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Year-round reproduction in a seasonal sea: biological cycle of the introduced ascidian *Styela plicata* in the Western Mediterranean

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Abstract The widely introduced ascidian *Styela plicata* is very common in the Western Mediterranean, an area that can act as a source for secondary introductions due to its high shipping activity. In order to understand the potential of this species to colonize new habitats, its reproductive features were assessed in the Western Mediterranean by means of monthly monitoring of two populations (Vilanova i la Geltrú 41°12′53″N, 1°44′11″E; Blanes 41°40′29″N, 2°47′56″E) from January 2009 to December 2010. The reproductive activity of this species was assessed through gonad histology and a gonad index. Population size-structure was measured monthly in order to study recruitment dynamics. No clear seasonal pattern was observed, and mature gametes and recruits were present all year long. Spawning was potentially continuous, although it seemed punctuated with pulses of gamete release, particularly in spring. A prolonged reproductive period is likely to confer a competitive advantage on *S. plicata* in temperate seas, where most species reproduce seasonally, and may promote recurrent introductions as larvae are available for settlement on transport vectors over much of the year.

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Introduction

Countless marine species travel daily from their source of origin to new locations, either attached to ship's hulls or sea chests, in ballast waters, or being co-translocated with organisms associated with aquaculture activities (Allen 1953; Carlton and Geller 1993; Ruiz et al. 2000; Floerl and Inglis 2005; Blakeslee et al. 2010). Accordingly, introductions have increased notably during the last century, favored by maritime traffic and aquaculture (Carlton 1989; Vermeij 1996; Ruiz et al. 1997; Mack and D'Antonio 1998; Crooks 2002; Grosholz 2002). Although newly introduced species are often restricted to marginal habitats, such as harbors and aquaculture facilities, some may eventually spread to open habitats (Lambert 2002). Available information indicates that only one out of ten introduced species is able to survive and spread away from the introduced habitat, thus becoming invasive and causing serious alterations to native populations, communities, and ecosystems (Williamson and Fitter 1996). Invasive species are considered, after habitat loss and fragmentation, the second most important cause of species extinction (Zibrowius 1991; Mack et al. 2000; Clavero and Garcia-Berthou 2005).

Successful colonization of a new environment depends on the occurrence of adequate physical and biological conditions, both for adults and larvae (Blackburn and Duncan 2001; Stachowicz et al. 2002; Verween et al. 2007; Fowler et al. 2011; Zerebecki and Sorte 2011). Invasive species are often opportunistic, exploiting temporal windows of tolerable conditions to proliferate and occupy new habitats (McKinney 2002). Other characters that make introduced species prone to become invasive are adaptation to disturbance (Hobbs and Huenneke 1992; Altman and Whitlatch 2007), wide environmental tolerances (McMahon 1996; Marchetti et al. 2004), the ability to overcome local control

by resident species (Osman and Whitlatch 1998; Stachowicz et al. 2002), and high growth rates and reproductive output (McMahon 1996; Marchetti et al. 2004). Thus, in order to determine the invasive potential of a new introduced species and develop efficient management tools, it is necessary to acquire a better knowledge of their biological strategies and especially of their reproductive cycle (e.g., Grosholz and Ruiz 1996; Fine et al. 2001; Thornber et al. 2004).

Ascidians, or sea squirts, are conspicuous components of epibenthic marine communities all over the globe (e.g., Glasby 2001; Voultsiadou et al. 2007) and are among the most important marine invaders worldwide (Lambert 2002, 2007; Whitlatch and Bullard 2007). This group has the ability to severely modify the structure of coastal habitats by forming large aggregates that out-compete other organisms for resources (Zajac et al. 1989; Nandakumar et al. 1993; Lambert and Lambert 2003; Castilla et al. 2004; Agius 2007; Turon et al. 2007). *Styela plicata* is a solitary ascidian commonly found inhabiting harbors and salt marsh habitats of warm and temperate waters (i.e., Pacific, Indian, and Atlantic oceans, including the Mediterranean Sea), usually at high densities (Barros et al. 2009; David et al. 2010; Pineda et al. 2011, 2012). A recent genetic study has confirmed that this species has been present in these oceans for a long time, and that recurrent colonization events and shuffling among populations are determining its current genetic signature (Pineda et al. 2011). It is presently unclear where this species is endemic. Available evidence, albeit nonconclusive, points to an origin in the NW Pacific (Barros et al. 2009; Carlton 2009; Pineda et al. 2011). The introduction success of *S. plicata* has been attributed to its high tolerance of polluted waters (Naranjo et al.1996) and to moderately wide changes in temperature and salinity (Sims 1984; Thiyagarajan and Qian 2003; Pineda et al. 2012). The high genetic variability reported in *S. plicata* (Pineda et al. 2011) may also enhance this species' ability to adapt to new environments (Sakai et al. 2001) and displace indigenous species (Rius et al. 2009b).

