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Development of reproductive structures in the introduced green alga, *Codium fragile* ssp. *tomentosoides*, in the northern Adriatic Sea

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Biological invasions are among the most important drivers of global change. The introduced green alga, *Codium fragile* ssp. *tomentosoides*, has become an important component of low-shore assemblages on hard coastal defence structures along the north-west coast of the Adriatic Sea. We investigated the temporal dynamics, the spatial scales of variation and the effects of wave exposure on the abundance of reproductive structures of *C. fragile*. Furthermore, we assessed whether the reproductive output of *C. fragile* was correlated with the density of thalli. On the landward side of breakwaters, gametangia were found on thalli of *C. fragile* from June to September in both 2003 and 2004. Although there was some variation among breakwaters, the abundance of gametangia of *C. fragile* was greater on the landward than on the seaward side of breakwaters. Scales of variation in the number of gametangia and density of thalli of *C. fragile* were similar, with most of the variability occurring at a scale of tens of cm. Little variation emerged at larger (tens or hundreds of m) or smaller scales (within thalli, cm). The results of this study indicate that the provision of sheltered rocky habitats has been crucial for the establishment of *C. fragile* in the northern Adriatic Sea, enabling the alga to grow and reproduce. Processes operating at small spatial scales are responsible for patterns of variation in the number of gametangia and density of thalli of *C. fragile*. The tendency for a positive correlation between density of thalli and reproductive output in *C. fragile* in August suggests that greater densities of plants could ameliorate harsh environmental conditions during daytime low tides, fostering reproduction.

Key words: artificial habitats, *Codium fragile* ssp. *tomentosoides*, density-dependent regulation, invasive species, reproduction, spatial variation, wave exposure

Introduction

Biological invasions are acknowledged to be among the most severe threats to biological diversity in terrestrial and marine environments (Kareiva, 1996; Williamson, 1996; Grosholz, 2002). Exotic species can dramatically alter the functioning of recipient ecosystems, with repercussions on their ability to provide goods and services to humans (Williamson, 1996; Pimentel *et al.*, 2000). Along with the physical and biological attributes of recipient environments, life-history traits of the invader have been indicated as key determinants of successful invasions (Ceccherelli & Cinelli, 1999; Byers, 2002; Kennedy *et al.*, 2002; Stachowicz *et al.*, 2002).

The siphonaceous green alga, *Codium fragile* (Sur.) Hariot ssp. *tomentosoides* (van Goor) Silva,

has become an important component of intertidal and shallow subtidal rocky assemblages of many temperate seashores in the northern and southern hemisphere (Trowbridge, 1998; Campbell, 1999; Chapman *et al.*, 2002; Castilla *et al.*, 2005). The extensive coastal urbanization and related construction of hard human-made structures (e.g. breakwaters, jetties, groynes) has enabled *C. fragile* ssp. *tomentosoides* (hereafter referred to as *C. fragile*) to establish viable populations also along sandy coasts of the north Adriatic Sea (Bulleri & Airoldi, 2005; Bulleri *et al.*, 2006). The distribution and dynamics of *C. fragile* and the mechanisms underlying its establishment (i.e. interactions with the native mussel, *Mytilus galloprovincialis*) in this area have been described by Bulleri & Airoldi (2005). These authors have shown that thalli of *C. fragile* are not perennial and are generally found on breakwaters from early spring (March) to autumn (October, November), although resting stages of the alga, such as

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perennial holdfasts or filamentous forms (vaucheri-ioid stage) are likely to persist throughout the year. Wave-sheltered sides of breakwaters provide highly suitable habitats for *C. fragile*, resulting in the formation of a lush canopy at low-shore levels during summer months. In contrast, on the seaward side of breakwaters, thalli of *C. fragile* are readily dislodged by wave action when their size increases and only few scattered thalli are generally found in late summer (Bulleri & Airoldi, 2005).

Vegetative propagation, through fragments of the plant, single utricules, vegetative buds or medullary filaments (Borden & Stain, 1969), has been suggested to facilitate the long-distance dispersal of *C. fragile*. Although gametangia containing two types of biflagellate cells of different sizes have been reported for *C. fragile*, the existence of functional male gametes is controversial (Prince & LeBlanc, 1997) and repro-duction in *C. fragile* appears to be exclusively parthenogenetic (Trowbridge, 1998; Prince & Trowbridge, 2004). At a local scale, partheno-genetic reproduction could increase chances of establishing viable populations, even when at relatively low densities (Trowbridge, 1998). The number of reproductive structures on plants of *C. fragile* and the timing of their formation exhibit large seasonal fluctuations in some popula-tions (Churchill & Moeller, 1972). So far, however, there is no information about spatial variation in the abundance of reproductive structures of *C. fragile* and whether their development is affected by wave exposure.

