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LARVAL SETTLEMENT, EARLY GROWTH AND SURVIVAL OF *HALIOTIS TUBERCULATA COCCINEA* USING SEVERAL ALGAL CUES

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ABSTRACT Settlement of *Haliotis tuberculata coccinea* larvae was examined in the presence of crustose coralline algae (CCA), *Ulvella lens,* and *Ulva rigida.* Germlings of the 2 green macroalgae of different age, enrichment level, and in combination were tested as settlement cues, and CCA was tested as a positive control. Larval settlement was the highest on CCA (61%) tailed by a 45-day-old mix of *U. lens* and *U. rigida* (52%) and 45-day-old *U. rigida* (46%). Settlement was the lowest (about 3%) on a mix of 4-day-old *U. lens* and *U. rigida* and 45-day-old enriched or unenriched *U. lens*. In all treatments, postlarvae were fed for 4 wk with a mix of diatoms (*Amphora* sp., *Proschkinia* sp., *Nitzschia* sp., and *Navicula incerta*); postlarval growth was the best on the 45-day-old mix of *U. lens* and *U. rigida*. This substrate was also the best of the green macroalgae germlings substrates tested for settlement induction and provided good survival rates. The substrate protein content correlated negatively with larval settlement and survival. The algal cues were differentiated by their fatty acid composition. Fatty acids such as 18:1n-7, 18:2n-6, 16:4n-3, arachidonic acid, and eicosapentaenoic acid were suggested to affect settlement and survival. The fatty acids correlated with settlement were different from the ones associated with survival rates, except eicosapentaenoic acid, which correlated with both. The results of this study show the high value of *U. rigida* for *H. tuberculata* postlarvae, and the influence of substrate age on the settlement success.

KEY WORDS: abalone, crustose coralline algae, *Ulvella lens*, *Ulva rigida*, settlement, early growth and survival, biochemical composition, *Haliotis tuberculata coccinea*

INTRODUCTION

Knowledge relevant to settlement cues is critical for abalone breeding (Roberts 2001), because adequacy of the selected cues directly determine settlement rates directly and early postlarval survival (Searcy-Bernal et al. 1992, Slattery 1992, Daume et al. 1999, Roberts 2001, Takami et al. 2002). Cues from a wide range of sources are reported to induce settlement of *Haliotis* larvae, including crustose coralline algae (CCA) (Morse et al. 1980, Morse & Morse 1984, Moss & Tong 1992, Takami et al. 1997, Daume et al. 1999, Daume et al. 2000, Roberts et al. 2004, Williams et al. 2008), films of benthic diatoms (Seki & Kan-No 1981, Kawamura & Kikuchi 1992, Daume et al. 1999, Roberts et al. 2007), abalone mucus trails (Searcy-Bernal et al. 1992, Slattery 1992, Seki & Taniguchi 1996, Seki 1997, Bryan & Qian 1998, Laimek et al. 2008), bacterial films (Bryan & Qian 1998, Roberts 2001), or several purified chemicals like γ-amino butyric acid (Morse et al. 1980, Morse & Morse 1984, Morse 1985, Trapido-Rosenthal & Morse 1986, Searcy-Bernal et al. 1992, Bryan & Qian 1998, Stewart et al. 2008).

Despite the established use of benthic biofilms, consisting of bacteria and mixed diatom species, in abalone hatcheries worldwide (Daume 2006), larval settlement rates on such substrates can be very low (1–10% of larvae) (Daume et al. 2000, Courtois de Viçose et al. 2010). Alternatively, the germlings of the green alga *Ulvella lens* have been tested and used successfully to induce abalone settlement (Takahashi & Koganezawa 1988, Daume et al. 2000, Daume & Ryan 2004, Daume et al. 2004). Because each abalone species responds differently to settlement cues (Daume et al. 1999), in previous studies a range of cues were tested for *Haliotis tuberculata coccinea* and showed that *U. lens* induced higher settlement rates than diatom biofilms (Courtois de Viçose et al. 2010). Daume (2006) raised the hypothesis that age and biochemical characteristics of *U. lens* influences abalone larval settlement based on the clearer preference of *Haliotis rubra* and *Haliotis laevigata* larvae for older, rather than for younger, *U. lens*. However, this hypothesis has not been tested on other abalone species.

The potential of various macroalgae to affect settlement of invertebrate larvae has been tested and indicates that settling and metamorphosing larvae are highly influenced by chemical, biological, and physical cues—characteristics of each algae (Walters et al. 2003). Invertebrate larvae were shown to respond to surface-associated or waterborne cues from macroalgae (Pawlik 1992) and recruit specifically in response to compounds produced by different algal species (Krug & Manzi 1999, Williamson et al. 2000), and were reported to vary among the different species (Hay 1996, Karsten et al. 1999). Planktonic larvae of sea urchins have also been reported to settle and metamorphose in response to polyunsaturated fatty acids (PUFAs) (Kitamura et al. 1993) and dibromomethane (Taniguchi et al. 1994).

Green macroalgae, other than *Ulvella lens,* have also been studied for their potential for abalone settlement induction and postlarval nutrition, but have yet to be investigated thoroughly as a source of potential settlement cues, taking into account their prevalence in the adult and postlarval habitat. For instance, Seki (1997) reported that the foliose green algae *Ulva* sp. induced metamorphosis of *Haliotis discus hannai,* and Strain et al. (2006) suggested that germlings of *Ulva* sp. could provide a suitable food source for abalone juveniles based on their nutritional and structural properties. Besides, the settlement induction of *Haliotis rubra* larvae by the green algae *Ulva australis* and *Ulva compressa* was related primarily to the alga and linked to chemical compounds produced by the algae or their surface texture and topography (Huggett et al. 2005). However, to the

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best of our knowledge, the use of germlings of *Ulva* spp. or macroalgae other than *U. lens* as abalone settlement inducers has not been investigated until now.

To improve the efficiency of *Haliotis tuberculata* breeding techniques and production, and, more specifically, of *Haliotis tuberculata coccinea,* further research was needed to improve settlement induction as well as postlarval growth and survival. The current study tests the germlings of *Ulva rigida* as settlement inducers of *H. tuberculata coccinea* and investigates the effects of age in addition to proximate biochemical and fatty acid composition of *Ulvella lens* and *U. rigida* germlings, and CCA, on larval settlement, and postlarval survival and growth of *H. tuberculata coccinea*.