As is usual in solitary ascidians, *S. plicata* is hermaphroditic and oviparous, with development involving a non-feeding, short-lived larval stage (Svane and Young 1989). *S. plicata* is reported to become sexually mature at about 40-mm body length (Tucker 1942; Yamaguchi 1975). The reproductive cycle or the settlement patterns of *S. plicata* have been determined for populations in Japan (Yamaguchi 1975) and the Eastern Mediterranean (Sabbadin 1957; Sciscioli et al. 1978; Tursi and Matarrese 1981). These reports point to an arrest of reproductive activity during winter months and to either a prolonged settlement during summer (June–September, Sciscioli et al. 1978), a restricted settlement period in summer (August–September, Tursi and Matarrese 1981), or a variable number of discrete generations per year (Sabbadin 1957; Yamaguchi 1975).

These differences in the reproductive cycle could be due to a number of factors, including temperature, location, and the genetic structure of the investigated populations. To date, no life-cycle data are available for populations in Western Mediterranean, where *S. plicata* is abundant in most harbors and marinas. Considering the high maritime traffic in some of those harbors (e.g., Barcelona, Alicante, and Marseilles) and the existence of smaller marinas all along the coast, this area could act as a source for recurrent introductions in the Mediterranean and in other oceans and thus deserves further investigation.

The goal of this study was to assess the reproductive features and population dynamics of the introduced ascidian *S. plicata* in the Western Mediterranean to assist in predicting the spreading potential of this species. To achieve this goal, the reproductive cycle was determined for two populations from the NE coast of Spain through examination of the gonad histology and calculation of a gonad index (GI) over a 2-year period. We also took monthly measurements of the population size-structure at one of the sites, in order to determine population dynamics and recruitment patterns. We hypothesized that *S. plicata* has a seasonal cycle of reproduction coupled to the strong seasonality of environmental parameters in the Mediterranean.

Materials and methods

Study site, sampling, and size-structure

The study was undertaken in two harbors of the NW Mediterranean coast: Vilanova i la Geltrú (41°12′53″N, 1°44′11″E), the larger and more polluted harbor, and Blanes (41°40′29″N, 2°47′56″E) located ca. 100 km NE from the former. Both harbors sustain a variety of marine-related activities including recreational boating and commercial fishing. From January 2009 to December 2010, ten adult *S. plicata* (>40 mm, Yamaguchi 1975) were collected monthly at each site from depths of 0–1 m by pulling up harbor ropes or removing individuals from submersed docks. The ascidians were immediately fixed in 4 % formaldehyde and stored at room temperature until used for histology and morphometric analyses. Water temperature was automatically registered at 1-m depth every hour with HOBO® loggers (0.001 °C precision), Onset Computer Corporation, Massachusetts. The size-structure of this species was determined from November 2009 to December 2010 in Vilanova i la Geltrú, the population where *S. plicata* was more abundant. Every month, multiple ropes located in the same area of the harbor were pulled from the water, and the height of 150 randomly selected individuals was measured directly on the ropes with a calliper. Clumps of individuals and ropes were scrutinized to detect small

recruits (<10 mm). After measurement, the ascidians were left on the ropes and put back into the water. We used different ropes on each sampling date. Individual lengths recorded monthly were used to plot size–frequency histograms using 2-mm size classes.

Morphometric variables

Once in the laboratory, the individuals kept in 4 % formaldehyde were carefully cleaned to remove as many epibionts as possible from their tunics. For each individual, we measured the height (maximal distance from base to tip) as a proxy for size using a calliper. As we worked with fixed, contracted organisms, the length measured was an underestimation of the lengths of live, relaxed individuals. However, the tough tunic in this species only allows for slight contraction of the siphons, and we assume that individuals contracted to a similar degree and thus that measurements were comparable. The tunic was cut open to separate the mantle and dissect it along the ventral side to remove the branchial sac and to expose the gonads. Each individual possessed 2–11 gonads attached to the right side of the body wall and 1–3 gonads to the left side. Each gonad has a central, elongated ovary covered with testis follicles (Tucker 1942). From each specimen, a small piece (<1 cm long) of one gonad from the right side was cut, weighed, and kept in 4 % formaldehyde for histology; the remaining gonadal tissue was dissected, weighed, and placed in an oven at 60 °C for 48 h to obtain its dry weight. To obtain the total dry weight (DW) of the gonads, we estimated the DW of the removed piece for histological purposes using the observed wet/dry weight ratio obtained for the other gonads. Wet and dry weights were also obtained for the tunic and mantle. A gonadosomatic index (hereafter referred to as gonad index, GI) was calculated as the gonad DW divided by the mantle DW.