Knowledge of the scales over which repeatable patterns are observed provides a focus for the scales at which ecological processes can be expected to differ. Understanding the scales of variation in the reproductive output of introduced species becomes critical when attempting to identify the mechanisms governing their establish-ment and spread, or to predict localities susceptible to invasions. Several authors (Underwood & Chapman, 1998; Menconi *et al.*, 1999; Benedetti-Cecchi, 2001; Fraschetti *et al.*, 2005) have shown great variation in patterns of distribution and abundance of intertidal organisms at small spatial scales, suggesting that processes operating at these scales (e.g. settlement, recruitment, competition, grazing) can be more important than those operating at larger scales. Comparable small-scale variation in patterns of abundance, growth and recruitment of *C. fragile* has been documented on coastal defence structures (Bacchiocchi & Airoldi, 2003; Bulleri & Airoldi, 2005). The processes underlying these patterns may also influence the scales of variation in the development of repro-ductive structures of *C. fragile*.

The aim of this study was to analyse the temporal dynamics, the spatial scales of variation and the effects of wave exposure on the abundance of reproductive structures of *C. fragile* in the northern Adriatic Sea. Specifically, we tested the hypotheses that the development of reproductive structures occurred during summer months, that their abundance was greater on the sheltered (landward) than on the exposed (seaward) side of breakwaters and that most of the variation in their abundance occurred at a small scale. Finally, since understanding population dynamics of invasive species can enhance our confidence in predicting the outcome of future invasions, we assessed whether the reproductive output of *C. fragile* was regulated by density-dependent mechanisms. Several studies have shown that the density of plants can have important ecological consequences on the survival, growth and reproductive output of individuals (Antonovics & Levin, 1980; Schiel & Choat, 1980; Rice, 1990; Viejo & Åberg, 2001). Since studies in intertidal habitats (Schiel & Choat, 1980; Schiel, 1985; Bertness & Leonard, 1997) suggest that crowding can enhance the reproduc-tive output of plants by ameliorating physical conditions (e.g. desiccation, heat), we predicted a positive relationship between the density of thalli and the abundance of reproductive structures.

Materials and methods

Study system

The north-western Adriatic Sea is a flat alluvial system, extending almost uninterrupted for about 300 km and characterized by, in comparison to other regions of the Mediterranean, a large tidal amplitude (about 1 m). Severe erosion has led to the proliferation of hard coastal defence structures, resulting in the reinforcement of about 60% of the coast. Further details about the study area can be found in Bacchiocchi & Airoldi (2003) and Airoldi *et al.* (2005). This study was carried out in Cesenatico (44°11′N; 12°24′E). Here, breakwaters are built with quarried blocks, about 1–3 m in diameter, deployed on a sandy bottom at a distance of about 220 m from the shoreline. These breakwaters, about 100 m in length and separated by gaps of about 20 m, run parallel to the shoreline and thus provide both wave-exposed (seaward side) and wave-sheltered (landward side) substrata. Extending about 2–3 m above and below the mean level of low water, breakwaters provide both intertidal and subtidal habitats for marine life.

Spatial and temporal patterns

Temporal and spatial patterns in the number of reproductive structures of *C. fragile* were investigated on the landward side of breakwaters, where the alga generally attains large densities (Bulleri & Airoldi, 2005). Three randomly chosen breakwaters (hundreds of m

apart) were sampled at monthly intervals from May to September. Sampling was repeated in both 2003 and 2004. Each time, 10 thalli of *C. fragile*, each representing one individual and up to 50 cm apart, were collected at random from each of three randomly chosen blocks, tens of m apart. Thalli were brought to the laboratory and preserved in 5% neutral formalin in seawater for subsequent analysis of reproductive structures. For each thallus, three 5-cm-long apical sections selected at random (cm apart) were cut from the branches (lateral branches were removed along these sections when necessary). The number of reproductive structures per section (hereafter referred to as gametangia) was determined with a slight modification of the method described by Churchill & Moeller (1972). Each apical section was homogenized in 50 ml of water for 25 s in a blender. Homogenization did not seem to damage or reduce the number of recognizable reproductive organs significantly (see also Churchill & Moeller, 1972). The homogenate was then stirred on a magnetic stirring plate and the number of gametangia in a 1-ml fraction was quantified in a counting chamber, under a dissecting microscope at 40× magnification. Because of differences in the diameter of thalli, the wet weight of apical sections, measured with a precision Ohaus scale (model: Galaxy TM100) after blotting off excess water with absorbent paper, varied between 0.16 and 0.64 g. The abundance of gametangia was, therefore, expressed per unit weight rather than length of apical sections.

