

Original Article

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Recruitment of the limpet *Patella ulyssiponensis* and its relationship with crustose coralline algae: patterns of juvenile distribution and larval settlement[†]

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Abstract

Recruitment of the limpet *Patella ulyssiponensis* was investigated in relation to the presence of living crustose coralline algae (CCA) in rocky-shore habitats. Juvenile limpets (≤ 10 mm maximum shell length) were counted in CCA-present and CCA-absent habitats, on three shores in SW Portugal during summer 2007 and winter 2009. Furthermore, the settling response of laboratory-reared larvae of *P. ulyssiponensis* to CCA-covered substratum, and bare-rock, was examined. Across the intertidal zone, we found a clear association between the distribution and abundance of juveniles and the presence of CCA. Although the presence of CCA was not an absolute requisite for juvenile occurrence, null juvenile densities were mostly recorded in CCA-absent areas. The highest juvenile densities (maximum of 64 individuals in 15×15 cm) were consistently found in CCA-dominated habitats, namely steep wave-exposed areas at low-shore and rock-pools. The hypothesis of CCA-enhanced settlement was not supported, as settlement intensities of laboratory-reared larvae were similar between chips of rock encrusted by CCA and chips of bare-rock. From the overall number of settlers onto CCA-encrusted rock chips, 51% were found in tiny pits lacking CCA. This was the first study of the settlement patterns of larvae of the genus *Patella* using naturally occurring rocky substrata. These results are preliminary and should be confirmed with choice-experiments and improved monitoring of the position of settlers. We suggest that CCA plays a role in the recruitment of *P. ulyssiponensis*, potentially promoting survivorship of early benthic stages, but possibly not enhancing settlement.

Introduction

Biotic interactions involving crustose coralline algae (CCA) are widely recognized as key ecological processes affecting the patterns of distribution and abundance of associated species (e.g. de Figueiredo *et al.*, 1996; Vermeij, 2005; Maneveldt *et al.*, 2006). These interactions can drive the structure and persistence of communities within marine benthic ecosystems (reviewed by Nelson, 2009).

Recruitment success, and consequent population demographic dynamics, of many benthic invertebrates may be dependent on the availability of CCA-covered substrata (sponges: Whalan *et al.*, 2012; corals: Sebens, 1983; Morse *et al.*, 1988; Tebben *et al.*, 2015; polychaetes: Gee, 1965; asteroids: Barker, 1977; Johnson *et al.*, 1991; sea urchins: Rowley, 1989; Pearce & Scheibling, 1990; chitons: Barnes & Gonor, 1973; Rumrill & Cameron, 1983; abalone: Morse *et al.*, 1979; Morse & Morse, 1984; Roberts *et al.*, 2010; and limpets: Steneck, 1982; McGrath, 1992; Guallart *et al.*, 2017). This relationship is based on field observations of the association of young individuals with CCA (e.g. McGrath, 1992), and/or evidence of the ability of larvae to detect chemical or biological cues at the CCA surfaces and trigger selective settlement (e.g. Morse *et al.*, 1979). The case study of abalone larvae and their highly specific requirement for a unique CCA inducer of settlement and metamorphosis (a small water-soluble peptide similar to the neurotransmitter gamma-aminobutyric acid (GABA)), provides the most complete model for the behavioural, cellular and molecular mechanisms involved in the control of the process of habitat selection during gastropod settlement (reviews by Morse, 1991; Roberts, 2001).

In intertidal limpets, mutual or commensal interactions with CCA or other crustose algae have been documented worldwide, establishing a common link between the abundance of recruits and the presence of algal species (Branch, 1975a; Steneck, 1982; Quinn, 1988; McQuaid & Froneman, 1993). In the North-east Atlantic, a taxonomically difficult CCA complex named 'Lithothamnia' (*sensu* Hawkins & Jones, 1992) is frequently the most abundant space-occupier within shallow rock-pools (e.g. Kooistra *et al.*, 1989). These 'Lithothamnia'-lined rock-pools and other rocky-shore habitats where CCA are conspicuously



present have been considered important nurseries for limpets of the family Patellidae (Lewis & Bowman, 1975; Bowman, 1985; Bowman & Lewis, 1986; McGrath & Foley, 2005).

'Lithothamnia'-induced settlement has long been suggested for patellid larvae, based on the spatial patterns of distribution of the smallest field-detectable recruits (Bowman, 1981; Kooistra et al., 1989; McGrath, 1992). However, settlement cues for patellid limpets remain unknown. This is partly because settlers of these species (only detectable by microscopic examination), have rarely been found in nature (but see Bowman, 1985; Gardner, 1986; McGrath & Foley, 2005). Rearing of larval patellids to settlement and metamorphosis is also very rare (but see Smith, 1935; Dodd, 1957; Wanninger et al., 1999; Ferranti et al., 2018). Laboratory assays with *Patella* embryos and larvae are often referred to as methodologically difficult (Dodd, 1957; Smaldon & Duffus, 1984; Espinosa et al., 2010; Pérez et al., 2016), which has probably impaired the progress of laboratory settlement studies.

The rough limpet, *Patella ulyssiponensis* (Gmelin, 1791), formerly known as *Patella aspera* Röding, 1798 (currently in use only for Macaronesia: see Weber & Hawkins, 2005), is an abundant and conspicuous herbivorous gastropod on rocky shores in the North-east Atlantic (Norway to Mauritania) and Mediterranean. Along with other widely distributed patellids, it has long been considered a 'keystone' species in rocky intertidal community structure and functioning (e.g. Branch, 1981; Hawkins & Hartnoll, 1983; Hawkins et al., 1992; Coleman et al., 2006). It occurs in the low intertidal zone of wave-exposed sites (Thompson, 1979), and at higher shore levels but mainly restricted to 'Lithothamnia'-dominated rock-pools (Guerra & Gaudêncio, 1986; Kooistra et al., 1989; Firth & Crowe, 2008).