MATERIAL AND METHODS

Larval Rearing

Captive *Haliotis tuberculata coccinea* broodstock were kept in 60-L tanks placed in a flow-through system at the Instituto Canario de Ciencias Marinas (Canary Islands, Spain). Ripe *H. tuberculata coccinea* were induced to spawn (male-to-female ratio, 1:2) by UV-irradiated seawater (Kikuchi & Uki 1974), and larvae were obtained from gametes of 8 males and 14 females. Larvae from 1 mixed batch were used to conduct the study and were estimated competent for settlement when the third tubule of the cephalic tentacle could be observed (Hahn 1989, Courtois de Vicose et al. 2007).

Algal Cultures

Four and 45-day-old *Ulvella lens* and *Ulva rigida* germlings were obtained from mature, spore-producing *U. lens* and *U. rigida* thalli. The germlings of *U. lens* and *U. rigida* were obtained according to previously described production methods, and their cultures were adapted (Daume et al. 2004, Strain et al. 2006). The cultures of old germlings (45 days old) from *U. lens*, *U. rigida,* and a mix of both were maintained aerated under a natural photoperiod during the 45 days and the f/2 culture medium was renewed weekly. As opposed to the old *U. lens* germlings, enriched, 45-day-old *U. lens* germlings were enriched at twice the f/2 concentration during the entire culture period. Germlings grown for 4 days and 45 days presented different developmental stages on plates and reached diameters ranging from 10–75 μm 75–300 μm, respectively. In addition, 45-day-old germlings showed developed sporangia. In the CCA treatment, settlement plates remained in nursery tanks to be colonized by CCA until reaching 46.67 ± 7.88% coverage.

Four species of diatoms—*Navicula incerta*, *Proschkinia* sp., *Nitzschia* sp., and *Amphora* sp.—were provided as diets to the postlarvae. All diatoms were cultured in 40-L horizontally laid algal bags at initial inoculums of 10^5 cells/mL and were grown for 5 days in f/2 medium supplemented with 1 mg/L silicate (Guillard, 1975) at ambient temperature and under continuous light of 62 ± 8 μmol photon/m^2/sec. Photon flux density (irradiance) was measured using a digital light meter (HT170N, HT ITALIA, Italy). The cultures were not axenic. Diatom cell concentration was evaluated weekly with a Neubauer hemocytometer. Prior to the counts, ultrasound (1 min or 3 min) was applied to the diatoms samples to avoid possible cell aggregations. Equal cell numbers of each diatom were combined to obtain the 200-mL diatom mixture fed weekly to the postlarvae.

Estimates of Algal Cover

Ten randomly chosen fields of view on the settlement plates, for each treatment, were photographed at a magnification of 400× at the time of larval settlement. Percent coverage of CCA, *Ulvella lens,* and *Ulva rigida* were then calculated by processing the images with Image J Software (Image J 1.42q; National Institutes of Health, Bethesda, MD).

Larval Settlement Experimental Protocol

The settlement study aimed at evaluating the effect of different algal types and of different age and biochemical composition on the settlement rate of *Haliotis tuberculata coccinea*. Eight treatments were tested: young (4 days old) and old (45 days old) *Ulvella lens*, enriched old *U. lens* (45 days old), young (4 days old) and old (45 days old) *Ulva rigida,* a combination of *U. lens* and *U. rigida*, both young and old, and, last, CCA as the control treatment. Each treatment settlement plate (50-cm^2 plastic squares) was colonized by the respective substrate. Each type of substrate was tested in triplicate and each replicate consisted of 4 settlement plates placed vertically in 12-L containers ($n = 12$/treatment). Larvae were introduced in the containers at a density of 200 larvae/12-L container, representing 1 larvae/cm^2 of substrate, their number being estimated by counting them in 3 3-mL subsamples drawn from the entire larval batch.

Twelve-liter containers were filled with 1-μm filtered seawater supplied with low aeration. Water was renewed at a rate of 1%/h, 24 h after the introduction of larvae, and was finally increased up to a 20% exchange per hour after 72 h.

Outlets were fitted with 125-μm mesh screens to prevent the loss of larvae. Seawater temperature in the rearing containers was 21 ± 0.5°C. An artificial photoperiod (12 h light/12 h dark) was provided at a light intensity of 2,000 Lux and measured using a digital light meter (HT170N, HT ITALIA).

Larvae attached permanently to the substrate after complete metamorphosis (loss of the velum) were considered as settled larvae and were counted on every plate of each replicate ($n = 12$/treatment) under a dissecting microscope 48 h after larvae introduction to the containers. Plates were kept immersed in seawater during microscopic observation and were replaced immediately thereafter.

Postlarval Growth and Survival

Three days after the introduction of the larvae, and after larvae completed their metamorphosis, a mixture of 4 different diatoms (*Navicula incerta*, *Proschkinia* sp., *Nitzschia* sp., and *Amphora* sp.) were added as food to the 12-L containers. Postlarvae were subsequently fed weekly with 200-mL inoculums of the diatom mixture (10^6 cells/mL). Postlarvae growth and survival were monitored for 4 wk after settlement. The number of live postlarvae was counted on every settlement plate of each replicate ($n = 12$/treatment) under a dissecting microscope at weekly intervals. The shell length of 10 randomly selected postlarvae per replicate was measured weekly with a profile and measuring projector (model PJ-H3000; Mitutoyo, Japan). Plates were kept immersed at all times and were replaced immediately after observation and measurement.

Daily growth rate (DGR) was calculated according to the formula:

$$\frac{Lf - Li}{t}$$

where *Lf* is the final shell length measured in micrometers, *Li* is the initial shell length measured in micrometers, and *t* is time measured in days.