Data were analysed by means of a mixed model ANOVA, with the following factors: Year (random), Date (random and nested within year), Breakwater (random and crossed), Block (random and nested within Date (Year) × Breakwater) and Thallus (random and nested within Block (Date (Year)) × Breakwater). Heterogeneity of variances before ANOVAs was checked by means of Cochran's test and data were appropriately transformed when necessary (Underwood, 1997).

Relationships with wave exposure

Relationships between the number of gametangia and wave exposure were analysed in September 2004 by comparing the number of gametangia on thalli of *C. fragile* collected from the landward (sheltered) and the seaward (exposed) sides of each of the three breakwaters. Although by late summer, *C. fragile* had virtually disappeared from the seaward sides (Bulleri & Airoldi, 2005), breakwaters were thoroughly searched in September 2004 for any remaining thalli. A minimum of nine thalli was found on the seaward side of the breakwaters. The number of gametangia was determined for each of five apical sections per thallus as described previously. Data were analysed by means of a mixed model ANOVA, including the factors: Exposure (fixed), Breakwater (random and crossed) and Thallus (random and nested within the interaction Exposure × Breakwater). The SNK test was used for *a posteriori* comparisons (Winer *et al.*, 1991).

Scales of spatial variation

In order to test models concerning relevant scales of variation in the distribution of gametangia, we compared estimates of spatial variability on the landward side of breakwaters at increasing spatial scales: (i) cm, corresponding to the distance among apices within each thallus; (ii) tens of cm, corresponding to the distance among thalli within each block; (iii) tens of m, corresponding to the distance among blocks within each breakwater; and (iv) hundreds of m, corresponding to the distance among breakwaters. Estimates of variation were obtained by running a 3-way ANOVA on number of gametangia independently for each date of sampling (except the first one in both 2003 and 2004 when no gametangia were found) and extracting the relevant components of variance from the observed mean square for each factor. This was done by equating the observed mean squares to the expected mean square derived from the linear model of the analysis (Winer *et al.*, 1991; Underwood, 1997). This procedure resulted in eight replicate estimates of variation for each of the scales investigated. Occasionally, negative estimates of variance were obtained. In these cases, variances were set to zero, assuming that they were sample under-estimates of small or zero variances (Searle *et al.*, 1992; Underwood, 1996).

Density data (number of thalli per 400 cm^2) were collected at the same sampling times and from the same breakwaters and blocks of this study by using quadrats of 20 × 20 cm (these density data are reported in Bulleri & Airoldi, 2005). Estimates of variance in the density of thalli of *C. fragile* within blocks, among blocks and among breakwaters were calculated with the same procedure described previously and compared visually to estimates of variance in the number of gametangia.

Relationships between number of gametangia and density of thalli

We tested the null hypothesis of no correlation between the density of thalli and the number of gametangia of *C. fragile*. The average densities of thalli and numbers of gametangia per block were calculated and used as input to the analyses. Data from sampling times within the same month (July, August and September) were merged between years 2003 and 2004 to increase the number of points in the correlation; data from June were not analysed because numbers of gametangia were still very small at that time of the year.

Results

Gametangia of *C. fragile* were found on the landward side of breakwaters from June to September in both 2003 and 2004 (Fig. 1). Numbers of gametangia were generally small at the beginning of the season (June) and increased in the following months, but trajectories through time varied greatly among breakwaters (Table 1) and there was no clear seasonal peak (Fig. 1).

The analysis also showed a large variability among both blocks and thalli (Table 1).

Numbers of gametangia differed between the landward and seaward sides of breakwaters in September 2004, but there was significant variation among breakwaters (Fig. 2, Table 2). On two breakwaters, there were significantly larger numbers of gametangia on the landward than on the seaward sides, but no difference was found on the third breakwater (Fig. 2, Table 2).

