Growing or reproducing in a temperate sea: Optimization of resource allocation in a colonial ascidian

Journal:	<i>Invertebrate Biology</i>
Manuscript ID:	Draft
Manuscript Type:	Original Manuscript
Date Submitted by the Author:	n/a
Complete List of Authors:	López-Legentil, Susanna; University of Barcelona - Faculty of Biology, Department of Animal Biology (Invertebrates) Erwin, Patrick; University of Barcelona, Department of Animal Biology (Invertebrates) Velasco, Marta; University of Barcelona, Department of Animal Biology (Invertebrates) Turón, Xavier; Center for Advanced Studies of Blanes,
Keywords:	Tunicate, Growth rate, Maturity index, Mediterranean Sea, Temperature

SCHOLARONE™
Manuscripts

view

Formatted for: Invertebrate Biology

2

3

**Growing or reproducing in a temperate sea: Optimization of
resource allocation in a colonial ascidian**

6

Susanna López-Legentil^{1,*}, Patrick M. Erwin¹, Marta Velasco¹ and Xavier Turon²

8

¹*Department of Animal Biology, University of Barcelona, Diagonal Avenue 643,
08028 Barcelona, Spain*

²*Center for Advanced Studies of Blanes (CEAB-CSIC), Accés Cala S. Francesc 14,
17300 Blanes, Girona, Spain*

13

14

*Corresponding author: Susanna López-Legentil. Tel. +34 93 403 1150; Fax. +34 93
403 5740; Email. slopez@ub.edu

17

Running title: Life cycle of the colonial ascidian *Didemnum fulgens*

19

Abstract. To date, there is still little information about the life cycles of ascidians in temperate seas. Here, we investigated the biological cycle of the colonial ascidian *Didemnum fulgens*, a dominant species in some shallow localities of the NW Mediterranean Sea. Growth rates and frequency of fission/fusion events were calculated over a period of 13 months and the reproductive cycle determined after 32 months of observation. For reproduction analyses, zooids were dissected in the laboratory and classified in 5 reproductive categories used to calculate a maturity index. For growth analysis, underwater photographs of marked colonies were used to quantify the surface area of *D. fulgens* colonies, calculate monthly growth rates, and document fusion and fission events. Clear seasonal patterns in reproduction and growth were observed in *D. fulgens*, with distinct periods of investment in each function. Gonad maturation started in winter and larval release occurred in early summer, just before maximal sea temperatures were reached. After reproducing, colonies shrank and aestivated during the warmer summer months. Growth occurred during the cooler months, with maximal and minimal growth rates observed in winter and summer respectively. Fusions and fissions occurred year-round although fissions were more frequent in fall (coincident with high growth rates) and fusions in spring (coincident with reproduction). These results add to mounting evidence that ascidian life cycles in temperate seas are characterized by a trade-off between investment in reproduction and growth, triggered by seasonal temperature shifts and constrained by resource availability during summer.

Additional key words: Tunicate, growth rate, Mediterranean Sea, maturity index, temperature

Introduction

Ascidians or sea-squirts (Chordata; Tunicata) are sessile, filter-feeding organisms that occur in benthic ecosystems across a broad range of depths and latitudes (Lambert 2005). These animals are notorious for their detrimental effects on aquaculture facilities, as fouling organisms attached to ship hulls (Turner et al. 1997; Rodriguez and Ibarra-Obando 2008; Hewitt et al. 2009; Carman et al. 2010), and as introduced and invasive species (e.g. *Didemnum vexillum*, Lambert 2009; Stefaniak et al. 2009, 2012). In addition, ascidians have been utilized as bioindicators of pollution (Agell et al. 2004; Carman 2007; Azumi et al. 2008) and as a source of bioactive secondary metabolites for anti-cancer drug research (Erwin et al. 2010). Thus, while their ecological and biotechnological importance is well known, comparatively little is known about their biology and, in particular, their growth and reproductive strategies.

