observe the crab behaviour at the time of settlement came with the fortuitous collection of *P. gibbesi* megalopae with light traps during a research survey on fish postlarvae [34].

In our study we set up an experimental mesocosm to investigate substrate preference and behaviour of *P. gibbesi* megalopae during the settlement phase. First, we tested the null hypothesis that megalopae have no preference among different substrates. Our prediction is that preference would be given to substrates that offer a suitable shelter. Second, based on the assumption that the faster the arrival to a suitable shelter, the higher the probability to escape predation and reduce pre-settlement mortality, we predict that the number of megalopae that make a direct and fast substrate selection would be higher than those that explore or hesitate before selection.

Methods

Sample collection

Megalopae of *P. gibbesi* were collected in the Gulf of Castellammare (NW Sicily, southern Tyrrhenian Sea, approximately 38.07°N, 13.02°E) during October 2014. Sampling was carried out with light traps (CARE® Ecocean) [35] during a fish postlarvae sampling survey [34]. Each light trap consisted of a 7 W battery-powered LED lamp housed in a waterproof floating case with a 2 mm mesh conical net hanging vertically beneath the case. The traps, floating under the surface, were moored on a 25 m deep sandy-muddy bottom at ca. 1.5 km from the coast and were deployed at night during new moon phases. The light traps were retrieved before sunrise and the collected organisms carefully transferred into aerated tanks and transported to the CNR-IAMC facilities in Castellammare del Golfo. The alive megalopae (carapace length = 0.67 ± 0.04 cm) were sorted, maintained in small aerated aquaria filled with seawater collected at the sampling site and used in the experiment within 24 h from collection. The identification of megalopae was based on [31].

Substrate preference and behaviour monitoring

An experiment was performed to determine the substrate preference of the megalopae. The experiment was conducted in two independent identical 150 l PVC aerated round containers (80 cm diameter, 36 cm height) filled with seawater at 18 °C, located under natural light conditions with sand on the bottom. The sides of the containers were opaque to avoid any external interference. The bottom was virtually divided into three roughly equal sectors, each one characterized by one of the following natural substrates placed upon the sand: gravel (0.3–3 cm in diameter), two roundish cobbles (13 cm each in diameter) and two flat stones (ca. 20 × 13 × 3 cm each). The arrangement of substrates in each container was random in order to minimise any effect of the working environment on the experiment. The central bottom area of each tank contained only sand. In order to create narrow spaces potentially useful as a shelter, cobbles were placed touching side by side while the flat stones where put one on top of the other. In each trial a lidded Petri dish containing a megalopa randomly taken from the maintenance aquarium was carefully placed on the sand at the centre of a container. After a 5-min acclimation period, the lid was removed by a remotely controlled nylon string previously glued on it to avoid any interference of the operator with the larval behaviour, and the megalopa was left free to move in the containers for a trial duration of 30 min. The substrate where the megalopa was found at the end of each trial was considered the preferred one as long as the individual spent at least 50 % of the trial time on it, as ascertained from the examination of video recordings (see later). The duration of trials, acclimation period and release system were set after a pre-survey conducted with 20 individuals. Eighteen trials were conducted in each container using 36 megalopae.

A video-recording system was installed above each container to record the behaviour of megalopae during each trial. An ethogram was built a priori with three different categories: direct selection, hesitation and exploration (Table 1). Hesitation and exploration are defined according to Zimmermann et al. [36] (actually our hesitation corresponds to Zimmermann's immobility category) while direct selection is defined as the act of moving directly to a specific substrate after release without performing any other behaviour.

The behaviour performed for the longest time by each megalopa during the trial before the definitive substrate selection was defined as the dominant behaviour and was recorded through ad libitum sampling [37]. The dominant behaviour was not recorded in those individuals that did not choose any substrate. For all megalopae that preferred a substrate, the time to selection [TS (in s)] was measured during each trial.