Analytical Methods

Triplicate samples of algae from each treatment were collected by scraping the surface of settlement plates at the start of the settlement experiment. The settlement plates used for the biochemical analysis of the substrates were colonized and cultured in the same conditions as the ones used for the study of larval settlement rate. The samples were cleaned, washed with freshwater to remove salts, and stored at –80°C prior to analysis for total lipids, protein, carbohydrate, ash, and total fatty acids. Before analysis, all samples where homogenized with mortar and pestle before being weighed for further analysis. Dry matter was calculated from weight loss after drying for 24 h at 105°C. Total protein was calculated from total Kjeldahl nitrogen according to AOAC (2005) standard methods. Ash content was determined gravimetrically after incinerating the samples at 600°C during 24 h. Total lipids were analyzed gravimetrically after extraction with chloroform–methanol (2:1) (Folch et al. 1957). Total carbohydrate content was calculated by difference. Fatty acids in the lipid extracts were ''transesterified'' to methyl esters with 1% sulfuric acid: methanol complex (Christie, 1982). Fatty acid methyl esters (FAME) samples were extracted into hexane and stored at –80°C. Fatty acids were analyzed in a Thermo Finnigan-GC Focus gas chromatograph equipped with a flame ionization detector (260°C). Fatty acid methyl esters were separated with a capillary column (Supercowax 28 m × 0.32 mm × 0.25 i.d.) using helium as the carrier gas under the conditions described by Izquierdo et al. (1989). Individual FAME were identified by reference to well-characterized external standards (Sigma), and the relative amount of each fatty acid was expressed as a percentage of the total amount of fatty acids in the analyzed sample.

Data Analysis

Statistical analysis was performed using the Statgraphics Plus 5.1 software. (Manugistics, Rockville, MD). Assumption of normality and homogeneity of variance were assessed with standardized skewness and kurtosis, and Bartlett's test. Analyses of variance (1-way ANOVAs) were performed to compare proximate biochemical composition as well as settlement, survival rate, and DGR among treatments. Data showing significant differences ($P < 0.05$) were analyzed by paired comparisons using Tukey's HSD test. Multiple regression analyses were carried out to explain the variation in settlement, growth, and survival among treatments. The proximate biochemical composition of the algal cues—proteins, lipids, ash, and carbohydrate contents—were the factors selected for the analyses. Principal component analysis was performed on the data of fatty acids composition of the algal cues, and principal components were identified. Kendall rank correlation between the main principal components and settlement, growth, and survival data was then performed to identify the effects of fatty acid composition and their variations among treatments.

RESULTS

Estimates of Algal Cover

The percent coverage of the different substrates, independent of age and algal species, ranged between 39.85 ± 7.43% and 46.67 ± 7.88% at the time of larval settlement. The differences in algal percent coverage among treatments were not significant ($F_{7,72} = 1.40$, $P = 0.22$).

Larval Settlement

The number of settled larvae was significantly higher on CCA and the combination of old *Ulvella lens* and *Ulva rigida* treatment ($F_{7,16} = 207.51$, $P < 0.001$; Fig. 1), whereas the latter did not induce significantly higher larval settlement than the old *U. rigida* treatment ($P > 0.05$). Forty-eight hours after the introduction of the larvae, an average settlement rate of 61 ± 6% was achieved with CCA (Fig. 1), followed by settlement rates of 52 ± 0.6% and 46±7% with the combination of old *U. lens* and *U. rigida* and old *U. rigida* treatments, respectively. In contrast, larval settlement rates on young *U. lens* and young *U. rigida* were not significantly different ($P > 0.05$), reaching 14 ± 2% and 10 ± 0.6%, respectively, and were significantly lower ($P < 0.01$) than the settlement rates induced by CCA, the combination of old *U. lens* and *U. rigida,* and old *U. rigida* treatments. Finally, the lowest larval settlement rates ($F_{7,16} = 207.51$, $P < 0.001$) were observed on young *U. lens* and *U. rigida* combination, old *U. lens* and enriched old *U. lens* treatments, with insignificantly different values ($P > 0.05$) of 3 ± 1.5%, 3 ± 0.6% and 4 ± 0.7% respectively (Fig. 1).

Postlarval Growth and Survival

After 4 wk of the experiment, the young and old combinations of *Ulvella lens* and *Ulva rigida* produced the largest postlarvae, with an average of 1,144 ± 113 μm and 1,249 ± 133 μm in shell length, respectively (Fig. 2).

Daily growth rates of postlarvae weekly fed 200 mL of 10^6 cells/mL diatom mix (*Navicula incerta*, *Proschkinia* sp., *Nitzschia* sp., and *Amphora* sp.) during the 4 wk were significantly higher

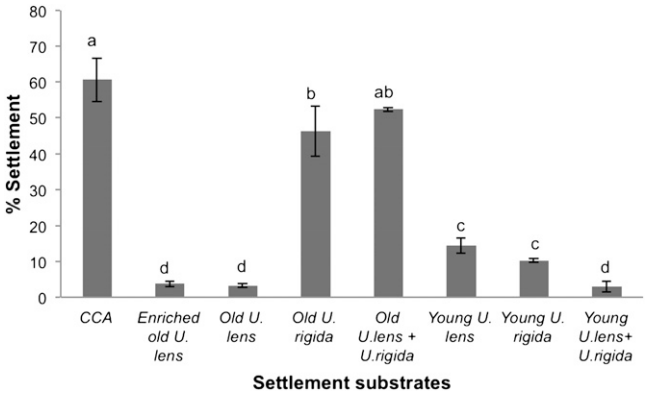

Figure 1. Percentage settlement of *Haliotis. tuberculata coccinea* after 48 h on crustose coralline algae (CCA), enriched old *Ulvella lens* (*U. lens*), old and young *U. lens,* old and young *Ulva rigida* (*U. rigida*), as well as old and young *U. lens* and *U. rigida*. Vertical bars indicate SD. Values with different letters are significantly different ($P < 0.05$, $n = 3$).