Ascidians are hermaphroditic and mostly reproduce through sexual cross-fertilization, resulting in the production of free-living, non-feeding larvae that must find a suitable substrate to settle and metamorphose in a matter of hours to days (Svane and Young 1989; Rius et al. 2010). Solitary ascidians are normally oviparous, while colonial ascidians usually brood their larvae. Colonial ascidians can also experience fission and fusion events. Fission is a phenomenon by which an existing colony divides to generate two or more smaller colonies and is considered an asexual method for colony proliferation. In contrast, fusion is the union of two or more colonies that results in rapid growth. In didemnid ascidians, colonies appear to fuse indiscriminately (Bishop and Sommerfeldt 1999), while in botryllids fusion is regulated by genetic histocompatibility (Rinkevich and Saito 1992; Saito et al. 1994; Rinkevich 2005). In fact, within colonial ascidians, most of available information on

1
2
3 71 reproductive ecology and colonial dynamics refers to botryllid ascidians (e.g. Newlon
4
5 72 III et al. 2003; Bates 2005; Rinkevich 2005; Marshall et al. 2006; Westerman et al.
6
7 73 2009). In contrast, much less is known about enterogonid families of colonial
8
9
10 74 ascidians (e.g. Bishop and Sommerfeldt 1999; Dias et al. 2008; Shenkar et al. 2008).
11
12 75 In general, marine species that live in warmer waters reproduce and grow
13
14 76 throughout the year, while those that live in temperate seas tend to exhibit seasonal
15
16 77 life cycles (Coma et al. 2000; Hernández-Zanuy et al. 2000; Fukuda and Hirose 2008;
17
18 78 Shenkar and Loya 2008; Ritzmann et al. 2009). In the Mediterranean, most colonial
19
20 79 ascidian species reproduce before maximal sea temperatures are reached in summer
21
22 80 (Turon 1988; Turon and Becerro 1992; Molin et al. 2003; López-Legentil et al. 2005),
23
24 81 while a few do so during the cooler months of the year (e.g. *Aplidium* spp., Turon
25
26 82 1988; Turon and Becerro 1992). Similarly, most of the colonial ascidians studied
27
28 83 experience positive growth during fall and winter (Turon and Becerro 1992; López-
29
30 84 Legentil et al. 2005; Pérez-Portela et al. 2007) and no species has been observed to
31
32 85 actively grow during the summer months. Millar (1971) proposed temperature as the
33
34 86 main factor regulating the life cycles of temperate ascidians, but other parameters,
35
36 87 such as limited resource availability (Bates 2005; Lambert 2005), turbidity (Millar
37
38 88 1971), currents and wave exposure (Shenkar and Loya 2008), or competing
39
40 89 invertebrate species (Dias et al. 2008; Stocker and Underwood 1991) may also
41
42 90 influence their life cycles. A trade-off between reproduction and growth has also been
43
44 91 observed in some ascidians, suggesting a partitioning of resources to specific
45
46 92 biological functions (López-Legentil et al. 2005; Pérez-Portela et al. 2007). In fact,
47
48 93 López-Legentil et al. (2007) showed that reproduction was the main factor driving
49
50 94 both growth and defense production in a temperate ascidian species.
51
52
53
54
55
56
57
58
59
60

95 Aestivation periods and resting forms are also common in ascidians living in
96 temperate seas. Resting periods normally occur during the unfavorable season, which
97 for most Mediterranean filter-feeders is summer (Coma et al. 2000). The function of
98 aestivation is not well understood in ascidians and several hypotheses have been
99 suggested for different species. Berrill (1951) described a regression period after
100 reproduction that involved tissue reorganization. Burighel et al. (1976) suggested an
101 excretory role of degenerative residues. Turon (1992) proposed a ‘rejuvenative’
102 process that involved the regression of old thoraces and budding of new ones, and
103 Molin et al. (2003) suggested a propagative budding to generate new zooids after
104 reproduction. Although the reason for aestivation and resting is not clear, this
105 phenomenon appears to be widespread among ascidians in the Order
106 Aplousobranchia, affecting many species from several different families (e.g.
107 *Polysyncraton lacazei*, Didemnidae, Turon 1992; *Pseudodistoma crucigaster*,
108 Pseudodistomidae, Turon and Becerro 1992; *Polycitor adriaticus*, Polycitoridae,
109 Molin et al. 2003; and *Pycnoclavella* spp., Clavelinidae, Pérez-Portela et al. 2007).

110 Didemnid ascidians can be dominant organisms in sublittoral communities
111 (e.g. Bak et al. 1981; Ryland et al. 1984; Stocker 1991), especially when they become
112 invasive (e.g. Kremer et al. 2010; Lambert 2009; Smale and Childs 2012). Thus, the
113 study of their population dynamics and life cycles is useful to explain processes such
114 as space occupation and community diversity patterns (Dijkstra et al. 2007; Dijkstra
115 and Harris 2009; Fiore and Cox Jutte 2010). During the last decade, populations of the
116 didemnid ascidian *Didemnum fulgens* (MILNE-EDWARDS 1841) have increased in
117 the NE coast of Spain, from a rarely reported species to a common ascidian in rocky
118 infralittoral communities (*pers. obs.*) and on the rhizomes of the seagrass *Posidonia*
119 *oceanica* (Balata et al. 2007). The recent expansion of *D. fulgens*, in terms of