Variability in the number of gametangia and density of thalli of *C. fragile* occurred at the same spatial scales (Fig. 3). Most of the variation occurred within blocks, at a scale of tens of cm (67.70% for the number of gametangia and 71.95% for density of thalli), while variation at larger or smaller spatial scales was generally very small (Fig. 3).

In August, there was a significant positive correlation between number of gametangia and density of thalli of *C. fragile* (Fig. 4).
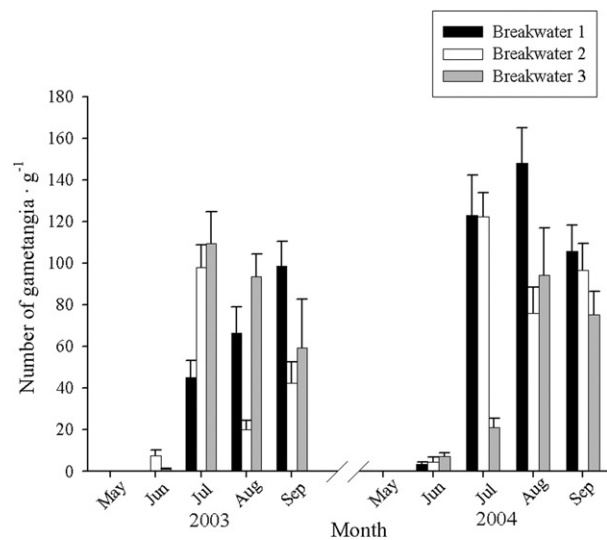
This correlation was not significant after two outliers were eliminated from the analysis ($r = 0.24$; $p = 0.40$), but a trend for a positive relationship between these variables was still evident. A similar trend emerged in July, but the correlation was weaker and not significant. Finally, there was no relationship between the two variables in September, when densities of thalli did not exceed five plants per $400\,cm^2$.

Discussion

In sheltered habitats provided by breakwaters, gametangia of *C. fragile* started developing in June and were found in relatively large numbers from late July to September, when the density of erect thalli generally decreased because of breakage by storms (Bulleri & Airoldi, 2005). Temporal dynamics in the abundance of gametangia differed among breakwaters and there was not a clear peak. Several factors are known to control the
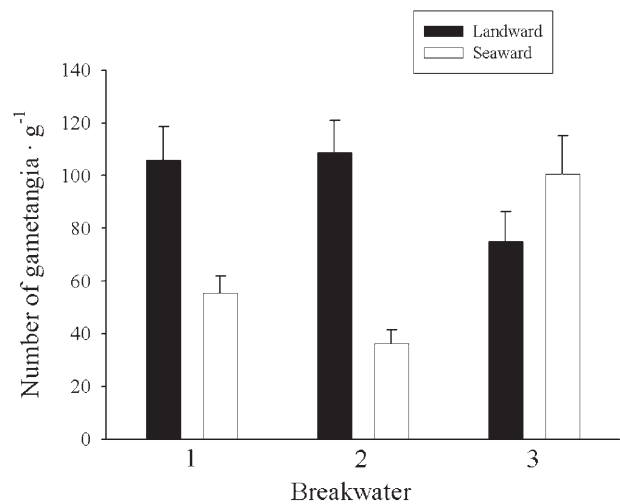

Fig. 1. Number of gametangia per g of tissue on the landward side of each of three breakwaters from May to September of 2003 and 2004. Data are means $\pm$ SE ($n = 27$).

Fig. 2. Number of gametangia per g of tissue on the landward and on the seaward side of each of three breakwaters in September 2004. Data are means $\pm$ SE ($n = 9$).

Table 1. Analysis of the effect of Year, Date, Breakwater, Block and Thallus on the number of gametangia per g of tissue in *C. fragile* ssp. *tomentosoides* on the landward side of breakwaters. Pooling procedures were applied according to Winer *et al.* (1991).