CCA is a critically important substratum for the early life stages of *P. ulyssiponensis*. In the British Isles, Bowman (1981, 1985) observed post-settlement phases of *P. ulyssiponensis* (0.2–0.5 mm transparent individuals, larval shell still attached) as well as newly emerged *P. ulyssiponensis* (individuals with an adult calcified shell reaching a maximum shell length – MSL = 1 mm) solely within habitats dominated by 'Lithothamnia'. High densities of *P. ulyssiponensis* recruits (MSL < 3 mm) have also been found occupying the 'Lithothamnia' surface or in its close vicinity around the edges of *Mytilus* and *Corallina* clumps, within rock-pools or wet runnels located at lower tidal levels (Bowman, 1981; Guerra & Gaudêncio, 1986). Unlike other congeneric species, whose older individuals (MSL of 6–10 mm) seem to migrate to emersed open-rock (Bowman, 1981; Guerra & Gaudêncio, 1986; Delany et al., 1998), 'Lithothamnia'-dominated rock-pools may provide a habitat for *P. ulyssiponensis* until adulthood (Delany et al., 1998; Firth & Crowe, 2008). In addition, adults exploit 'Lithothamnia' as a food source (Hawkins et al., 1989; Delany et al., 2002). This life-long relationship between *P. ulyssiponensis* and 'Lithothamnia' reinforces the hypothesis of enhanced larval settlement by CCA-covered substrata and/or the existence of potential adaptive advantages for the maintenance of this habitat association. Adaptation to CCA-dominated habitats, which are usually permanently wet or submerged, would benefit *P. ulyssiponensis*, given its low tolerance of desiccation (Davies, 1969) and limited metabolic flexibility (Davies, 1967), compared with other patellids.

Presently, there is no published description of larval development of *P. ulyssiponensis* nor protocols to culture this species *in vitro*. However, Ribeiro (2008) studied the effect of temperature on the survival and development of laboratory-reared larvae of *P. ulyssiponensis*, *Patella depressa* (Pennant, 1977) and *Patella vulgata* (Linnaeus, 1758) and included data on settlement assays. The substrata used in those assays had been previously subjected to manipulation such as hard mechanical brushing or defaunation (Ribeiro, 2008; Ribeiro, personal communication). To our knowledge, our study is the first to address settlement patterns of

P. ulyssiponensis larvae in relation to unmanipulated (i.e. naturally occurring) rocky substrata, in the presence or absence of CCA.

Here, we examined the spatial variation in recruitment patterns of *P. ulyssiponensis* in SW Portugal and its potential relationship with the presence of CCA in rocky-shore habitats. The CCA taxon in this study was the complex 'Lithothamnia', mostly composed of *Lithophyllum incrustans* (Philippi, 1837) in the intertidal zone of the South-west Portuguese coast (Berecibar E., personal communication); hereafter it will be referred to as 'CCA'. We aimed to characterize differences in field patterns of distribution and abundance of juveniles (MSL ≤ 10 mm) of *P. ulyssiponensis* among a series of intertidal habitats, with and without CCA. We also report on a laboratory settlement experiment with larvae of *P. ulyssiponensis* exposed to chips of CCA-covered or bare-rock substrata. Specific hypotheses tested were: (1) patterns of juvenile densities with respect to habitat would vary depending on the presence or absence of CCA, predicting higher densities in habitats where CCA is present; and (2) larval settlement would be higher on chips of rock encrusted by CCA in comparison with chips of bare-rock.

Materials and methods

Field surveys to assess juvenile distribution

Two surveys, focusing on the search for small limpets (*Patella ulyssiponensis* juveniles – MSL ≤ 10 mm), were undertaken in low water spring tide periods. The first survey was carried out during August–early September 2007 (summer 2007) and the second during February 2009 (winter 2009). These reflect the trough and peak in the yearly reproductive cycle of this species in SW Portugal (Guerra & Gaudêncio, 1986; Castro, 2004). Spring/summer reproductive inactivity is followed by several peaks of gonadal development and spawning from late summer to early spring, and settlement mainly in late autumn to winter/early spring. Surveys were performed across a wide range of intertidal habitats present at three rocky shores with diverse geomorphology, located in the region of Sines, south-west coast of continental Portugal (see Figure 1). The study shores (Figure 1) were: (i) a natural shore, formed by steeply sloping basaltic massifs, adjacent to the breakwater of the General Cargo Terminal of the Port of Sines, hereafter named Port, (ii) Vale Marim, composed of metamorphic rocks interspersed with shale intrusions, boulders and pebbles and (iii) Oliveirinha, characterized by extensive and gently sloping shale platforms. All shores had wave-exposed platforms extending across the intertidal zone, with a heterogeneous mixture of open-rock, rock-pools, crevices and other patchy habitats, where limpets (*Patella* spp. and *Siphonaria pectinata*) are commonly found. The tidal regime is semi-diurnal, with maximum amplitude of ~3.5 m during spring tides.

In each survey, a series of habitats (Table 1), categorized as 'CCA-present' or 'CCA-absent' (named using subscripts 'p' and 'a', respectively, following the habitats' abbreviation names – see Table 1), was searched. Four (replicate) randomly sampled areas were assessed in each survey.

Within each sampling area, all limpets (≤10 mm MSL) were collected using dissecting forceps, needles, magnifying lens and lamps. This field search was complemented with further observations in the laboratory of material removed by destructive sampling in the case of complex three-dimensional or multi-layered habitats (i.e. calcareous substrata of the habitat 'urchin burrows' (U_p) and thalli/shells of *Lichina/Mytilus/Fucus* (L_a, M_a, F_a)).

Sample area differed among habitats: in the case of U_p, each area consisted of a 15 × 15 cm quadrat within large lower–mid-shore rock-pools including at least 10 adult *Paracentrotus lividus*,

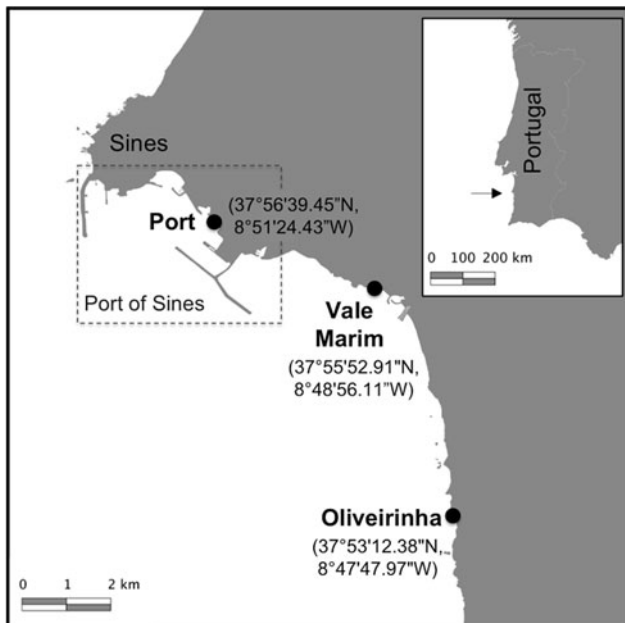


Fig. 1. Map of the study region and shores sampled.