The mean gonad index over time was correlated between sites and with temperature variations using monthly means and cross-correlation analyses (Pearson's coefficient). In these analyses, values of one variable were correlated with values of the other at different time lags (months). Correlation at time lag 0 corresponds to the usual Pearson's correlation, positive lags correlate values in the first series to values in the second series that number of lags afterward, and negative lags relate values in the first series to previous values in the second one. All analyses were performed using the software SYSTAT v. 12 (©SYSTAT Software, Inc. 2007), and SigmaStat v. 3.11 (©SYSTAT Software, Inc. 2004).

Histological analysis

Among the individuals used for morphometric analysis, each month, a piece of gonad from at least 5 individuals per population was dehydrated, embedded in paraffin, sectioned, and stained. Methylene blue staining was used in preliminary sections to assess the gross morphology of the gonads and to guide further sectioning. For detailed histological observation, hematoxylin-eosin staining was used following standard procedures. The gonad sections were observed under a microscope equipped with a micrometer. The diameter of 100 oocytes sectioned through the nucleolus was measured per individual following Bingham (1997). In some individuals fewer than 100 oocytes could be found with nucleolus visible, but in all cases, we measured a minimum of 200 oocytes month^{-1}. In total, 7,504 oocytes were measured for individuals from Vilanova i la Geltrú and 6,602 from Blanes. We calculated oocyte size-frequencies each month using 50-μm size classes as follows: <50 μm (pre-vitellogenic), 50–100 μm and 100–150 μm (vitellogenic), and >150 μm (mature, Sciscioli et al. 1978). For the testes, a categorical maturity index was established, according to the development of the male follicles (i.e., 1 = immature, only spermatogonia, 2 = mature sperm, 3 = spawning, empty spaces within the lumen).

Results

Population structure

The introduced ascidian *S. plicata* was present all year round in both harbors, Vilanova i la Geltrú and Blanes, on hanging ropes and virtually any hard substrate available. This species was often found forming aggregates of individuals attached to each other's tunics. During the study period, this species was more abundant and formed larger aggregates in Vilanova i la Geltrú than in Blanes. Height was a good descriptor for size, as it showed a high correlation with total dry weight in the individuals collected (Pearson's $r = 0.735$, $p < 0.001$).