Source of variation	df	MS	F	p	Denominator
Year = Y	1	51.69	0.30	> 0.05	D(Y)
Date(Year) = D(Y)	6	171.71	7.63	< 0.01	D(Y) × B
Breakwater = B	2	17.39	0.77	> 0.05	D(Y) × B
Y × B	2	6.52	Eliminated	–	–
D(Y) × B	12	22.50	2.32	< 0.05	Block (D(Y) × B)
Block(D(Y) × B)	48	9.68	1.94	< 0.01	Thallus (Block (D(Y) × B))
Thallus(Block(D(Y) × B))	144	5.00	29.84	< 0.001	Residual
Residual	432	0.17			

Transformation: Ln($x + 1$); Cochran's test: $p > 0.05$.

development of propagules in seaweeds (reviewed by Santelices, 1990), including total radiation, light quality, photoperiodism and nutrient concentration. Temperature seems to be particularly important in regulating the development of reproductive structures in *C. fragile* (Churchill & Moeller, 1972). It has been suggested that a critical temperature, ranging between 12 and 16°C, should be exceeded before reproduction can occur (Churchill & Moeller, 1972). In the study region, surface seawater temperatures tend to rise above 12°C around May (Regione Emilia Romagna, 2005). Appearance of gametangia in June in both 2003 and 2004 could, therefore, support a temperature-dependent development of reproductive structures in *C. fragile* (Churchill & Moeller, 1972).

In September 2004, the abundance of gametangia of *C. fragile* was greater on the landward than on the seaward side of breakwaters. It is possible that the exposed conditions on the seaward sides are less favourable for the development of gametangia, or that fertility is hampered on the seaward sides because, in these habitats, *C. fragile* never reaches relatively large sizes, thalli rarely weighing more than 10 g (Bulleri & Airoldi, 2005). Differences in the size of thalli of *C. fragile* between exposed and sheltered habitats were negligible in September 2004. Nonetheless, the small size of thalli at this time of the year was the result of different processes on opposite sides of the breakwaters, such as dehiscence of thalli and pruning of weakened lateral branches on the landward side and limited growth on the seaward side (Bulleri & Airoldi, 2005). *C. fragile* has been cited as an example of an alga in which maximum growth and reproduction are achieved simultaneously (Santelices, 1990) and, on Argentinian shores, Rico & Perez (1993) found that only half of the thalli of *C. fragile* weighing less than 50 g were fertile. To date, however, there is no information about the size at which thalli of any subspecies of *C. fragile* become fertile (Trowbridge, 1998). Whatever the cause, the landward sides of the breakwaters offered ideal conditions for *C. fragile* ssp. *tomentosoides*, enabling the alga to grow and reproduce better than on the seaward sides. Inconsistencies among breakwaters indicate, however, that factors other than wave exposure contribute to regulating the development of reproductive structures in *C. fragile*.

Most of the variability in the number of gametangia on thalli of *C. fragile* occurred within blocks, at a scale of tens of cm. Although large variation in the distribution and abundance of intertidal organisms at small spatial scales has been widely documented on rocky shores (Underwood & Chapman, 1998; Menconi *et al.*, 1999; Benedetti-Cecchi, 2001; Fraschetti *et al.*, 2005), scales of variation in the development of reproductive structures have received little attention. Both biological (e.g. settlement, recruitment, competition, grazing) and physical (features of the substratum such as the shape of blocks and topography) factors have been shown to promote small-scale variability in the settlement and recruitment of algal propagules (Foster, 1975; Harlin & Lindberg, 1977; Denny, 1988, Santelices, 1990). Some of these factors, affecting the physiological status of *C. fragile*, might indirectly regulate its reproductive output. Independently of the mechanisms operating, small-scale variation in the development of gametangia can have important implications for the spread of the alga. The reproductive mode is, in fact, among the traits determining the likelihood of successful establishment in exotic macroalgae (Nyberg & Wallentinus, 2005). Although vegetative re-growth from fragments of thalli has been recognized as the main mechanism for long-distance dispersal (Trowbridge, 1998), the establishment of a viable population after a new region has been inoculated is likely to take place through recruitment of parthenogenetic zygotes. Under these circumstances, small-scale variation in the development of reproductive structures could play a critical role in the early phases of establishment. Indeed, small-scale variation in physical and biological conditions, controlling the development of reproductive structures on pioneering thalli, would determine the success of new introductions of *C. fragile*.

Table 2. Analysis of the effect of Exposure, Breakwater and Thallus on the number of gametangia per g of tissue in *C. fragile* ssp. *tomentosoides*.