Table 1 The ethogram used in the behavioural analysis

Direct selection	The larva moves directly to a substrate after leaving the Petri dish
Hesitation	The larva shows a period of hesitation over the sandy area before selecting a substrate
Exploration	The larva explores the container, before selecting the definitive substrate

Data analysis

Substrate preference and behavioural category were measured as percent frequencies of outcomes from the trials. A priori Pearson's Chi squared tests were conducted to check the effect of containers on the variables related to substrate preference (Chi squared test, $\chi^2 = 0.14$, $P > 0.05$) and to behavioural categories (Chi squared test, $\chi^2 = 1.53$, $P > 0.05$). A priori Wilcoxon–Mann–Whitney test was used to detect differences in TS values between the two containers (Wilcoxon-Mann–Whitney test, $U = 103$; $P > 0.05$). Since no differences were found data from the two containers were aggregated for the subsequent analyses.

Data were analysed comparing observed versus expected frequencies by Chi squared test. In order to identify which preference or behaviour contributed to statistical significance, the standardized residuals were analysed [38]. TS data were fourth root transformed and analysed by a one-way ANOVA with behaviour as fixed factor with three levels (direct selection, exploration, hesitation). Homogeneity of variances was checked with a Cochran's C test [39]. Where appropriate, pairwise comparisons were performed by means of a Student–Newman–Keuls (SNK) test. All analyses were performed by means of the R software package (R Ver. 3.2.0) [40].

Results

Overall, the megalopae of *P. gibbesi* showed a clear preference for hard substrates (Fig. 1a), which were selected by 28 individuals (Chi squared test, $\chi^2 = 12.2$, $P < 0.01$). Fifteen megalopae found a shelter in between the cobbles, 13 in between the flat stones and 2 in the gravel. Six megalopae did not choose any of the available substrates and spent the trial time either immobile or moving around the container during the full trial period.

Figure 1b shows the behaviour displayed by megalopae. After release, 17 megalopae displayed a direct selection of substrate, nine explored the container and four hesitated on the sand before reaching the definitive substrate. The Chi squared test highlighted statistical differences among the displayed behaviours ($\chi^2 = 8.17$, $P < 0.05$). Standardized residuals analysis highlighted that hesitation provided the higher contribution (standardized residuals >|2|) to the differences among the three behavioural categories. The TS needed by megalopae to select the preferred substrate was significantly shorter after direct selection than after exploration and hesitation (Fig. 1c; Table 2). Table 3 shows the average TS values for each selected habitat and behavioural category. The six megalopae that did not choose any of the available substrates were not added because of their erratic behaviour that was not possible to categorize. Two megalopae selected the gravel after a mean TS of 1 s and remained on that