which were removed to examine their surrounding substrata; for habitats with well-defined boundaries, such as rock-pools (PM_p , PMS_p , PHL_p , PHR_a) and patches of $L_a/M_a/F_a$, the total surface area of each replicate was sampled and estimated from scaled digital photographs; along crevices (CM_a , CMS_a , CH_a), replicates were 50×2 cm transects; and finally, for open-rock (OL_p , OL_a , OLM_a , OM_a) and vertical walls (VN_p , VS_a), 50×50 cm quadrats were sampled, using sub-sampling when densities of juvenile limpets exceeded 15 individuals per 15×15 cm.

All collected limpets were field-preserved in 96% ethanol and stored in the lab at 4°C , to be later identified under a dissecting stereomicroscope following Bowman (1981), and their MSL measured to the nearest $100 \mu\text{m}$ using a calibrated eyepiece.

All juvenile *P. ulyssiponensis* densities presented here were standardized to a 15×15 cm area and their records on sampling areas of all habitats were treated as the response variable in analyses for each shore and survey.

Larval settlement in laboratory conditions

Larval rearing was carried out in a temperature-conditioned room, using $0.2 \mu\text{m}$ Millipore-filtered and UV-sterilized natural seawater (FSW). Larval breeding proceedings were generally based on Ribeiro (2008 and references therein), although several methodological aspects were modified after pilot assays to increase the production of larvae.

During one of the peaks of the annual reproductive period of this species in SW Portugal (Castro, 2004), live *P. ulyssiponensis* with $MSL > 30$ mm were collected during low tide on 13 February 2011, at low-shore open-rock in Oliveirinha. Specimens were carefully removed from the substrata and transported to the laboratory in refrigerated conditions on top of wet stones and kept alive in an aquarium with aerated seawater at 17 – 18°C . After 2 h, collected specimens were dissected and a set of eight females and five males showing maximal reproductive development stages (stage V defined by Orton *et al.*, 1956) was selected immediately following dissection, to provide gametes.

All artificial fertilization procedures were made in a controlled-temperature room at 18°C . Whole female gonads were carefully dissected out and placed in a bowl with 150 ml of FSW; each ovary was then gently pulled apart and agitated to release oocytes.

The suspension of oocytes was subsequently stirred with a plastic pipette, so that clean oocytes could be collected from the bottom of the bowl and then separated from debris by transferring them to a clean glass dish with FSW. After repeating this washing procedure twice, oocytes were treated with alkaline FSW (made up to pH 9 using NH_4OH) for 10 min, according to Hodgson *et al.* (2007). Following alkalization, oocytes were returned to clean FSW to be rinsed twice using the same washing procedure, and then distributed among four beakers with 500 ml of FSW. Whole male gonads were dissected out, placed in a Petri dish containing a few drops of FSW and cut into pieces so that sperm were released. Suspended sperm were then collected and transferred with a plastic pipette into a 50 ml Falcon tube, adding FSW to bring the total volume to 50 ml. The suspension of sperm was mixed and finally, to activate sperm prior to *in vitro* fertilization (Hodgson *et al.*, 2007), it was maintained for 30 min at 4°C . For fertilization, a total of 5 ml of motile sperm (motility verified under the microscope) was estimated to be added to each 500 ml-beaker containing oocytes, to obtain an ideal concentration of 10^6 sperm ml^{-1} (checked using a Neubauer improved hemocytometer chamber), determined for *P. ulyssiponensis* by Hodgson *et al.* (2007). Therefore, the contact between gametes was made through the sequential addition of 1, 1.5 and 2.5 ml of the sperm suspension in three 10-min separated times, followed by manual and gentle stirring of the beaker volume during 30 min. The fertilization time was considered as the time of the first sperm addition.

Two and a half hours post-fertilization, sperm were washed off by replacing the water with 500 ml of clean FSW and eggs were incubated during a 15-h period for development of trochophore larvae (easily detected by swimming movements near the water surface), followed by a second water change. After the hatching of swimming trochophores, cultures were left undisturbed at a constant temperature of 20°C . Culture vessels were not stirred, since stirring techniques (airlift-droplet system – Strathmann, 1987; standard aeration and magnetic stirring) tested during our pilot assays did not yield a higher number of larvae compared with still water. Cultures were not fed (cf. Dodd, 1957; Ribeiro, 2008; Aquino de Souza *et al.*, 2009), as *Patella* larval development can be lecithotrophic (Wanninger *et al.*, 1999). Water was changed regularly every 12 h until the third day post-fertilization, when larvae reached competency. The stage of competency was detected by observations of larval behaviour and morphology: post-torsional veligers swimming close to the bottom surface or moving in fast upward bursts followed by passive sinking; competent larvae were capable of withdrawing into the protoconch and had an operculum, cephalic tentacles and bi-lobed foot. All culture water changes included the passage of the whole volume of each beaker through a stack-filter formed by a $250\text{-}\mu\text{m}$ -mesh sieve (used to catch flocculant waste and debris) placed on top of a funnel, which bottom end was positioned inside a permanently submerged $50\text{-}\mu\text{m}$ -mesh sieve, where eggs or larvae were retained and transferred into a 500 ml beaker with fresh isothermal FSW.