Source of variation	df	MS	F	p	Denominator
Exposure = E	1	6.71	0.61	> 0.05	E × B
Breakwater = B	2	0.23	0.10	> 0.05	Thallus (E × B)
E × B	2	11.06	4.79	< 0.05	Thallus (E × B)
Thallus (E × B)	36	2.31	28.90	< 0.001	Residual
Residual	108	0.08			

Transformation: $Ln(x+1)$; Cochran's test: $p > 0.05$. SNK tests (Exposure × Breakwater) Breakwater 1: Landward > Seaward; Breakwater 2: Landward > Seaward; Breakwater 3: Seaward = Landward.
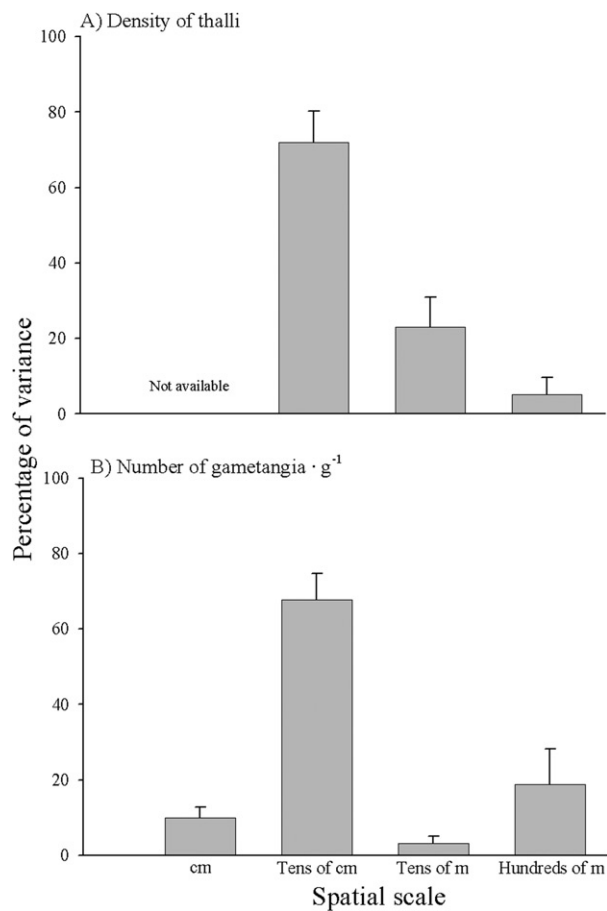
Fig. 3. Percentage of variance (+SE) in the density of thalli (A) and in the number of gametangia per g of tissue (B) at different spatial scales. Data are means across sampling dates ($n = 8$). For further details see text.

The importance of small-scale variation in the development of gametangia on the spread of the alga would, however, be dependent on the pressure of propagules (Lonsdale, 1999), as chances for fragments of the alga to re-generate thalli in favourable spots increase at increasing rates of immigration. The occurrence of most of the variation at a small scale suggests, on the other hand, that average conditions for the development of reproductive structures do not change much over broad scales and that the entire region might sustain a viable population of the alga (Bulleri & Airoldi, 2005).