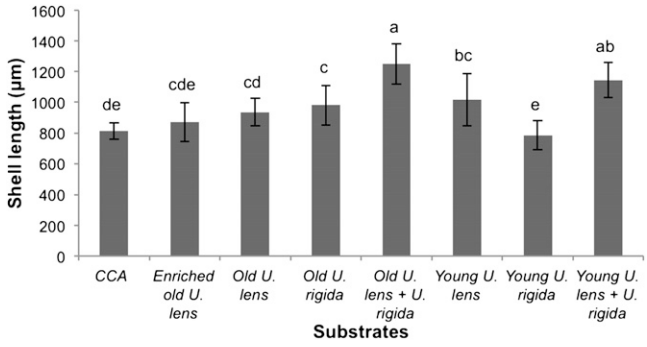

Figure 2. Early growth of *Haliotis tuberculata coccinea* postlarvae at 4 wk postsettlement on the 8 algal cues tested. Vertical bars indicate SD ($n = 10$). CCA, crustose coralline algae; *U. lens, Ulvella lens; U. rigida, Ulva rigida.*

on the young and old combinations of *Ulvella lens* and *Ulva rigida* ($F_{7,72} = 22.77$, $P < 0.001$). Postlarvae exhibited the lowest DGR on young *U. rigida* treatment with 23 ± 4 µm. This value was significantly lower than those observed for the young and old combinations of *U. lens* and *U. rigida* ($F_{7,72} = 22.77$, $P < 0.001$; Table 1).

Postlarval survival, 4 wk after settlement, was significantly higher ($F_{7,16} = 127.16$, $P < 0.001$) for the young combination of *Ulvella lens* and *Ulva rigida*, with 92 ± 7%. In contrast, survival rates were significantly lower ($F_{7,16} = 127.16$, $P < 0.001$) on old *U. lens,* young *U. rigida,* and enriched old *U. lens,* but not significantly different among the 3 substrates ($P > 0.05$; Table 1).

Biochemical Composition of Algae and Outcome on Settlement, Survival, and Growth

The proximate biochemical composition of the different settlement cues is shown in Table 2. Overall, CCA showed significantly the lowest protein ($F_{7,16} = 360.3$, $P < 0.001$) and lipid contents ($F_{7,16} = 146.87$, $P < 0.001$), whereas enriched old *Ulvella lens* had the highest protein content ($F_{7,16} = 360.3$, $P < 0.001$), as well as the highest lipid content together with old *U. lens*. Accordingly, CCA had the highest ash content whereas enriched and unenriched old *U. lens* had the lowest ash levels ($F_{7,16} = 670.83$, $P < 0.001$). The highest carbohydrate content was obtained in the combined old *U. lens* and *Ulva rigida* treatment, without significant differences from old *U. lens* or old *U. rigida* as opposed to the young *U. rigida* treatment, which showed a significantly lower carbohydrate content ($F_{7,16} = 227.91$, $P < 0.001$).

TABLE 1.

Mean daily growth rate (DGR) and survival rate of *Haliotis tuberculata coccinea* postlarvae on the 8 algal cues tested, ($n = 10$ and $n = 3$).

Treatment	Survival rate at week 4 (% ± SD)	DGR at week 4 (µm/day ± SD)
CCA	63 ± 0.8^{c}	25 ± 4.3^{de}
Enriched old *U. lens*	41 ± 3.5^{d}	27 ± 3.7^{cde}
Old *U. lens*	35 ± 2.4^{d}	30 ± 3.7^{cd}
Old *U. rigida*	68 ± 1.7^{bc}	32 ± 5.6^{c}
Old *U. lens* + *U. rigida*	76 ± 1.0^{b}	44 ± 5.5^{a}
Young *U. lens*	70 ± 0.7^{bc}	33 ± 4.5^{bc}
Young *U. rigida*	36 ± 3.3^{d}	23 ± 4.4^{e}
Young *U. lens* + *U. rigida*	92 ± 7^{a}	39 ± 4.8^{ab}

Different superscripts in each column indicate means that differ significantly at the 95% level (ANOVA, Tukey's test, $P < 0.05$). CCA, crustose coralline algae; *U. lens, Ulvella lens; U. rigida, Ulva rigida.*

The mean amounts of fatty acids for each algal cue tested are given in Table 3. The fatty acids found in highest proportion in all treatments were saturated fatty acid (particularly, 16:0 (palmitic acid)), monounsaturated fatty acids (including 16:1n-7 (palmitoleic acid), 18:1n-9 (oleic acid), 18:1n-7), and PUFAs 18:2n-6 (linoleic acid), 18:3n-3 (linolenic acid), 20:4n-6 (arachidonic acid (ARA)), 20:5n-3 (eicosapentaenoic acid (EPA)), and 22:6n-3 (docosahexaenoic acid (DHA)). The fatty acid profile varied among the treatments, highlighting the difference in the CCA treatment in comparison with the green algae by presenting the lowest amount of monounsaturated fatty acids—particularly, 18:1n-9 and 18:1n-7—and the highest amount of PUFAs, especially ARA and EPA. Old *Ulvella lens* and young *Ulva rigida* were the 2 green macroalgae treatments that presented the highest EPA content. The enriched old *U. lens* treatment presented the lowest amount of saturated fatty acid and DHA as well as the highest amount of 16:1n-7, 18:1n-7, and 16:4n-3. In general *U. rigida,* both at young and old ages, was higher than *U. lens* in n-6 fatty acid contents—particularly, ARA and linoleic acid. In both species, the older the algal culture the higher the ARA content and the lower the linoleic acid content. In contrast, nutrient enrichment of the culture medium increased the levels of linoleic acid in enriched old *U. lens* in comparison with old *U. lens,* but reduced other polyunsaturated fatty acid contents such as ARA, EPA, or DHA. Last, old *U. rigida* was also characterized by high 18:1n-9 and 22:1n-9 contents, which were also reflected in the fatty acid composition of the mixture of old *U. rigida* and old *U. lens*.