Cultures were sampled after the first two water changes (2.5 and 15 h post-fertilization), so that fertilized eggs and trochophores could be observed and counted under a stereo dissecting microscope. Eggs were counted in three subsamples of 1 ml using a Bogorov Plankton Counting Chamber and trochophores were counted in three subsamples of 2.5 ml transferred into a Petri dish. The total numbers of competent larvae produced were counted, by observing the contents of the $50\text{-}\mu\text{m}$ -mesh sieve after the last filtration of the volume of each culture beaker. Only eggs and larvae with normal development were considered in all counts. Larval survival rate until the developmental stages of trochophore and competency was estimated by dividing the

Table 1. List of intertidal habitats surveyed ('x') during searches for juvenile *P. ulyssiponensis* carried out in summer 2007 and winter 2009, at three shores (Port, Vale Marim, Oliveirinha) on the SW coast of Portugal, organized in 'CCA-present' or 'CCA-absent' categories (subscripts 'p' and 'a', respectively, following the habitats' acronyms)

Habitat description	Category	Summer 2007			Winter 2009		
		Port	Vale Marim	Oliveirinha	Port	Vale Marim	Oliveirinha
U _p – Urchin burrows – 'Lithothamnia' areas surrounding <i>Paracentrotus lividus</i> within medium-depth rock-pools at low-shore	CCA-Present			x			x
PM _p – Rock-pools at mid-shore – dominated by 'Lithothamnia'	CCA-Present	x	x	x	x	x	x
PMS _p – Rock-pools at mid-shore within shale intrusions – dominated by 'Lithothamnia'	CCA-Present		x			x	
PHL _p – Rock-pools at high-shore – dominated by 'Lithothamnia'	CCA-Present	x		x	x		x
VN _p – Vertical north-facing walls – shaded platform ridges, moist bare-rock among mixed assemblages of encrusting algae (<i>Ralfsia verrucosa</i> , <i>Lithophyllum tortuosum</i> , 'Lithothamnia', <i>Codium adhaerens</i>) and the barnacle <i>Perforatus perforatus</i>	CCA-Present			x			x
OL _p – Open-rock at low-shore – the main occurrence zone of <i>P. ulyssiponensis</i> , at Port is dominated by 'Lithothamnia'	CCA-Present	x			x		
OL _a – Open-rock at low-shore – the main occurrence zone of <i>P. ulyssiponensis</i> , in Vale Marim and Oliveirinha is characterized by foliose algae and algal turf interspersed with <i>Nemoderma tingitanum</i> and/or other encrusting algae (e.g. <i>Codium adhaerens</i>)	CCA-Absent		x	x			x
OLM _a – Open-rock at lower-mid shore level	CCA-Absent	x	x	x			
OM _a – Open-rock at mid-shore – the main occurrence zone of large-size <i>P. depressa</i> limpets and the barnacle <i>Chthamalus montagui</i>	CCA-Absent	x	x	x			
PHR _a – Rock-pools at high-shore dominated by bare-rock	CCA-Absent	x	x	x	x	x	x
CM _a – Crevices at mid-shore	CCA-Absent	x	x	x	x	x	x
CMS _a – Crevices at mid-shore within shale intrusions	CCA-Absent		x				
CH _a – Crevices at high-shore	CCA-Absent	x	x	x	x		x
L _a – <i>Lichina</i> patches – rock underneath and bordering <i>L. pygmaea</i> thalli at the high-shore	CCA-Absent	x	x	x			
VS _a – Vertical south-facing walls – sun-oriented platform ridges, with overhangs and cracks, dominated by bare-rock and the barnacle <i>Chthamalus montagui</i>	CCA-Absent			x			x
M _a – <i>Mytilus</i> patches – mussel shells and bordering rock within overhangs at the upper mid-shore	CCA-Absent			x			
F _a – <i>Fucus</i> patches – rock underneath and bordering <i>F. spiralis</i> thalli at the upper mid-shore	CCA-Absent			x			

mean number of larvae that had reached that stage in 500 ml by the mean number of eggs ascertained for the same volume.

Around 69 h post-fertilization (on 16 February 2011), a no-choice settlement experiment began, in which competent larvae were introduced to small glass dishes (4 cm tall, 9 cm diameter), each one containing 100 ml of FSW at 20°C and a single type of substratum at the bottom (set-up). The number of competent larvae introduced in each dish was variable (between 35 and 70 larvae, 51 on average). Two contrasting treatments were considered: rock encrusted with live CCA from mid-shore rock-pools (referred as 'CCA-chips', five replicate dishes) and untouched bare-rock from mid-shore open-rock (referred to as 'rock-chips', eight replicate dishes). Both treatments consisted of 3 × 3 × 0.5 cm chips of natural substratum, which were collected in Oliveirinha using a hammer and chisel, a few hours prior to the settlement assay. Chips of substrata were transported to the laboratory within separate containers in damp refrigerated conditions and maintained submersed in aerated seawater without additional manipulation before use. Following the introduction of competent larvae, dishes were monitored under a dissecting microscope, and notes were taken regarding larval behaviour

and morphology 24 and 48 h after set-up (four to five days post-fertilization). At these two observation times, all non-swimming larvae were counted within each dish: settlers (onto the chip of substratum) and individuals attached to the glass surface. The percentage of settlers at the end of the 24-h and the 48-h periods relative to the initial number of larvae introduced in each dish was taken as the response variable and compared between the two treatments separately for each time. In the case of the treatment 'CCA-chips', apparent aggregations of settlers were observed in particularly small pits of substratum lacking any CCA cover (each pit measuring a maximum of 1–2 mm² and the sum of these pits within every single CCA-chip occupying far less than 5% of the total area of the chip). Therefore, for settlers found on CCA-chips, individual positions were distinguished between two zones, concerning their occurrence within the overall CCA surface or, alternatively, within pits not covered by CCA.

Statistical analyses

Analysis of variance (ANOVA) was used to test for differences in the distribution of juvenile *P. ulyssiponensis* in relation to

intertidal habitat. Six separate one-factor analyses were performed: one for each shore (Port, Vale Marim, Oliveirinha) in each survey (summer 2007 and winter 2009), using habitat as a fixed factor with number of levels variable among comparisons (due to different number of surveyed habitats in each shore/survey). Cochran's test was used to test for the assumption of homogeneity of variances and in cases where variances were heterogeneous, data were transformed using $\ln(x + 1)$ or $\ln(x + \text{constant})$. Student–Newman–Keuls (SNK) procedure was used to make post-hoc comparisons among levels when the term habitat was significant. WinGMAV[®] version 5 for Windows (designed, coded and compiled by A. J. Underwood and M.G. Chapman, Institute of Marine Ecology, University of Sydney, Australia) was used for computations.