Variation in the number of gametangia and density of thalli occurred at comparable spatial scales. The same small-scale processes might have produced, independently, similar patterns of variation in the two variables. Physical and biological conditions determining the recruitment and survival of thalli of *C. fragile* could be hypothesized to have a similar influence on reproductive output. Alternatively, patterns of distribution of thalli and development of gametangia may be linked by a cause–effect relationship. A weak but positive
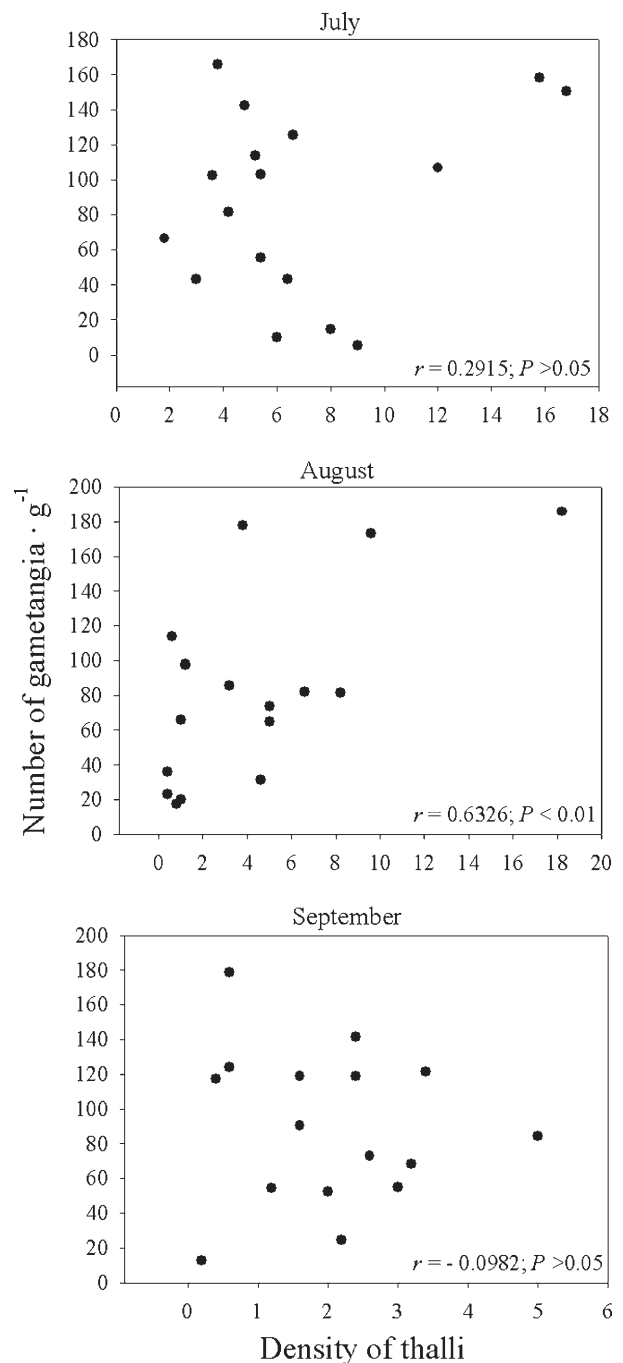

Fig. 4. Relationships between the mean number of gametangia per g of tissue and the mean density of thalli in July, August and September (data from 2003 and 2004 have been combined). Data are means across replicate thalli (for the number of gametangia) and quadrats (for the density) within blocks.

correlation between the number of gametangia and the density of thalli emerged in July and August. It could be argued that patterns in the distribution of thalli were generated by variation in the number of gametangia, but this is rather unlikely as it would imply very small variation in the dispersal, settlement and post-settlement mortality of parthenogenetic zygotes (Santelices, 1990). An effect of the density of thalli on their ability to develop

reproductive structures seems, therefore, a more plausible explanation. The effects of intraspecific competition in seaweeds can vary from positive to negative (Schiel & Choat, 1980; Cousens & Hutchings, 1983; Schiel, 1985; Dean *et al.*, 1989), with environmental conditions influencing the direction and strength of density-dependent inter-actions. In subtidal habitats, crowding can result in self-thinning of individuals within monospecific stands as a consequence of light limitation (Dean *et al.*, 1989). In contrast, increased densities of individuals in intertidal habitats can ameliorate physical conditions (e.g. desiccation, heat), with positive effects on growth, survival and reproduc-tion (Schiel & Choat, 1980; Schiel, 1985; Bertness & Leonard, 1997). Since the present study was conducted at low-shore levels, the period of time over which plants were exposed to aerial condi-tions was relatively short. Nonetheless, the ten-dency for a greater reproductive output at increased densities of thalli would suggest that the benefits of crowding, probably due to the provision of more benign physical conditions, prevailed over negative effects. Hence, sheltered habitats provided by breakwaters, fostering the density, growth and branching of thalli of *C. fragile* (Bulleri & Airoldi, 2005), would enhance the reproductive output and, ultimately, the estab-lishment and spread of the alga.

In conclusion, this study has shown that sheltered habitats on breakwaters represent highly suitable habitats for *C. fragile*, enabling the alga not only to attain large densities and sizes (Bulleri & Airoldi, 2005), but also to develop reproductive structures from mid-summer to autumn. Thalli of *C. fragile* can develop gametan-gia also on the seaward side of breakwaters, but, due to their limited persistence across the repro-ductive season (Bulleri & Airoldi, 2005), it could be argued that this species could not establish a viable population without the advantage of sheltered habitats. Variation in patterns of fertility of *C. fragile* seems to be related more to small-scale processes acting within blocks than to their position along the breakwaters or to processes operating at larger scales. There is large consensus on the need for expanding scales of ecological research (Naeem, 2006), but this does not diminish the importance of understanding small-scale processes, as their effects could scale up to regulate patterns of distribution of species over broad scales.

Acknowledgements

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