Figure 3 shows the results of the principal component analysis of the fatty acids composition from the different algal substrates. Based on the Eigenvalues, 7 components were extracted to describe the data. The first 2 components accounted for 50% of the total variance found among the different fatty acid composition of the algal cues, the first component being the most significant and explaining 33% of the total variance. Based on the first 2 components, Figure 3 shows that the fatty acids profiles of CCA, enriched old *Ulvella lens,* old *U. lens* in combination with old *Ulva rigida,* and old *U. lens* treatments are the furthest from each other. The first component presented the highest correlation level with the fatty acids 14:1n-7, 16:2n-6, 16:3n-1, 16:3n-3, 16:4n-3, 18:1n-7, 18:2n-6, 18:3n-3, 18:4n-3, ARA, and EPA, denoting that they were the ones likely to be involved in differentiating CCA from the enriched old *U. lens* treatment. The second component presented the highest correlation level with the fatty acids 18:1n-5, 18:1n-9, 18:2n-4, 18:2n-9, 20:1n-9 + n-7, 20:2n-9, 22:1n-9, 20:3n-6, and EPA, suggesting that they are likely to be involved in differentiating old *U. lens* in combination with old *U. rigida* from the old *U. lens* treatment.

The protein content of the algal substrate correlated negatively to the settlement rate (Fig. 4). The amount of protein was the only variable retained in the multiple regression model to explain the variation in settlement rates among the 8 experimental algal cues tested.

A Kendall rank correlation was carried out among larval settlement and the first components of the principal component analysis of the fatty acids (Fig. 3). The first component, differentiating

TABLE 2.

Proximate biochemical analysis (percent dry weight) of the 8 settlement substrates tested at the starting date of the settlement experiment (mean ± SD, $n = 3$).

Substrates	% Proteins (DW)	% Lipids (DW)	% Ash (DW)	% Carbohydrates (DW)
CCA	4.01 ± 0.27^{e}	0.30 ± 0.06^{e}	54.40 ± 0.86^{a}	41.29 ± 0.73^{c}
Enriched old *U. lens*	29.14 ± 0.92^{a}	7.71 ± 0.19^{a}	32.14 ± 0.16^{f}	31.00 ± 1.14^{e}
Old *U. lens*	14.97 ± 0.34^{c}	7.03 ± 0.67^{a}	32.85 ± 0.25^{f}	45.14 ± 0.30^{ab}
Old *U. rigida*	16.33 ± 0.76^{c}	4.35 ± 0.32^{c}	34.62 ± 0.41^{e}	44.70 ± 1.17^{ab}
Old *U. lens* + *U. rigida*	11 ± 0.63^{d}	5.79 ± 0.64^{b}	36.02 ± 0.42^{e}	47.18 ± 1.03^{a}
Young *U. lens*	10.70 ± 0.45^{d}	1.82 ± 0.28^{d}	43.43 ± 0.45^{d}	44.04 ± 1.18^{b}
Young *U. rigida*	26.32 ± 0.92^{b}	1.65 ± 0.15^{d}	48.53 ± 0.25^{c}	23.48 ± 0.92^{f}
Young *U. lens* + *U. rigida*	10.17 ± 1.32^{d}	1.56 ± 0.44^{d}	51.23 ± 1.17^{b}	37.04 ± 0.73^{d}

Different superscripts in each column indicate means that differ significantly from others at the 95% level (ANOVA, Tukey's test, $P < 0.05$). CCA, crustose coralline algae; DW, dry weight; *U. lens, Ulvella lens; U. rigida, Ulva rigida*.

CCA from enriched old *Ulvella lens*, was significantly correlated ($P < 0.05$) with settlement, indicating that composition of the substrate in the fatty acids 14:1n-7, 16:2n-6, 16:3n-1, 16:3n-3, 16:4n-3, 18:1n-7, 18:2n-6, 18:3n-3, 18:4n-3, ARA, and EPA could play a role in the settlement process.

Postlarvae survival rate correlated negatively to the protein content of the algal substrate (Fig. 5). Algal cues' protein content was retained as the parameter, from the model, accountable for survival rates' variations between the 8 experimental algal cues tested due to its elevated correlation with larval survival. The relationship between protein content and postlarvae survival rate was found to be significant (adjusted $R^2 = 0.39$, $F = 16.04$, $P < 0.01$).

The Kendall rank correlation carried out between growth and survival and the first components of the principal component analysis of the cues fatty acid composition showed that component 2 had a significant correlation ($P < 0.05$) with survival, reflecting the fact that survival could depend principally on the following fatty acid profile: 18:1n-5, 18:1n-9, 18:2n-4, 18:2n-9, 20:1n-9 + n-7, 20:2n-9, 22:1n-9, 20:3n-6, and EPA.

Growth was not correlated with the proximate composition of the substrates and was only moderately correlated with components 1 and 2, indicating that the proximate or fatty acid composition of the algal substrate does affect postlarval growth significantly.

DISCUSSION

The current study on *Haliotis tuberculata coccinea* showed the good potential of the germlings of *Ulva rigida* for settlement induction, and it investigated links among settlement attractiveness of the substrate, and postlarval growth and survival.

Larval Settlement

Larval settlement of *Haliotis tuberculata coccinea* was the highest on CCA, as shown in previous studies (Courtois de Viçose et al. 2010) and in many other abalones species (Daume et al. 1999, Daume et al. 2000). Larvae settled particularly well on plates colonized with old *Ulva rigida*, and with a mixture of *Ulvella lens* and *U. rigida* germlings grown for 45 days. Despite the fact that Huggett et al. (2005) reported that *Haliotis rubra* settle on *Ulva australis* and *Ulva compressa* plants, the current study reports for the first time that *H. tuberculata coccinea* exhibit a strong settlement response to germlings of *U. rigida,* which are to be considered settlement inducers of high potential.

In the current study, young *Ulvella lens* and young *Ulva rigida* treatments presented a high number of small individual patches, whereas the corresponding older treatments presented fewer larger patches. Although differences in algal developmental stages were observed among age-differentiated treatments, as reported in other studies (Daume et al. 2004), the algal percent coverage was similar in both young and old treatments. Despite the similarity in algal percentage coverage of all treatments, settlement rates of *Haliotis tuberculata coccinea* were significantly different among treatments and therefore illustrate the statement of Daume and Ryan (2004), suggesting that the developmental stage and maturity of *U. lens* is more important for settlement induction than its percent coverage. We could expect a similar algal developmental stage effect of *U. rigida* based on the higher settlement rates recorded on old *U. rigida* than on young *U. rigida* in the current study. Apart from algal development stage, additional factors could be involved in larval settlement by green macroalgae germlings. Effectively, a 14% settlement rate was recorded on young *U. lens* for *Haliotis laevigata* (Daume & Ryan 2004) and *H. tuberculata coccinea* in the current study, independent of the abalone species tested and age of *U. lens*. On the contrary, the settlement of *H. tuberculata coccinea* was lower on old *U. lens* than on young *U. lens,* in contrast to the settlement results obtained with *H. laevigata* (Daume & Ryan 2004). This suggests that settlement differences could also depend on algae species specificity, their surface texture or topography, and their proximate biochemical composition known to be affected by age and culture conditions of the algal substrate.