The percentages of settlers estimated at each observation time were compared between two groups of dishes, each group containing one of the two types of substrata ('CCA-chips' vs 'rock-chips') considered in the settlement experiment. These comparisons were analysed by means of *t*-tests for independent samples with equal variances, considering 11 degrees of freedom, after testing for homogeneity of variances between treatments.

Results

A total of 3359 juvenile *Patella ulyssiponensis* were field-collected, identified and measured (MSL in mm, mean \pm SE: 4.56 ± 0.04 ; 0.62–10.00 min-max). Abundances varied among habitats, shores and surveys, although shores and surveys were not formally compared and are treated separately (Figure 2). The presence of juvenile *P. ulyssiponensis* in relation to habitat varied between CCA-present and CCA-absent habitat types (Figure 2). In general, significantly higher densities of juveniles were found in habitats classified as 'CCA-present', compared with all the other habitats (Figure 2; Table 2, all SNK tests). Juvenile *P. ulyssiponensis* were most abundant in mid-shore rock-pools covered by CCA (PM_p of all shores in both time periods, and PMS_p in Vale Marim summer 2007) and/or in other CCA-dominated habitats, namely the urchin burrows in Oliveirinha (U_p – Figure 2; Table 2, SNK tests) and the steep low-shore open-rock at Port, where the maximum density of 64 juveniles per 15 \times 15 cm was recorded in summer 2007 (OL_p at Port; Figure 2; Table 2, SNK tests). In addition, no *P. ulyssiponensis* juveniles were found on mid-shore open-rock in any shore (OM_a) or on patches of *Lichina* (L_a) and *Fucus* (F_a) (Figure 2, summer 2007). Consistently at all sampled shores and surveys, densities registered at the other habitats classified as 'CCA-absent' were either null or very low (the highest mean density of 5.06 juveniles in 15 \times 15 cm was recorded in mid-shore crevices within shale intrusions – CMS_a in Vale Marim during summer 2007). The unique exception was the *Mytilus* habitat (M_a in Oliveirinha in summer 2007: Figure 2; Table 2, SNK test), for which the mean density of 12 juveniles in 15 \times 15 cm was found to be significantly higher than in all the other CCA-absent habitats and similar to two CCA-present habitats.

Regarding the laboratory larval rearing assay, the survival rate of healthy individuals relative to the initial number of eggs ($45,125 \pm 4399.67$ eggs in 500 ml – mean \pm SE, N = 4 beakers), until the trochophore and competent post-torsional veliger stages was $24.02 \pm 3.02\%$ and $0.34 \pm 0.07\%$ (mean \pm SE percentages), respectively.

Larvae of *P. ulyssiponensis* settled within all the 13 experimental dishes that were set up during the settlement experiment. From the total number of 673 competent larvae initially introduced, 75 (11.1%) and 115 (17.1%) settlers were recorded on the chips of substrata, as well as 95 (14.1%) and 54 (8%) individuals attached to the glass surfaces, respectively at the end of the 24-h and the 48-h observation periods.

Observed settlers were well-developed pediveliger larvae, with the cephalic region oriented downwards and the foot firmly attached to the substratum surface, either fixed or in some cases showing exploratory behaviour by crawling along the substrata. Nevertheless, observations of metamorphosis (defined as the complete loss of the swimming organ *vellum*) were residual (93.9% of settlers still maintained some cilia after 48 h of exposure to substrata). Morphology of settlers corresponded to the late larval stage described for *Patella vulgata* as the 'crawling larva with velum' (*sensus* fig. Id, p. 109 in Lebour, 1931) and measured \sim 200–220 μ m in cross-shell length.

The hypothesis of induction of higher settlement of *P. ulyssiponensis* larvae by CCA was not supported, as at both observation times, the percentage of settlers relative to the initial number of larvae was similar between chips of rock encrusted by CCA and chips of bare-rock (Figure 3 – 24 h: $t = 0.73$, $P = 0.48$; 48 h: $t = 0.75$, $P = 0.47$). The maximum percentage of larval settlement onto the chips of substrata was registered on bare-rock (34.8%).

Comparing the numbers of larvae settled onto the chips of substrata with the ones settled onto glass in each treatment and at each observation time, it was found that, from the total number of settled larvae in each set of dishes: at 24 h, most larvae settled onto CCA-chips (66%) while a minority settled onto rock-chips (36%); whereas at 48 h, the majority of larvae settled onto rocky substratum independently of being encrusted or not by CCA (80% on CCA-chips; 62% on rock-chips).

Furthermore, in the set of CCA-chips, 60 and 40% of the total number of settlers, observed at 24 and 48-h respectively, were recorded in pits of substratum not covered by CCA (Figure 4). Given the small area of these pits, observed settlers within each pit were positioned very close together.

Discussion

The focus of our study was the relationship between early life-history stages of *Patella ulyssiponensis* and CCA. Our results demonstrated differing patterns from settlers to juveniles in relation to CCA habitat presence. We found that juvenile *P. ulyssiponensis* (MSL < 10 mm) in SW Portugal occur almost exclusively in habitats characterized by the dominance of CCA. This species-habitat association was consistently found in all sampled shores and times and it is probably specific for *P. ulyssiponensis*, as it was not observed for other limpet species (*Patella depressa* and *Siphonaria pectinata*) sampled on the same surveys (Seabra *et al.*, in prep.).

The highest juvenile densities were found at the low-shore open-rock of Port (OL_p), with values nearly 20 times higher, on average, compared with the low-shore open-rock at the other sampled shores on both surveys (OL_a in Vale Marim and Oliveirinha). At Port, low-shore zones occur within particularly steep and wave-exposed platforms, where, in contrast with the other shores, foliose algae are scarce and CCA covers most of the space available (CIEMAR, 2018). Although vertically narrow, this CCA-dominated low-shore area supports an exceptionally large population of *P. ulyssiponensis* (CIEMAR, 2018). Moreover, observations on the shells of live large-sized *P. ulyssiponensis* (MSL > 30 mm) in the low-shore at Port indicated that the majority of these individuals were completely overgrown by well-developed rugose forms of CCA, also providing home for *P. ulyssiponensis* juveniles (personal observations). These population patterns parallel those described for the pear limpet *Scutellastra cochlear*. This patellid species dominates a characteristic 'cochlear zone' at the low-shore level of South African shores, where it occurs in clear association with the CCA *Spongites yendoi* (formerly referred as 'lithothamnion': e.g. Branch, 1975a). Through territorial gardening, *S. cochlear* excludes most algae