The population at Vilanova i la Geltrú was characterized by the presence of individuals of all sizes most of the year. Heights ranged from a minimum of 2 mm in October to 68 mm in an individual collected in August. Based on occasional observations of settled individuals on newly exposed ropes, we considered as recruits those individuals that were <15 mm high, which corresponded to an approximate age of 1 month (authors' pers. obs.). Recruits were especially abundant after mid-summer and absent only in May. Adults (>40 mm) were found all year round, although the largest sizes classes (>50 mm) were almost absent in December 2009 and in February and March 2010 (Fig. 1). Thus, size–frequency plots suggested higher recruitment in fall and winter coupled with slower growth and loss of the oldest individuals (Fig. 1). The presence of juveniles and adults all year long masked any evidence of a
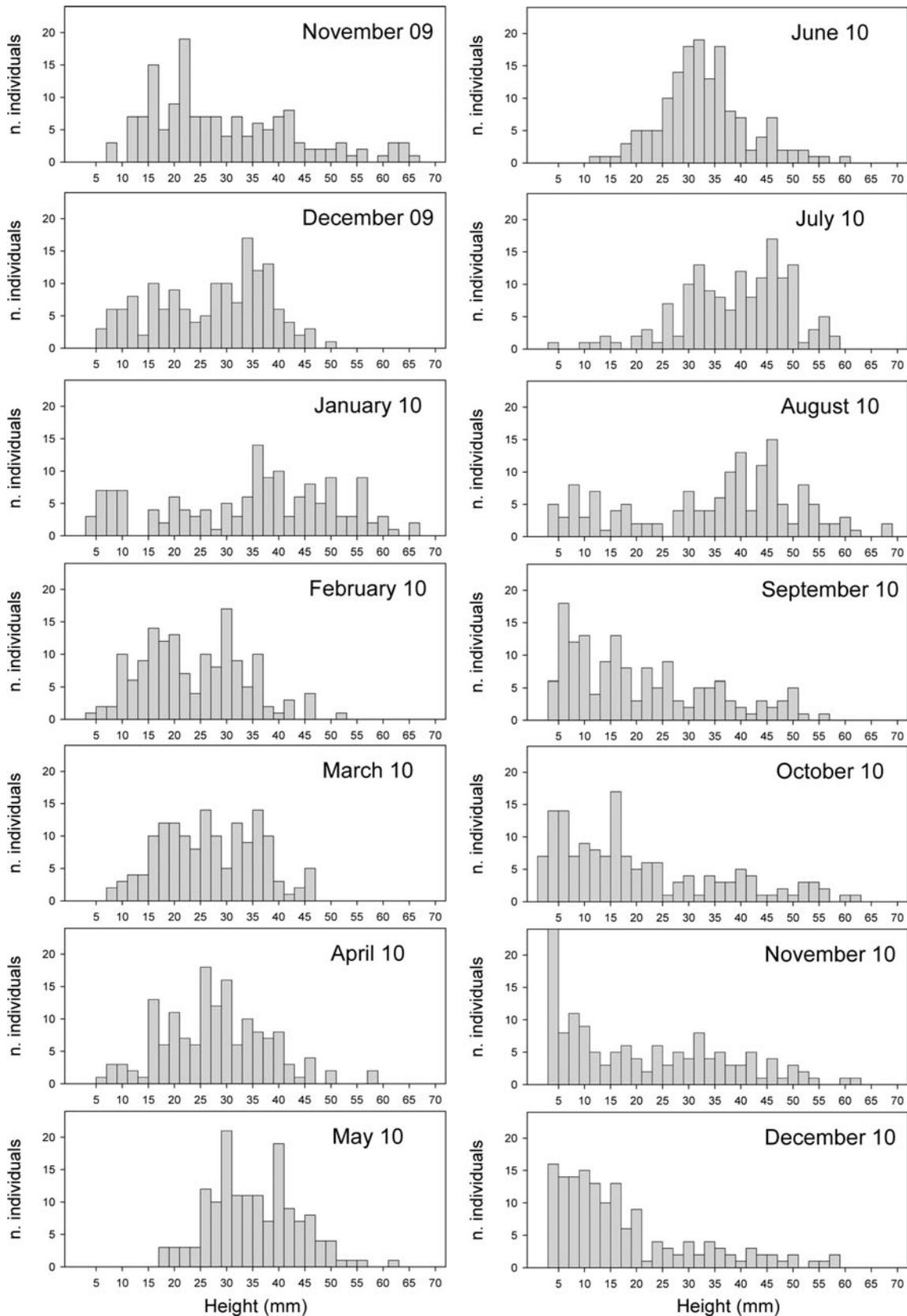
Fig. 1 Size–frequency histograms (height, mm) of *S. plicata* for each sampled month in 2009–2010 in Vilanova i la Geltrú
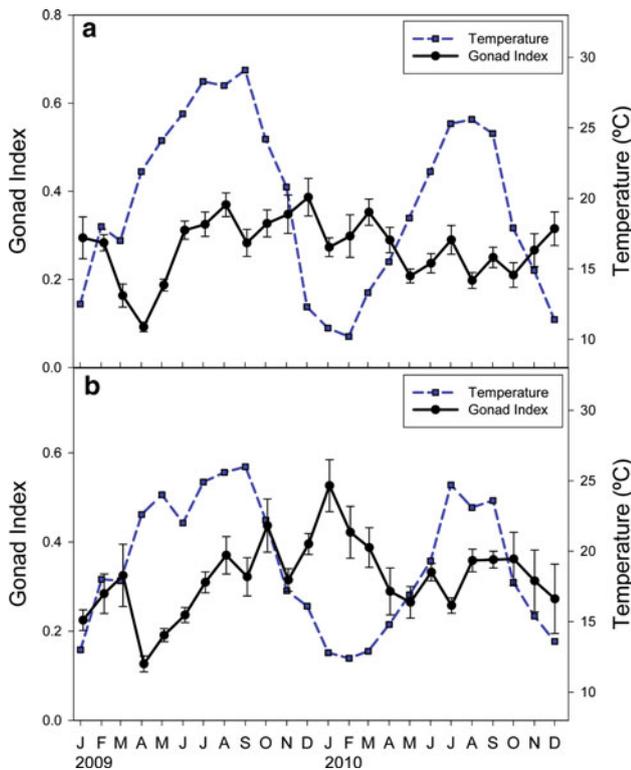
Fig. 2 Mean Gonad Index (±SE) and seawater temperature (°C) in 2009–2010 in **a** Vilanova i la Geltrú and **b** Blanes

cohort structure in the size–frequency histograms. Instead, the population structure seemed to result from overlapping, successive generations (Fig. 1).