120 population numbers and sizes, raises questions about the environmental shifts that
121 may have facilitated the spread of this species and the biological characteristics that
122 have allowed it to outcompete resident sessile organisms. The goal of this study was
123 to determine the seasonal cycles of reproduction and growth in the colonial ascidian
124 *D. fulgens* and assess whether a trade-off in resource allocation exists between these
125 two biological functions. To address these questions, we assessed the reproductive
126 cycle of *D. fulgens* by characterizing reproductive status monthly for over 2.5 years
127 and determined the growth rate and number of fission/fusion events monthly for 1
128 year using underwater transects and digital photography.

129

130 **Methods**

131

132 **Sample site and species identification**

133

134 This study was conducted in a coastal site of the Mediterranean Sea located between
135 l'Illa Mateua and Cala Montgó in L'Escala (NE Spain, 42° 7' 29'' N, 3° 7' 57'' E).
136 Since previous studies conducted in this area during the early 2000s, we have
137 observed the proliferation of an orange-pinkish didemnid that has rapidly become a
138 conspicuous species in shallow rocky communities reaching densities of ca. 7
139 colonies/m².

140 The species was identified following descriptions by Lafargue (1976), and
141 Lafargue and Wahl (1987). In short, *Didemnum fulgens* is characterized by bright
142 orange colonies, dense calcareous spicules (up to 50 µm in diameter) with short,
143 pointed rays (about 20 visible), zooids with small thoracic organs in the posterior part

144 of the thorax, a sperm-duct with 7-9 coils, and larvae with two adhesive papillae and
145 4-6 pairs of epidermal ampullae.

146 To barcode this ascidian species, one colony was preserved in 100% ethanol
147 and stored at -20°C until processed. Zooids were carefully separated from the tunic
148 under a stereomicroscope and DNA was extracted using the DNeasy Blood & Tissue
149 kit (Qiagen). The primer set Tun_forward and Tun_reverse2 described by Stefaniak et
150 al. (2009) was used to amplify a fragment of the Cytochrome *c* Oxidase subunit I
151 (COI) mitochondrial gene. Total PCR reaction volume was 25 µl, including 5 pmol of
152 each primer, 5 nmol of each dNTP, 1x reaction buffer (Ecogen), and 2.5 units of
153 BIOTAQ polymerase (Ecogen). PCR program included initial denaturing at 94°C for
154 5 min, followed by 40 amplification cycles of 94°C for 30 sec; annealing at 40°C for
155 30 sec; and extension at 72°C for 1 min, and a final extension at 72°C for 10 min.
156 Cleaning and sequencing reactions were carried out at Macrogen, Inc. (Seoul, Korea).
157 The resulting sequence has been deposited in the GenBank database (Acc. No.
158 JX846617) and BLAST searches revealed another didemnid species as best match (*D.*
159 *perlucidum* JQ731735, 88% similarity).

161 **Sample collection and monitoring**

162
163 To determine the reproductive cycle of *D. fulgens*, 5 colonies were collected and
164 immediately fixed in 10% formaldehyde every month from June 2009 to May 2012
165 by SCUBA diving. Colonies were separated by at least 5 m and collection depth
166 ranged from 4 to 10.5 meters. From April 2011 to May 2012, a permanent 3.5 m
167 horizontal transect at 7 m depth was established along a vertical wall and the position
168 of each colony of *D. fulgens* sketched underwater on a plastic slate. Each colony was

revisited monthly and photographed with an Olympus PT-027 underwater camera fixed to an aluminum frame (18.7 cm x 13 cm) to keep a constant focal distance. Seawater temperature was recorded using either a HOBO® Pendant Temperature Data Logger (Onset Computer Corporation) which registered temperature each hour, or a diving computer during the periods in which no datalogger was deployed. No sampling could be performed in December 2009, April 2010, September 2010, November 2010 and February 2012 because of unfavorable diving conditions. For these months, seawater temperatures were calculated as an average of the previous and following months' temperatures.

Reproductive cycle

Once in the laboratory, colonies fixed in 10% formaldehyde were dissected under a stereomicroscope and 10 zooids per colony were carefully observed to determine their reproductive status. Each zooid was scored according to the following categories: (1) immature, (2) presence of testes, (3) presence of testes and oocytes and (4) presence of oocytes. A maturity index (MI, Lopez-Legentil et al. 2005) was calculated for each colony by averaging the category number of the zooids examined, except when larvae were observed in the basal tunic. Colonies with larvae were scored with a maturity index value of 5 since larval production represents the culmination of reproduction and zooids regress to immature status during the brooding period. Monthly means were calculated by averaging the values of the colonies investigated that month.