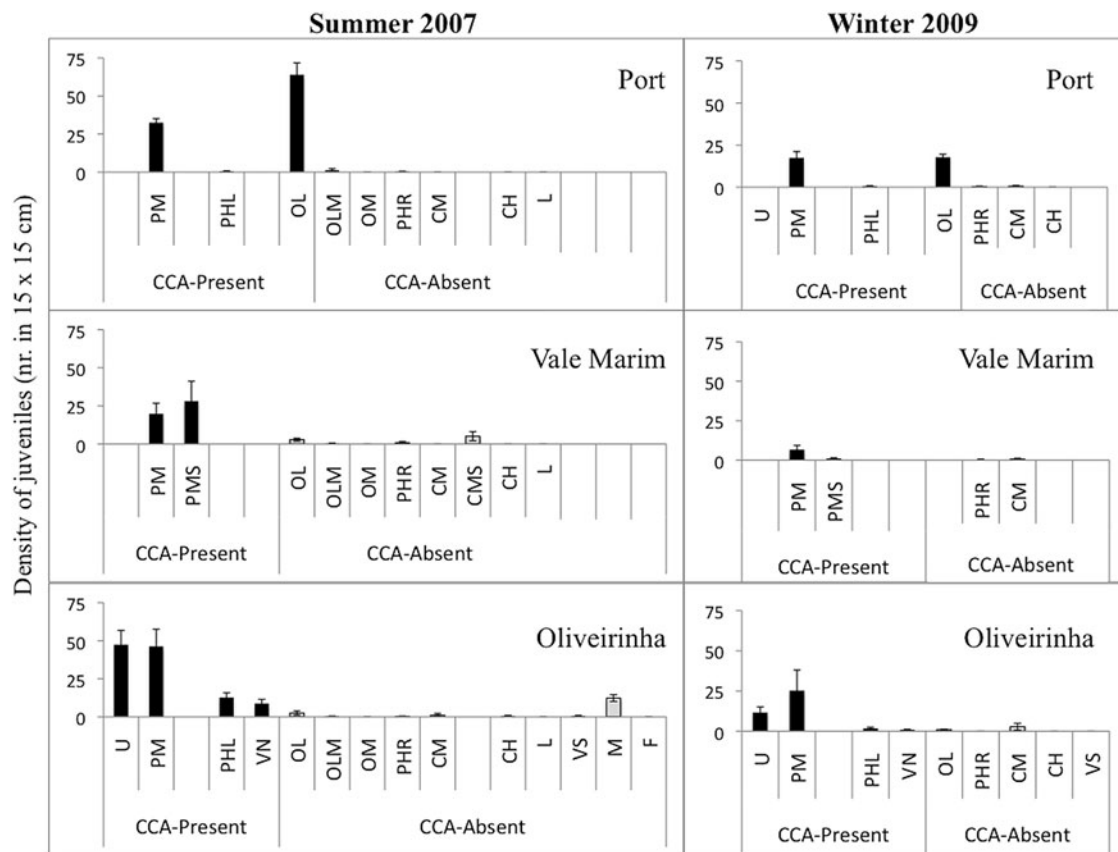


Fig. 2. Abundance of juvenile *Patella ulyssiponensis* (mean \pm SE, $N = 4$) on CCA-present (black bars) and CCA-absent (grey bars) habitats, at three shores (Port, Vale Marim, Oliveirinha) on the SW coast of Portugal, during two surveys (summer 2007 and winter 2009). Habitat acronyms listed in Table 1. Blank spaces displayed at the x-axes correspond to non-sampled habitats at a given shore/survey (should not be confounded with records of null/very low densities at sampled habitats, for which x-axes categories are labelled).

Table 2. Summary of results of analyses of variance to determine the distribution of juvenile *P. ulyssiponensis* along a range of intertidal habitats, within three shores (Port, Vale Marim, Oliveirinha) in SW Portugal, in summer 2007 and winter 2009

Period	Shore	Data transformation	df	MS	F	P-value	SNK
Summer 2007	Port	$\ln(x + \text{constant}_a)$	8	2.79	222.98	***	$OL_p > PM_p > \text{others}$
	Vale Marim	$\ln(x + \text{constant}_b)$	9	5.12	17.49	***	Undefined pattern but $(PMS_p = PM_p) > \text{others}$
	Oliveirinha	$\ln(x + 1)$	13	7.93	30.74	***	$(U_p = PM_p) > (PHL_p = M_a = VN_p) > \text{others}$
Winter 2009	Port	$\ln(x + 1)$	5	7.58	60.76	***	$(OL_p = PM_p) > \text{others}$
	Vale Marim	$\ln(x + 1)$	3	2.15	5.49	*	$PM_p > \text{others}$
	Oliveirinha	$\ln(x + \text{constant}_c)$	8	6.34	10.78	***	$(PM_p = U_p) > \text{others}$

df, degrees of freedom; MS, mean sum of squares; F, F-statistic; SNK, Student–Newman–Keuls test.

$\text{constant}_a = 8$; $\text{constant}_b = 1.2$; $\text{constant}_c = 0.5$.

Habitat acronyms listed in Table 1. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

and macrofauna (Branch, 1976), limiting the biodiversity in its vicinity to *S. yendoii* and to strips of filamentous red algae surrounding each limpet (Branch, 1975b; Plaganyi & Branch, 2000). Density of *S. cochlear* is positively related to wave action, so dense populations formed by uniformly spaced and closely packed individuals occur on wave-exposed shores, with juveniles mostly occurring on the backs of adults due to crowding (Branch, 1975a, 1975b). Similarly, the relatively greater wave action at Port probably contributes to the dominance of CCA and the high abundance of *P. ulyssiponensis* on the low-shore, by dislodging the less robust erect algae and releasing suitable substrata for limpet establishment and high recruitment rates. As in the *S. cochlear*–*S. yendoii* interaction, *P. ulyssiponensis* at Port probably feeds mainly on the ‘Lithothamnia’ present on

the rock and on conspecific shells, preventing overgrowth by other algae as settling spores are cleared away through limpet grazing activity.