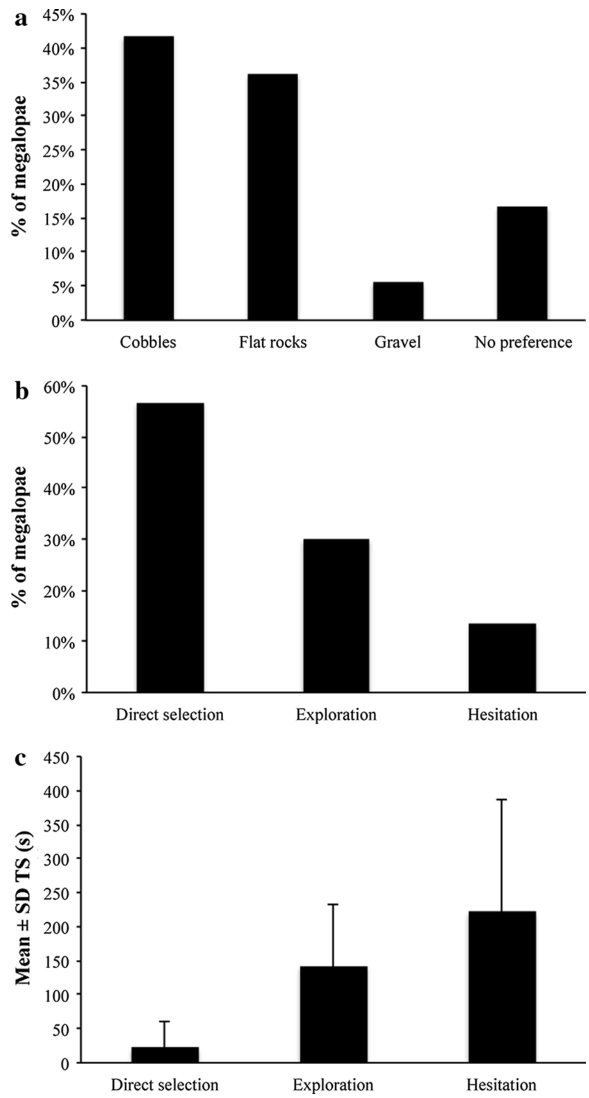

Fig. 1 Habitat preference and settlement response in megalopae of *P. gibbesi*. **a** Percent frequency of substrate preference, **b** percent frequency of dominant behaviour, **c** mean time to selection (TS) (in s) ± SD

Table 2 One way ANOVA performed on time to selection

Source of variation	*df*	Time to selection	
		MS	*F*
Behaviour	2	12.39	18.54***
Residuals	27	0.67	

Cochran's C test = 0.51

SNK test: direct selection < hesitation = exploration

*** P < 0.001

habitat for the entire duration of the trial. Seven megalopae selected directly the flat stones while eight selected directly the cobbles after a mean TS of 27.43 and 22.13 respectively. The TS values of the four megalopae that hesitated were the longest, with final selection occurring after 247.50 s on flat stones and 196.00 s on cobbles. The TS values of the nine individuals that explored were intermediate between hesitation and direct selection.

Discussion

The collection of megalopae in proximity to their natural settling ground is generally a very difficult task, due to their small size and to their tendency to hide in the refuges offered by a structured sea bottom as soon as they settle [41]. In our study, the light traps proved to be an effective collector of live *P. gibbesi* megalopae [34], which were caught off the coast and away from their settlement area during their pelagic phase and hence before any direct contact with a benthic habitat, making them suitable for a substrate preference experiment. One shortcoming of this sampling method is that, as opposed to artificially grown larvae, it does not allow to identify and select megalopae of a definite age. Having individuals of slightly different ages in the same lot might explain why some megalopae (arguably the youngest) did not show any selective behaviour, but this cannot be ascertained with the method employed.

The megalopae of *P. gibbesi* exhibited to a large extent a clear and active preference for a hard substrate i.e., cobbles and flat stones. Our results suggest that *P. gibbesi* prefers to settle on a physically stable substrate that offers narrow interstices for hiding rather than on mobile substrates like gravel or sand. Active substrate preference at settlement has been reported for other decapods crustaceans as American lobster [10], velvet crab [15] and red king crab [17].

The ability of *P. gibbesi* megalopae to discriminate and select among different substrates suggests that the presence of a suitable shelter is one of the cues that triggers the settlement. Other stimuli that we did not test, coming from habitat features (like e.g., biofilm composition) and from the presence of adult conspecifics, may play a major role in the settlement phase [9]. In particular the cues arriving from adult conspecifics might be crucial in the spread of a highly invasive benthic species, contributing to create vast and persistent populations. Nonetheless, laboratory experiments have shown that such cue does not work in other crustaceans, including another grapsoid crab like *Pachygrapsus transversus*, which also occurs in the Mediterranean [16].