The result of the multiple regression analysis of the settlement suggested that the protein content of the substrate may explain some of the variation in settlement rates, possibly associated with a variation in the amino acids content and their proportions. Amino acids have been recognized as strong feed attractants that facilitate fish larvae's food particle recognition (Kolkovski et al. 2009) and were found to affect growth and survival of *Haliotis rubra* juveniles because imbalanced proportions of some amino acids, in algal diets, could be limiting (Daume et al. 2003). Variation in amino acid content could also be related to peptide molecules found to be involved in settlement induction and metamorphosis regulation in reef-building corals (Iwao et al. 2002, Erwin & Szmant 2010) and proposed as

TABLE 3.

Fatty acid composition (percent total fatty acid) in the 8 settlement substrates tested at the starting date of the settlement experiments.

Fatty acids		CCA	Enriched old *U. lens*	Old *U. lens*	Old *U. rigida*	Old *U. lens* + *U. rigida*	Young *U. lens*	Young *U. rigida*	Young *U. lens* + *U. rigida*
Palmitic	14:0	0.88	0.96	2.42	0.92	2.24	3.50	2.57	4.55
	15:0	0.40	0.25	0.30	0.18	0.20	0.53	0.32	0.76
	16:0	24.33	19.35	29.05	31.30	30.16	33.95	21.48	34.03
	16:0ISO	0.04	0.28	0.23	0.22	0.05	0.03	0.04	0.08
	17:0	0.24	0.32	0.21	0.38	0.13	0.45	0.54	0.28
Stearic	18:0	2.98	0.75	1.49	1.37	1.25	1.83	2.69	1.99
	20:0	0.39	0.14	0.27	0.13	0.22	0.49	0.27	0.40
∑Saturated fatty acids		29.26	22.06	33.98	34.48	34.26	40.77	27.92	42.09
	14:1n-7	0.13	1.74	0.57	0.63	0.30	0.88	0.68	0.56
	14:1n-5	0.34	0.47	0.48	0.18	0.10	0.19	0.19	0.73
	15:1n-5	0.09	0.03	0.01	0.01	0.01	0.10	0.07	0.09
Palmitoleic	16:1n-7	2.56	16.60	4.63	6.16	5.14	3.08	7.37	2.42
	16:1n-5	0.23	0.67	0.79	0.49	0.23	0.37	0.46	0.39
Oleic	18:1n-9	4.79	8.67	18.31	21.91	27.25	15.51	9.69	18.48
	18:1n-7	2.58	7.68	7.09	5.99	3.81	5.18	6.06	4.56
	18:1n-5	0.25	0.25	0.23	0.14	0.12	0.17	0.21	0.21
	20:1n-9 + n-7	0.73	0.65	0.48	0.97	1.15	0.71	0.81	0.81
	20:1n-5	0.45	0.13	0.14	0.28	0.34	0.08	0.34	0.10
	22:1n-11	0.32	0.17	0.30	0.52	0.20	0.23	0.22	0.29
	22:1n-9	0.72	0.07	0.07	0.53	1.45	0.24	0.29	0.72
∑Monounsaturated fatty acids		13.19	37.13	33.10	37.80	40.11	26.74	26.41	29.36
Linoleic	16:2n-6	0.00	1.81	0.85	0.98	0.85	0.71	0.92	0.55
	16:2n-4	0.24	0.11	0.24	0.15	0.08	0.39	0.36	0.37
	16:3n-4	0.13	0.74	0.45	0.55	0.50	0.76	1.11	0.51
	16:3n-3	0.15	0.00	0.00	0.05	0.00	0.00	0.00	0.00
	16:3n-1	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	16:4n-3	0.00	6.84	1.54	2.62	2.05	3.04	4.21	2.48
	16:4n-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	18:2n-9	0.09	0.08	0.41	0.00	0.03	0.00	0.05	0.08
	18:2n-6	5.68	9.24	6.13	6.96	6.56	7.13	9.26	7.49
	18:2n-4	0.04	0.03	0.09	0.03	0.00	0.00	0.06	0.05
	18:3n-6	0.04	0.97	0.34	0.22	0.37	0.67	0.35	0.58
	18:3n-4	0.11	0.00	0.00	0.00	0.00	0.03	0.04	0.03
Linolenic	18:3n-3	0.68	9.57	5.44	6.99	7.70	10.38	10.73	5.42
	18:3n-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	18:4n-3	0.08	2.99	1.88	0.98	0.96	1.39	4.02	0.97
	18:4n-1	0.09	0.00	0.00	0.01	0.00	0.00	0.05	0.03
	20:2n-9	0.00	0.06	0.01	0.02	0.31	0.06	0.00	0.06
	20:2n-6	1.00	0.29	0.43	0.83	0.17	0.26	0.42	0.18
	20:3n-9	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	20:3n-6	0.21	0.13	0.72	0.22	0.06	0.12	0.16	0.18
	20:3n-3	0.00	0.18	0.04	0.31	0.30	0.22	0.38	0.18
ARA	20:4n-6	30.80	2.19	2.55	3.18	0.92	1.25	2.00	1.34
	20:4n-3	0.04	0.16	0.56	0.15	0.12	0.18	0.38	0.24
EPA	20:5n-3	15.53	3.99	6.91	2.06	1.50	3.63	6.11	3.88
	22:4n-6	0.15	0.05	0.05	0.08	0.17	0.02	0.06	0.04
	22:5n-6	0.10	0.44	1.33	0.15	0.38	0.49	0.51	0.36
	22:5n-3	0.18	0.11	0.29	0.07	0.23	0.25	0.94	0.82
DHA	22:6n-3	2.14	0.81	2.64	1.09	2.37	1.51	3.54	2.71
∑Polyunsaturated fatty acids		57.55	40.82	32.92	27.72	25.63	32.49	45.67	28.55