The other habitats where significantly higher juvenile densities of *P. ulyssiponensis* were found on all shores consisted mostly of CCA-covered rock-pools distributed across the intertidal zone (U_p in the lower levels, PM_p/PMS_p in the mid shore and/or PHL_p at the upper levels). Similar spatial patterns of association of small size-classes of *P. ulyssiponensis* with rockpool habitats dominated by CCA have been reported for the Portuguese coast (Guerra & Gaudêncio, 1986) and the North Atlantic in general (e.g. Bowman, 1981; Delany et al., 1998; Firth & Crowe, 2008).

The recruitment of *P. ulyssiponensis* is probably limited by its low desiccation tolerance (Davies, 1969) and by high susceptibility

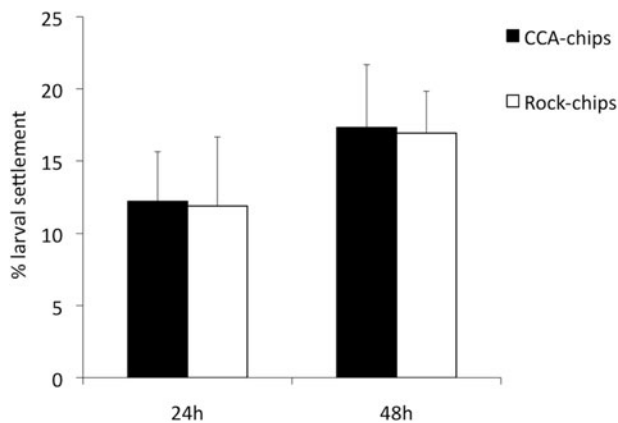


Fig. 3. Settlement of *Patella ulyssiponensis* (percentage of settlers relative to the initial number of competent larvae; mean \pm SE), 24 and 48 h after exposure to substrata, in CCA-chips (N = 5, black bars) and rock-chips (N = 8, white bars).

of recent benthic stages to temperature variation and storm damage (e.g. Thompson, 1979; Bowman & Lewis, 1986). Nevertheless, in our study, juvenile association with CCA-dominated habitats was apparently not an outcome of the limpet preference for submerged/damp/wave-protected habitats. For instance, during the summertime survey in Oliveirinha, significant differences were found between high-shore rock-pools with and without CCA (PHL_p vs PHR_a), which had similar dimensions (respectively, 318 cm² vs 331 cm² of surface area, on average; all <10 cm depth) and were closely located at the same rocky platforms and shore level. Overall, our results on juvenile distribution patterns suggest the importance of the 'Lithothamnia' itself for *P. ulyssiponensis* population recruitment, over other co-varying factors in the intertidal environment. This assumption is in agreement with the findings of a manipulative study carried out by Delany *et al.* (2002) on Clare Island (Ireland). They tested the effect of the presence of this living algal complex on the recruitment of juveniles and on the persistence of adults of intertidal limpets within rock-pools, separately for *P. ulyssiponensis* and *P. vulgata*. In contrast to *P. vulgata*, juvenile recruitment and adult immigration rates of *P. ulyssiponensis* were significantly reduced in rock-pools where 'Lithothamnia' had been killed by blowtorching the substratum (Delany *et al.*, 2002). Comparable importance has been given to biological interactions over physical features in dictating limpet population characteristics (such as distribution, size-structure, behaviour and feeding habits), in the case of *Patella vulgata* in Britain (Lewis & Bowman, 1975) and several *Patella* species in South Africa (Branch, 1976).

CCA presence is possibly not an obligatory condition of the nursery grounds of *P. ulyssiponensis* in SW Portugal, as juveniles occurred within a few CCA-absent habitats. Particularly, within *Mytilus* patches, sampled during our summertime survey in Oliveirinha, the abundance of juveniles was similar to those estimated in two CCA-present habitats. This is in agreement with the work of Delany *et al.* (2002), which was the first record of *P. ulyssiponensis* recruiting in the absence of any living CCA, despite its demonstrated importance for recruitment of this limpet.

Although not strictly essential for juvenile occurrence, our field surveys supported the idea that CCA do play a role in the settlement and/or recruitment of *P. ulyssiponensis* in SW Portugal. Our laboratory study on the settling response of *P. ulyssiponensis* was aimed to test the hypothesis that CCA would have some attribute detectable by *P. ulyssiponensis* larvae and capable of inducing higher rates of settlement, compared with other rocky substrata. Our results showed similar larval settlement between chips of CCA-encrusted rock and chips of bare-rock.

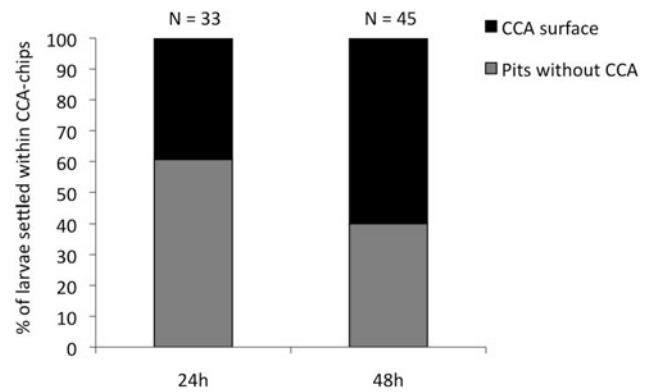


Fig. 4. Distribution of settlers of *Patella ulyssiponensis* in a set of five CCA-chips, 24 and 48 h after exposure to substrata, with respect to their occurrence within the overall CCA surface (bar portions in black) or within tiny pits of substratum lacking CCA cover occupying a maximum of 5% of the total area of each chip (bar portions in grey).

These two types of substratum were chiselled from rock-pools and open-rock at the same tidal level. Therefore, settlement patterns of laboratory-reared larvae did not match the distribution of juveniles across intertidal habitats, suggesting that the spatial variation in recruitment of this species is probably not the outcome of a specific CCA-inducement cue triggering larval settlement.