The ability to select the right settling habitat guarantees an advantage to the megalopa, increasing post-settlement performances and successful recruitment [e.g., 19, 20, 42, 43]. When the competent megalopae of the fiddler crab *Illyoplax pusilla* were experimentally kept without the sandy-muddy substrate they usually select

Table 3 Summary table of time to selection [TS (in s)] for each substrate and behavioural category

	Hesitation			Exploration			Direct selection		
	n	TS (s)	SD	n	TS (s)	SD	n	TS (s)	SD
GR							2	1	0
FS	2	247.50	123.74	4	126.75	39.17	7	27.43	45.91
CO	2	196.00	250.32	5	154.00	122.41	8	22.13	37.81

GR gravel, *FS* flat stones, *CO* cobbles

for settling, the rate of mortality increased and many of the resulting juvenile crabs showed malformations [44]. The video recordings allowed us to observe the different behaviours displayed by the megalopae during each 30-min trial. Seventeen out of 36 individuals reached the preferred substrate directly and within a short time after their release and remained hidden there for the duration of the trial. These megalopae selected the same type of substrate preferred by adults, making any successive ontogenetic movement to a different habitat unlikely. Differently, an ontogenetic habitat shift by early crab instars has been observed in other species [*Carcinus maenas*, 11; *Callinectes sapidus*, 45] as a strategy to improve post-settlement performances.

Nine individuals swam or walked throughout the containers exploring different substrates before the final selection, and so did the six individuals that did not make any substrate selection. Larvae are supposed to use all information collected through habitat exploration and to couple them to their endogenous state to decide when and where to settle, in order to maximize their fitness [46, 47]. However, although exploration can be considered a strategy to identify a suitable settling habitat, its excessive extension could prove detrimental considering that starvation and predation—two likely consequences of a settlement delay—are important causes of mortality during this critical phase [2].

Four megalopae hesitated on the sand after leaving the Petri dish, delaying the choice of a substrate more than the other individuals. In many species immobility is a possible defence strategy to avoid detection by visually-oriented predators [48]. Actually, a megalopa of *P. gibbesi* is potentially well visible to predators due to its remarkable size, so its permanence in the open space might increase the risk of predation. On the other hand, while hesitating out of a shelter, according to Steullet et al. [49] the megalopa could use its sensorial functions, exploiting all cues useful to identify a suitable habitat.

Our results showed that the megalopae displaying hesitation or exploration behaviour spent an average TS about ten and seven times longer respectively than those making a direct selection. Exploration or even hesitation could have a positive implication, in that megalopae that delay their settlement could contribute to the invasive success of *P. gibbesi* through a dispersion over a wider area than if they settled rapidly once in the vicinity of their preferred habitat. In this way also less suited areas like boulders with erect macroalgae or rocky walls can be colonized [25; pers. observ.].

The information collected in this study enriches the pool of hypotheses that explain the invasive success of *P. gibbesi* in the Mediterranean, which include: (1) efficient larval life strategy with a robust, large-sized megalopa able to settle also in exposed habitats [26]; (2) capability of rapid selection of a suitable habitat at settlement (present data); (3) reduction in the abundance of competitors, especially shallow-water rocky-bottom microherbivores [50]. All these possible explanations still need investigation, also in the light of the requirements of the EU Marine Strategy Framework Directive (2008/56/EC) that includes the assessment of non-indigenous species among the qualitative descriptors of good environmental status.

Abbreviations

W: Watt; TS: time to selection; SNK: Student–Newman–Keuls; SD: standard deviation.

Authors' contributions

AZ conceived and performed the experiment, wrote the first draft of the paper, performed the sampling and data analysis. FB contributed to the experimental design and setup of the study, co-wrote the introduction and reviewed drafts of the paper. VMG took care of the larvae in earlier phases and helped with the experiment. LM participated in the design and setup of the study and reviewed drafts of the paper. CP provided expertise on crab ecology and reviewed drafts of the paper. TVF contributed to the experimental design and setup and helped with data analysis. GD conceived the experiment with AZ, collected samples, helped with data analysis and reviewed drafts of the paper. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
The datasets analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
This article does not contain any studies with human participants performed by any of the authors. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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