Growth cycle

Since *D. fulgens* presents a thinly encrusting morphology, changes in the surface area over time were calculated as an estimate of growth. The surface area of each colony was measured using the image analysis software *ImageJ* v. 1.45s. Fission and fusion events were also recorded. The nature of these events, whether they occur among colonies with the same genotype or different genotypes (chimeras), is unknown for *D. fulgens*. In this study, we grouped together colonies resulting from a fusion or fission event into 33 groups or ‘growth units’. When fusion occurred, the areas of the previous colonies were added together to obtain a single value for each unit. When fission occurred, areas of the resulting colonies were added (Turon and Becerro 1992). Monthly growth rates (GR) were calculated as follows:

$$GR_m = \frac{(A_m - A_{m-1})}{A_{m-1}}$$

Where A_m and A_{m-1} are the areas at month m and at the previous month ($m-1$) respectively. These values were then smoothed to avoid noisy fluctuations using a weighed moving average: the value of the current month (GR_m) multiplied by 0.5 plus values of the previous (GR_{m-1}) and subsequent (GR_{m+1}) months multiplied by 0.25.

Cross-correlation analysis

Cross-correlation analyses were used to establish relationships between growth rate, maturity index, and temperature over time. In cross-correlations, two series of data are compared as a function of a time lag (n), using the Pearson correlation coefficient to measure relationships between values of the first data series and values of the second series n months earlier (in negative lags) or later (in positive lags). Correlation

coefficient values at lag 0 are equivalent to the standard Pearson correlation. We used
Systat v. 12.02 package for the analyses.

219

220 Results

221

222 Reproductive cycle

223

224 The time course of the main reproductive categories established for *Didemnum*
225 *fulgens* (Figure 1) showed a marked seasonal fluctuation in reproduction, with larval
226 release occurring mostly in spring and early summer. Immature zooids were recorded
227 all year round but were more abundant from July/August to December in the three
228 investigated years. Zooids with testes were present from January to April, zooids
229 combining testes and oocytes were found from March to May and zooids with only
230 oocytes were present from May to June. Larval release occurred in early summer before
231 the warmest sea temperatures were reached. Years with sharp increases in temperature
232 coincided with a shorter larval release period (e.g. one month in 2010) while slower
233 increases of temperature were accompanied by longer spawning events (e.g. three months
234 in 2011).

235 The maturity index (MI) increased in winter and achieved its maximum
236 around June each year, coinciding with the brooding period (Figure 2). After June, MI
237 decreased abruptly before seawater temperature reached its maximum, and maintained
238 low values until the following winter. Cross-correlation analysis showed a significant
239 negative correlation between the maturity index and the water temperature measured a
240 few months before (Figure 3). A MI rise was therefore preceded by a temperature
241 minimum one to four months before (time lag -1 to -4). The wave-like form of the cross-

242 correlation plot, with peaks separated by ca. 6 months, reflected the seasonal nature of
243 the evolution of both temperature and MI.

244 Most colonies entered into a resting state after reproduction that was
245 maintained during the warmer months of the year (June, July, and August). The
246 resting form was characterized by a closure of all inhalant siphons and a ‘leather-like’
247 appearance of the colonies (Figure 4). Most colonies resumed filtering (i.e. opened
248 inhalant siphons) in September and by October all colonies were actively feeding.

250 **Growth cycle**

251
252 Overall, the mean area of the colonies surveyed increased 261% (± 81 SE) during the
253 study period (Figure 5). Growth rates showed a clear seasonal trend, with lower
254 values in spring and summer and higher values in fall and winter (Figure 6). A total of
255 18 fusions and 24 fissions occurred in 51% of the colonies surveyed (Figure 7).
256 Fusions and fissions were recorded throughout the year although fusions were more
257 abundant in spring and fissions in fall (Figure 7). Fusions mainly occurred in May and
258 April after most of the colonies reached their maximal growth rate. Most fissions
259 occurred from June to January, corresponding with reactivation after summer
260 dormancy and lower levels of growth. Cross-correlation results indicated that growth
261 was significantly correlated with temperature, as growth rates increased in the colder
262 months and decreased in the warmer months (Figure 8A). There was a negative and
263 significant correlation at time lags -1, 0, 1 and 2 (Figure 8A). Thus, growth rates were
264 negatively correlated with the current water temperature and the temperature in the
265 previous and following one to two months.