The results of our settlement experiment are similar to previous results obtained in December 2010 (fewer replicates, data not shown). Nonetheless, our present results are preliminary because they should be confirmed by choice-experiments where CCA-chips, and rock-chips, are simultaneously presented to competent larvae. Our results provide estimates of larval settlement onto different natural substrata, and do not allow for inferences about selection or habitat preference of *P. ulyssiponensis* larvae in the field. Nevertheless, given 'the choice' between the chip of substratum and the glass surface within each dish, larvae settled mainly onto rocky substratum rather than onto glass, independent of the presence/absence of CCA (at 48 h, 71% of the total number of settled larvae, on average, were recorded onto substratum). Moreover, the fact that, within the area of CCA-chips, clustering of settlers was observed at both 24 and 48 h in very tiny pits lacking CCA-cover, suggests that *P. ulyssiponensis* larvae may have the ability to discriminate between different natural substrata at a very small spatial scale (few mm²). The occupation of 'CCA-free' pits by 51% of the total number of settlers recorded onto CCA-chips was impressive, especially if one considers the space-limiting nature of the pits. This observation supports our results on the absence of a positive effect of CCA presence on the larval settlement of *P. ulyssiponensis*. However, our results on the distribution of settlers should be confirmed in future experiments with frequent and accurate monitoring of individual positions of settlers (e.g. by time-lapse photography of settlement surfaces), to detect their timing and location at settlement, and to follow their possible movements after settlement.

Little information is reported in the literature regarding settlement and metamorphosis of patellids in controlled conditions. The conditions to promote larval settlement seem to vary among *Patella* species, e.g. presence of a conspecific adult cue for *P. caerulea* (Wanninger *et al.*, 1999; Ferranti *et al.*, 2018) and proximity to the air-water interface for *P. ferruginea* (Guallart *et al.*, 2017). Experimental investigation *in vitro* regarding settlement is hampered by the fact that the actual quantity of settlers and/or metamorphosed juveniles produced in laboratory studies was either very limited (Smith, 1935; Wanninger *et al.*, 1999; Guallart *et al.*, 2017) or not quantified (Dodd, 1957;

Ferranti *et al.*, 2018). Additionally, consistent evidence has been recently gathered concerning the need to supply settlers with a food suitable for their benthic mode of feeding (an algal film to be scraped by the radulae, i.e. microalgae and/or macroalgae sporlings and germlings, coating the surfaces available for settlement) to follow the development of metamorphosing specimens in the laboratory. Despite confirming the lecithotrophic nature of pelagic larvae of *Patella caerulea*, Ferranti *et al.* (2018) have demonstrated that larvae need to be fed as soon as the pediveliger stage is reached. In our study, the algal film naturally present on the surface of chips of substrata was possibly insufficient, given the observed lack of complete metamorphosis and the fact that a high mortality of settlers was observed 72 h after the start of the experiment.

Developing protocols for culturing larvae of *P. ulyssiponensis* was laborious, and not fully established during our pilot assays. In fact, 10 unsuccessful pilot assays were tried before the methods reported here. These observations were in agreement with the study of Espinosa *et al.* (2010) in which larvae of *Patella ferruginea* did not survive beyond the pre-torsional veliger stage, suggesting a common pattern of high sensitivity of lab-reared larvae at this development stage (also referred to by Smith, 1935) and/or a biological constraint of the genus during the ontogenic phase of torsion. Recently, Pérez *et al.* (2016) standardized a *Patella* spp. embryo-larval bioassay for use in ecotoxicological studies, which included data on *P. ulyssiponensis*, providing reliable protocols for larval rearing. However, the endpoint established in Pérez *et al.* (2016) was the percentage of normal trochophore larvae and therefore it would be important to apply the same approach to the latter stages of larval development. In contrast with Hodgson *et al.* (2007), it is possible to compare our results to those of Pérez *et al.* (2016) for rearing *P. ulyssiponensis* up to the trochophore stage because they assessed survival in the same way (percentage of normal trochophores relative to the initial number of fertilized eggs) and used the same incubation temperature (18°C). Our fertilization success was low (24%), although within the range of 21.6–46.3% obtained by Pérez *et al.* (2016) under optimized conditions. When applying these conditions in a very recent study (Pérez *et al.*, 2019), an even lower percentage of normal *P. ulyssiponensis* trochophores (11.56%) was recorded (stated as '%NL' in Table 1, p. 154 in Pérez *et al.*, 2019). Taken together these results point to the high variability in successful larval development and the need for further methodological improvement (Pérez *et al.*, 2016).

Clearly, more work is required to better understand the process of settlement in *P. ulyssiponensis*. Quantifying settlement in the field is often precluded by spatial patchiness, temporal unpredictability, and/or scarce numbers – all characteristic of broadcast-spawning intertidal gastropods (Underwood, 2004). Therefore, laboratory studies are probably the best alternative to study this process, notwithstanding the above-mentioned difficulties.

Overall, our study established different patterns of association with CCA in different stages of *P. ulyssiponensis* benthic life. This was due to the contrast found between larvae soon after settlement and juveniles, in terms of habitat fidelity. Settlers in our laboratory experiment were relatively similar-sized individuals with a maximum of two days post-settlement. In contrast, juveniles in the field showed greater variation in size and likely in age. Even juvenile patellids of the same size may belong to different age groups, due to substantial variance of individual growth rates depending on habitat (Lewis & Bowman, 1975). Therefore post-settlement age of the whole set of sampled juveniles may have spanned from a few months to more than a year (Bowman, 1981). Consequently, juvenile distributions at the time of surveys may not correspond to settlement locations. During the interval between initial settlement and the time at

which juveniles are large enough to be visible and identifiable in the field, distribution across habitats can change due to differential mortality or movement (e.g. Keough & Downes, 1982; Rodriguez *et al.*, 1993). We propose that the mismatch between juvenile distribution and larval settlement patterns is that recruitment densities of *P. ulyssiponensis* are not driven directly by the larval settling response to the presence of CCA, but instead from differential effects of selective pressures among different habitats causing spatial variation in post-settlement mortality. Accordingly, we suggest that the survivorship of *P. ulyssiponensis* settlers would be higher in CCA-dominated habitats, due to presence of potentially favourable conditions. Given the deep excavating capacity of the radula of *P. ulyssiponensis* (Hawkins *et al.*, 1989), the algal complex 'Lithothamnium' could be important for survival of early benthic stages of this species, possibly providing a reliable food resource. The next step should be exploring the mechanisms responsible for potentially higher survival in CCA.

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