Reproductive cycle

The mean gonad index (GI) and seawater temperature over the study period for Vilanova i la Geltrú and Blanes are shown in Fig. 2. Seawater temperature showed a clear seasonal cycle at both sites, with the lowest values at the end of winter (10.2 and 12.4 °C in February 2010 for Vilanova i la Geltrú and Blanes, respectively) and the highest in September 2009 (29.1 and 26 °C, respectively). Thus, temperatures in Blanes oscillated less (13.6 °C from lowest to highest) than in Vilanova i la Geltrú (18.9 °C). The mean GI did not show a clear seasonal pattern for either of the two populations, although a sharp decrease in the mean GI was observed in April 2009 in both (Fig. 2). Decreases in GI values were also observed in September 2009, and January, May, August, and October 2010 for Vilanova i la Geltrú (Fig. 2a), and September and November 2009, and January to May, July and December 2010 in Blanes (Fig. 2b). A noticeable peak was also observed in January 2010 for the population in Blanes (Fig. 2b).

Cross-correlation analyses were not significant for most time lags. Nevertheless, there was a clear wave-like pattern
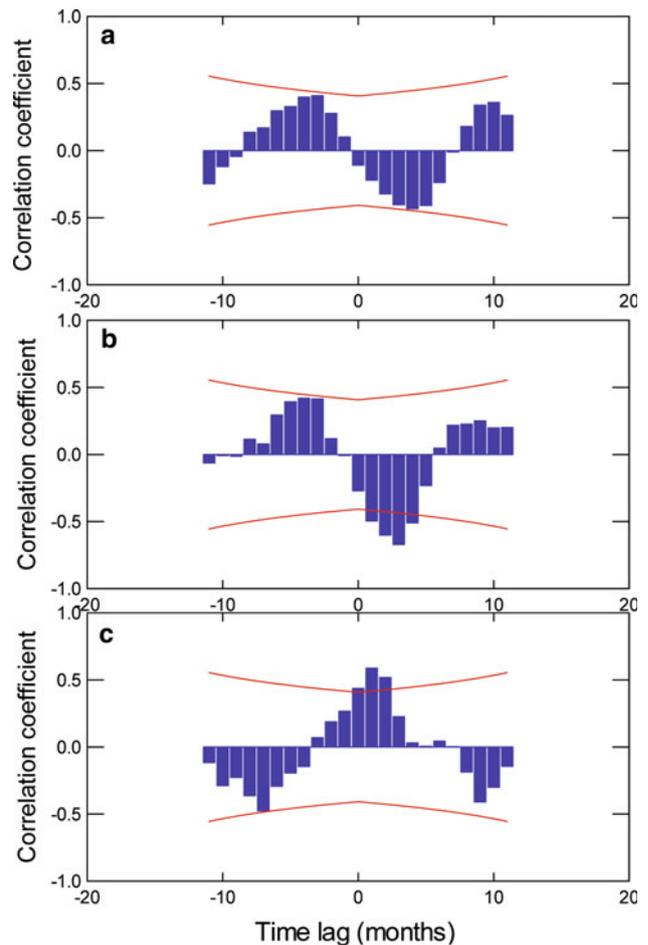

Fig. 3 Cross-correlation analyses of *S. plicata* **a** gonad index versus temperature in Vilanova i la Geltrú, **b** gonad index versus temperature in Blanes, **c** gonad index in Vilanova i la Geltrú versus gonad index in blanes. Curved lines represent 95 % confidence intervals of correlation coefficient

and positive correlations between gonad index and temperature in the previous months were found for both populations, while correlations were negative between GI and the temperature measured in subsequent months (Fig. 3a, b). This positive correlation indicated that GI tended to increase some months (peak of correlation at 2–3 month) after temperature increases. Correlations at time lag 0 were small and negative. Mean GIs followed similar patterns in both localities, as indicated by a significant correlation coefficient (cross-correlation analysis at time lag 0, Fig. 3c). The relationship was also positive and significant between the mean GI in Vilanova i la Geltrú and the values in Blanes the two subsequent months (time lags +1 and +2, Fig. 3c).

Gonad histology showed, in cross-section, the presence of a central core of female follicles with oocytes surrounded by peripheral male follicles (Fig. 4a). Mature oocytes had test cells inside the chorion and were surrounded by two layers of follicle cells (Fig. 4b). Male