ARA, arachidonic acid; CCA, crustose coralline algae; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; *U. lens, Ulvella lens; U. rigida, Ulva rigida.*

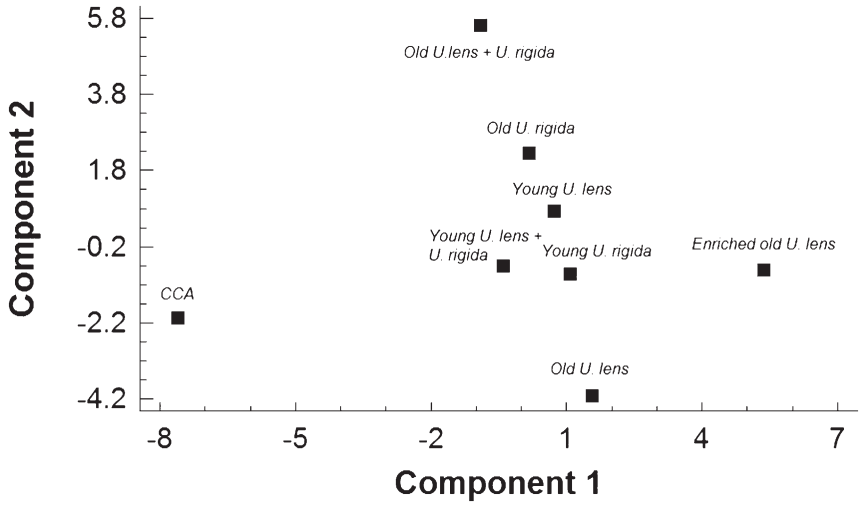

Figure 3. Graphic representation of the principal component analysis carried out on the fatty acid profiles of the different algal treatments used as settlement substrates. *U. lens, Ulvella lens; U. rigida, Ulva rigida.*

possible chemical cues for abalone larval settlement (Morse & Morse 1984, Morse 1985). Settlement rates on old and old enriched *Ulvella lens* (3% and 4%, respectively) were lower compared with those of 20%, 40%, and 61% reported for *H. rubra* and *Haliotis laevigata* in other studies (Daume et al. 2000, Daume & Ryan 2004, Daume et al. 2004). Both old and old enriched *U. lens* presented the highest lipid content, the latter being thought to be detrimental for abalone growth (Britz & Hecht 1997), and variations in a substrate's lipid content also affects its fatty acid profile. A well-balanced biochemical composition, rather than high individual components contents, seems to be necessary to obtain good settlement rates, as suggested for older abalone stages (Strain et al. 2006).

Fatty acids play an important role as membrane constituents (Jensen et al. 1990) and were reported to play a role in induction of larval metamorphosis by disturbing the cell membranes. In the current study, the algal substrates were differentiated by their fatty acid composition, and only a few fatty acids were correlated to the settlement process. Studies on sea urchin settlement have isolated fatty acids from coralline red algae and *Ulvella lens* responsible for settlement induction and highlighted the positive effect of certain fatty acids (Kitamura et al. 1993, Takahashi et al. 2002). In the current study, 18:1n-7, 18:2n-6, 16:4n-3, 18:3n-3, ARA, and EPA contents in CCA, *U. lens* and *Ulva rigida* correlated positively with settlement and were common to the ones identified by Kitamura et al. (1993) and Takahashi et al. (2002) for settlement induction of sea urchin. Particularly, ARA was highest in CCA and old *U. rigida,* leading to the highest settlement rates, whereas it was lower in young *U. rigida*, leading to lower settlement rates.

This study showed that *Haliotis tuberculata coccinea* larvae may distinguish between algal substrates, their different developmental stages, and biochemical composition, and that germlings of the green algae *Ulva rigida* are suitable for improving larval settlement.

Postlarval Growth and Survival

Settlement-inducing substrates were also considered for their effects on early postlarval growth and survival. Postlarval growth was not correlated to the proximate biochemical composition of the substrate or its fatty acid composition, which could be explained by the fact that at 4 wk postsettlement, postlarvae growth is dependent primarily on diatom ingestion and it is likely that postlarvae are not yet capable of accessing larger particles, such as macroalgae germlings, from the substrate

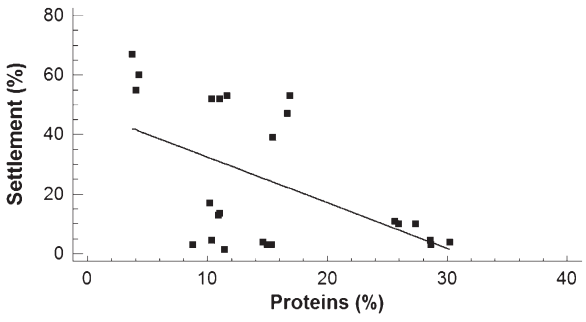

Figure 4. Relationship between protein content of algal substrate and settlement rates on the 8 experimental substrates tested ($n = 24$). The regression is significant (adjusted $R^2 = 0.25$, $F = 8.62$, $df = 1$, $P < 0.01$).

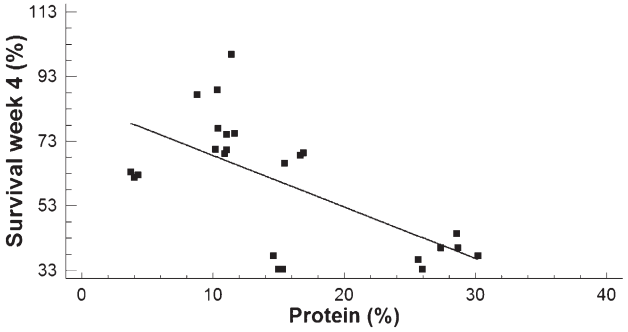

Figure 5. Parameters affecting postlarvae survival during the first 4 wk postsettlement for the 8 experimental substrates tested. Relationship between protein content of algal substrates and survival rates ($n = 24$; $R^2 = 0.39$, $df = 1$, $F = 16.04$, $P < 0.01$).