Fig. 4 Light micrographs of **a** a gonad histological section stained with hematoxylin-eosin (*MMF* mature male follicle, *DO* developing oocyte, *MO* mature oocyte. **b** female gonad stained with methylene blue (*TC* test cells, *FC* follicle cells, *N* nucleolus). **c**, **d**, **e** male follicles stained with hemotoxylin-eosine (c: immature, d: mature, e: mature and spawning). *Scale bars* = 200 μm (**a**), 50 μm (**b**), 100 μm (**c**, **d**, **e**)
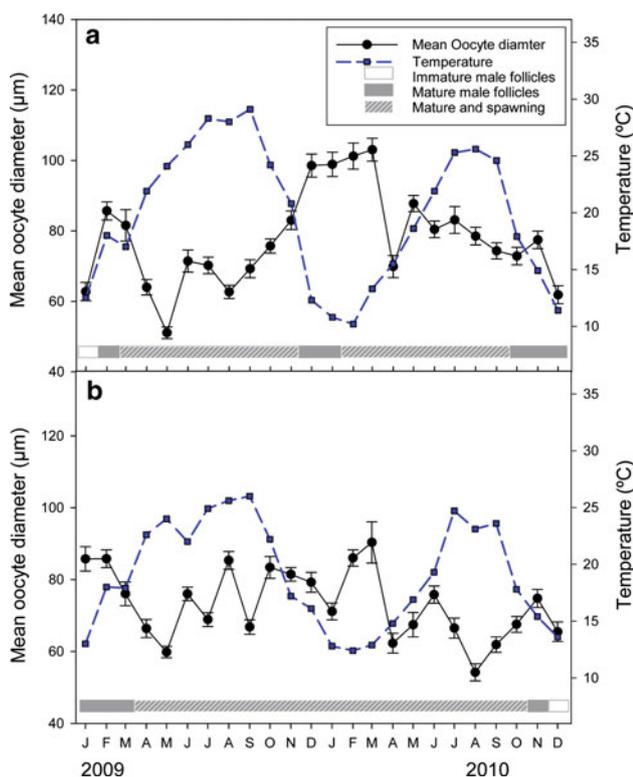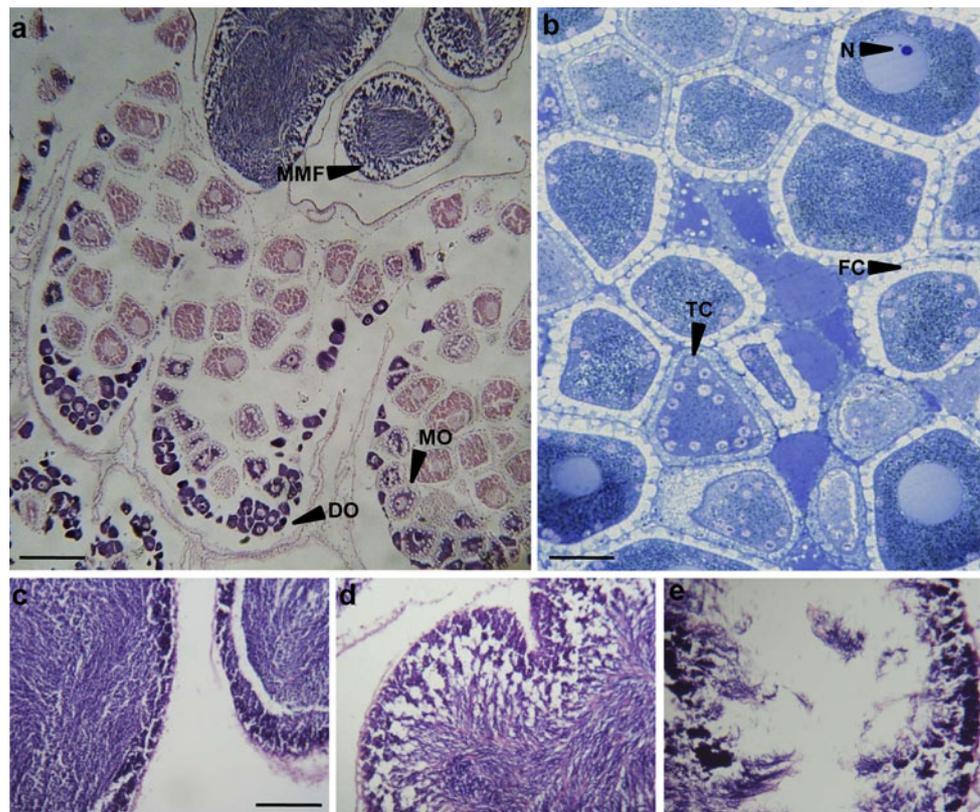

Fig. 5 Mean oocyte diameter (±SE) in *S. plicata* and seawater temperature in **a** Vilanova i la Geltrú and **b** Blanes. *Horizontal bars* show male maturation state over study period

follicles were characterized by a thick wall of germinative epithelium and a lumen occupied by developing spermatozoa (Fig. 4a, c, d) or by empty spaces after spawning (Fig. 4e). We found considerable variability in the maturation state of the male gonads within the same month and population, while mature oocytes were always present in at least some individuals. These observations confirmed the year-round reproductive cycle previously found with the GI (Fig. 5). A sharp decrease in the mean oocyte diameter was also observed in spring 2009 and 2010 in both populations (Fig. 5). Minimum values for the mean oocyte diameter were also recorded in August 2009 and December 2010 for the Vilanova i la Geltrú population, while for the Blanes, population minima were recorded in July and September 2009, and January, April, August and December 2010 (Fig. 5). Mature male follicles and follicles with partially empty lumens, due to the release of sperm, were also found over most of the year except for some of the coolest months (January 2009 in Vilanova i la Geltrú and December 2010 in Blanes), when gonads had mostly immature male follicles (Fig. 5).

The oocyte size-frequencies showed that pre-vitellogenic oocytes (<50 μm) were present all year round, but increased in proportion concomitantly with the decreases in mean oocyte diameter (Fig. 6). Mature oocytes (>150 μm) were also present all year, but were especially abundant during winter. In the individuals collected from Vilanova i
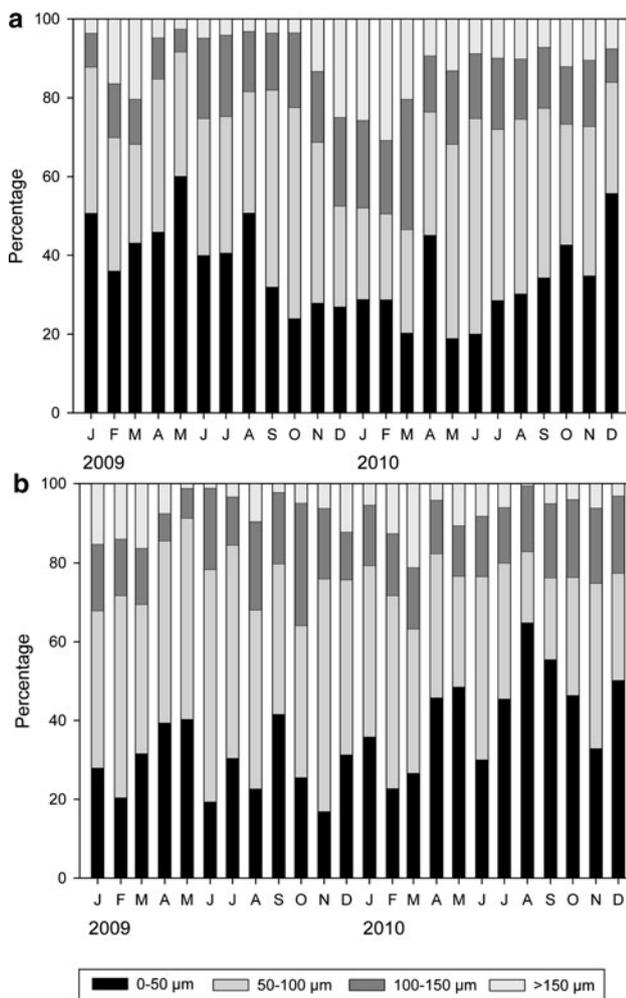
Fig. 6 Percentage of each oocyte size-class (previtellogenic, 0–50 μm; mature, >150 μm) in *S. plicata* in 2009–2010 in **a** Vilanova i la Geltrú and **b** Blanes

la Geltrú, the proportion of mature oocytes peaked in winter-early spring followed by a sharp decrease in April (Fig. 6a). In Blanes, the proportion of mature oocytes was more variable, with minimum values in May, June, and September 2009, and April, August, and December 2010 (Fig. 6b).

Discussion

The introduced ascidian *S. plicata* was present year round in the two studied populations from the Western Mediterranean. Individuals were 2–68 mm in height. There was no clear seasonal cycle for reproduction in Vilanova i la Geltrú or Blanes in 2009–2010. In fact, this species had mature oocytes and male follicles all year round. The mean gonad index and the mean oocyte diameter showed marked decreases in spring 2009 and, to a lesser degree, in spring

2010. Gonad index decreases alone may not correspond to spawning events, as shown by a lack of correlation between GI and oocyte diameter in some ascidian species (e.g., Bingham 1997; Wong et al. 2011). Mobilization of reserves stored in the gonads in periods of shortage, as noted also in other groups (e.g., echinoderms King et al. 1994; Lozano et al. 1995), can occur in ascidians (Bingham 1997). However, the coincidence of several parameters (GI drop, oocyte diameter drop, and minima in proportion of mature oocytes) suggests that pulses of spawning occur in spring in *S. plicata*. Less confidence can be placed on secondary peaks of GI as indicative of spawning, as they are often not coincident with changes in oocyte diameter. A combination of several approaches (histology and gonad indices) seems the best way to obtain a complete picture of the reproductive cycle in ascidians (Becerro and Turon 1992; Sahade et al. 2004). In our case, results indicate that *S. plicata* reproduces throughout the year in the Western Mediterranean, although more intensely so in spring. This pattern of pulses of spawning against a background of continuous reproduction has been described in other temperate ascidians (Svane and Lundälv 1981, 1982; Bingham 1997).