as food source. Daume (2006) stated that *Ulvella lens* is not ingested effectively by abalone with a shell length smaller than 3 mm; this could also be applied to *Ulva rigida*. Daume et al. (2000) showed that the green alga *U. lens* is not nutritionally adequate for the initial growth of *Haliotis rubra* postlarvae, with a growth rate of 13 μm/day, and that it was necessary to enhance *U. lens* with diatoms to sustain postlarval growth. Growth rates obtained for *U. lens* and *U. rigida* in the current study are within the range of those reported on *U. lens* supplemented with diatoms by Daume and Ryan (2004) and Daume et al. (2004) (26–40 μm/day). Growth rates are also in agreement with the results obtained by Daume et al. (2000) (27–39 μm/day) and Gordon et al. (2006) (20–35 μm/day), and within the range of those (25–37 μm/day) reported by Kawamura and Takami (1995) and Daume et al. (1999) for postlarvae feeding on diatoms. As suggested earlier, these results indicate that postlarval growth, in this experiment, was sustained by diatoms and not by the macroalgal substrate; therefore the significant growth differences observed among treatments were linked to the effects of the settlement substrate on diatoms ingested by the postlarvae.

Daume et al. (2004) demonstrated a strong negative correlation between the percent coverage of *Ulvella lens* and the diatom density and associated biofilm on settlement plates as older plates, presenting a lower *U. lens*, showed higher diatoms densities resulting from the competition for space between the algae.

Taking into account that postlarvae of all treatments were reared in the same conditions, fed an identical mixture of 4 diatoms weekly, and that all the substrates tested presented a similar percent algal coverage, the differences in postlarval growth observed among algal substrates are unlikely to be attributable to space availability for diatoms to grow or original number of diatoms cells inoculated in each treatment. The differences in postlarval growth among treatments are therefore suggested to be influenced by the macroalgal substrate itself and linked to its effect on the extracellular polysaccharide secreted by the diatoms or on associated bacteria known to influence diatom growth (Holmstrom et al. 1996) and nutritional value (Kawamura 1996, Roberts et al. 1999, Daume 2006).

Protein content and various identified fatty acids involved in the differentiation of the different algal substrates were correlated to *Haliotis tuberculata coccinea* postlarval survival, as observed for settlement rates. Their effect on postlarval survival could have been the result of the recognized antialgal or antibacterial properties of peptides and fatty acids (Alamsjah et al. 2008, Dorrington & Gomez-Chiarri 2008, Desbois & Smith 2010). These properties possibly affected diatom growth and reduced exposure of postlarvae to pathogens or bacterial infections, as was observed for *Artemia nauplii* (Defoirdt et al. 2006), and therefore influenced the survival of *H. tuberculata coccinea* postlarvae. The fatty acids correlated with survival were different from the those associated with settlement, with the exception of EPA. This PUFA is correlated with macroalgae food value, therefore promoting growth in *Haliotis discus hannai* and *H. tuberculata* juveniles (Mai et al. 1995, Dunstan et al. 1996, Mai et al. 1996), and was found to accumulate in abalone foot muscle independently of species, age, and diet (Dunstan et al. 1996). Other identified fatty acids, such as 18:2n-9 and 20:1n-9+1n-7, may have been used as energy sources and consequently affected survival.

It would of interest to investigate further the effect of fatty acids from green algae germlings on abalone settlement and to evaluate their nutritional impact on postlarval growth and survival because they are among the best algal diets identified for several abalone species (Mai et al. 1996, Daume et al. 2004, Strain et al. 2006).

Biochemical Composition of Algae

Algae proximate biochemical and fatty acid compositions differed among species, culture conditions, and algal developmental stages, which is in agreement with previous studies (Thompson et al. 1993, Fabregas et al. 1996, Shpigel et al. 2000). The differences in proximate biochemical composition of *Ulvella lens* and *Ulva rigida* treatments observed here provide a good illustration of this phenomenon. Protein content of both old and young *U. lens* was lower than that observed for the same species (34.3%) by Daume and Ryan (2004), whereas lipid content was higher in old *U. lens* (7%) in the current study. These differences can be related to variations in the culture conditions between both studies or in the algal developmental stage, because in our study older algal cultures were associated with increased lipid and reduced ash content. The increased protein and lipid contents observed for enriched old *U. lens* (29.1% and 7.7%, respectively) compared with the unenriched old *U. lens* (14.9% and 7%, respectively) demonstrate the effects of the culture conditions on the algal biochemical composition. The alga *U. rigida* presented higher protein content than *U. lens* in similar growing conditions as well as lower protein and lipid content than those reported by Strain et al. (2006) for other *Ulva* species (32.3% and 7.1%, respectively). Such differences in proximate biochemical composition could be linked to species specificity, genetic differences, or differences in environmental conditions.

CONCLUSIONS

The effects of germlings of the green macroalgae *Ulva rigida* on settlement, growth, and survival of *Haliotis tuberculata coccinea* were evaluated for the first time. Larvae showed high settlement rates on CCA, on a combination of mature green algae *Ulvella lens* and *U. rigida* as well as on mature *U. rigida* alone. Postlarval growth rates were also the highest on a combination of mature *U. lens* and *U. rigida*. A dual combination of green algal cues was beneficial for *H. tuberculata coccinea* larval settlement, growth, and survival. The study also showed that the biochemical composition of the algal substrates is affected by developmental stages, as well as the culture conditions, of the algae and that the biochemical composition is likely to impact postlarval settlement and survival.

These results suggest that green macroalgae play an important role in the early life of abalone, and a better understanding of their role is required because it could have an implication in the recruitment of abalone larvae to benthic populations and it could improve abalone culture performance. Ulvaceae species present great potential because they are ubiquitous, fast growing, and easily cultured, in contrast to the slower growing CCA.

Further research investigating development techniques for Ulvaceae spores culture and green algae may provide strong settlement cues for larvae, influence diatom characteristics positively to sustain early postlarval growth, and provide a

diet for juvenile abalone in hatcheries, such as allowing the development of reliable applicable techniques for *Haliotis* spp. settlement and nursery rearing. Such advances could, consequently, improve the sustainability of abalone postlarvae production.

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