Prolonged reproductive activity of *S. plicata* from spring to autumn was previously reported in the Eastern Mediterranean (Sabbadin 1957; Sciscioli et al. 1978). These authors considered that this species did not actively reproduce during the coldest months, as no recruitment was detected in winter on artificial panels (Sciscioli et al. 1978; Tursi and Matarrese 1981). Sabbadin (1957) sets the low temperature threshold for *S. plicata* reproduction at 10 °C, a seawater temperature that was easily reached during the winter months in the Lagoon of Venice (Italy). Below 10 °C, this species not only was unable to reproduce but could also disappear (Sabbadin 1957). Such low temperatures are seldom reached in the Western Mediterranean (Margalef 1985; Coma et al. 2000) and, although there was a short period in which male follicles were not mature in the coolest months, mature individuals with large oocytes were found in winter in both of our study populations. Moreover, small recruits were observed in Vilanova i la Geltrú during the winter months. Thus, taken together, our results indicate that *S. plicata* is also reproductively active in the winter months in the Western Mediterranean. These results are in agreement with Panagiotou et al. (2007), who reported the presence of *S. plicata* recruits all year in Thessaloniki Bay (Greece). In Tokyo Bay (Japan), where the temperature regime is more similar to the Western Mediterranean, Yamaguchi (1975) observed that individuals of *S. plicata* were ripe in winter, although they did not spawn during the coldest months of the year. Finally, previous studies have reported the existence of several generations per year for this species in central Japan

(Yamaguchi 1975) and Eastern Mediterranean (Sabbadin 1957). Our results did not show any clear-cut succession of generations for populations in Western Mediterranean harbors. Rather, individuals of a wide range of sizes were present all year round, suggesting that there were several overlapping cohorts of recruits each year. Interannual differences in the number and intensity of GI peaks and mean oocyte diameter changes were also observed in the two monitored harbors. Temperature has been suggested as the main factor triggering not only ascidian reproduction (e.g., Millar 1971; Turon 1988), but also the reproductive cycles of many marine invertebrates in temperate seas (Orton 1920). Accordingly, temperature differences between years and sites could explain the similarities and differences found. For instance, there was an unusual and sharp increase in seawater temperature in February 2009 (6 °C). This anomaly could in turn explain the spawning event observed for both populations 2 months later (April 2009). Similarly, the smaller spawning episode recorded in spring 2010 could be due to a later and more gradual increase in seawater temperature (March).

In the Mediterranean, most marine invertebrates present seasonal biological cycles (Coma et al. 2000), including colonial (Turon 1988; De Caralt et al. 2002; López-Legentil et al. 2005) and solitary ascidians (Becerro and Turon 1992; Panagiotou et al. 2008; Vafidis et al. 2008; Rius et al. 2009a). Summer is an unfavorable season for many sessile invertebrates in the Mediterranean, due to food shortage (Coma et al. 2000) and high densities of macroalgae competing for space (Ballesteros 1989). Therefore, the continuous presence of this species and the ability to reproduce all year round may confer a competitive advantage on S. plicata compared to seasonally reproducing invertebrate species. Extended reproductive cycles and fast growth to maturity have been reported for several invasive ascidians (Bourque et al. 2007; Shenkar and Loya 2008; Wong et al. 2011). Thus, based on our current results, this species could become a threat to local biota if it spreads to natural habitats. However, although the species has been found outside harbors, to date its abundance has always been low and it never monopolizes the substrate as it does inside harbors, marinas, or on artificial structures (authors' pers. obs.). Other factors controlling the spread of the species to natural substrates, such as predation (Sutherland 1974), competition, or the effects of hydrology, should be investigated.

Besides conferring a competitive advantage, a continual reproductive period could also allow S. plicata to exploit temporal windows of favorable conditions. It has been demonstrated that adults of this species can respond to changes in temperature and salinity by increasing the production of stress proteins (Pineda et al. 2012). However, the embryos and larvae of S. plicata, although relatively resistant to pollutants such as copper, are very sensitive to changes in temperature or salinity (Pineda 2012). Based on these observations, it is likely that some reproductive episodes do not result in local recruitment and spreading them over time would increase the probability of some larval recruitment. In addition, the existence of multiple batches of larvae over the year ensures a plentiful supply of juveniles on ships' hulls, ready to spread to other marinas, and facilitating recurrent introduction of the species. This fact, coupled with a large genetic pool (coherent with the genetic structure described for this species, Pineda et al. 2011), would ensure the persistence of S. plicata populations living under harsh conditions such as those usually encountered in enclosed man-made habitats.

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