Relationships between growth and reproduction were investigated to look for potential trade-offs in the allocation of energy resources. Our results showed opposite trends for the two variables: when the maturity index increased, growth rates decreased and vice versa (Figure 8B). Cross-correlation analyses further confirmed this observation revealing a significant negative correlation between growth rate and the maturity index at time lags -1 and -2, indicating that higher investment in reproduction in the previous one and two months was related with lower current growth (Figure 8B).

Discussion

The colonial ascidian *Didemnum fulgens* presented a clear seasonal life cycle alternating between reproduction and growth. Maturation of the gonads started in winter and culminated with larvae release in early summer, just before maximal sea temperatures were reached. After spawning, colonies went into aestivation coinciding with the warmer months of the year. Growth started in fall and continued during winter, with a decrease in spring at the time of the rise of the maturity index. Reproduction was positively correlated with seawater temperature in the previous months, while growth rates were negatively correlated with current temperature and with investment in reproduction during the preceding months.

Temperature has often been reported as the main abiotic factor triggering ascidian life cycles in temperate seas (e.g. de Caralt et al. 2002; López-Legentil et al. 2005; Pérez-Portela et al. 2007). Likewise, both the reproduction and growth cycles of *D. fulgens* were tightly correlated with temperature shifts over time. In the Mediterranean, ascidians reproduce either before or after maximal seawater temperatures are reached, with reproductive timing related to the biogeographical

291 distribution of each ascidian species. Atlanto-Mediterranean species that have cold-
292 water affinities reproduce during winter, while endemic or Senegalian species
293 reproduce in spring-early summer (Turon 1988). Moreover, the duration of the
294 reproductive period in ascidians may also be related to the rate at which maximal
295 seawater temperatures are reached each year. For instance, in our study we found that
296 years with sharp increases in temperature coincided with a shorter larval release
297 period (e.g. 2010) while slower increases of temperature were accompanied by longer
298 spawning events (e.g. 2011). Similarly, Pérez-Portela et al. (2007) showed that when
299 seawater temperature increased sharply, *Pycnoclavella aurilucens* incubated larva for
300 two months, while incubation could last up to four months when maximal
301 temperatures were reached more progressively. To date, there is less than a handful
302 studies describing ascidian life cycles during more than two years. Although long-
303 term monitoring studies are labor-intense and time consuming, they remain necessary
304 to acquire a thorough understanding of the factors that influence ascidian life cycles in
305 temperate seas.

306 Aestivation periods and resting forms are common in Mediterranean ascidians
307 and have been ascribed to unfavorable conditions, such as high seawater temperatures
308 and decreases in food availability for filter-feeding organisms (Coma and Ribes 2003;
309 Coma et al. 2000). *D. fulgens* was also observed to acquire a leather-like appearance
310 following reproduction events and stopped filtering throughout summer. Similarly,
311 most Mediterranean ascidians with resting forms reproduce before summer (e.g.
312 *Polysyncraton lacazei*, Turon and Becerro 1992; *Clavelina lepadiformis*, de Caralt et
313 al. 2002; *Cystodytes dellechiaiei*, López-Legentil et al. 2005; *Pycnoclavella brava*,
314 Pérez-Portela et al. 2007), while the few exceptions correspond to Atlanto-
315 Mediterranean species (e.g. *Applidium* aff. *conicum*, Turon and Becerro 1992;

1
2
3 316 *Pycnoclavella communis*, Pérez-Portela et al. 2007). What happens while a colony is
4
5 317 resting is unknown in most cases and depends on the species (Burighel et al. 1976;
6
7 318 Turon 1992). For the only didemnid ascidian investigated in the Mediterranean,
8
9 319 *Polysyncraton lacazei*, Turon (1992) suggested that a 'rejuvenative' process took
10
11 320 place during the resting period and involved the regression of old thoraces and
12
13 321 budding of new ones. Further studies are needed to establish whether the same
14
15 322 phenomenon occurs in *D. fulgens*. A thorough understanding of the physiological
16
17 323 processes involved in the development of resting forms, will cast light on the
18
19 324 implications of these processes for ascidian health, long-term survival, and space
20
21 325 occupation and maintenance.
22
23
24

25 326 Colonial ascidians are characterized by their capacity to divide and form
26
27 327 smaller colonies (fission) and to grow rapidly by merging previously independent
28
29 328 units (fusion). Fragment dynamics are high in some tropical and temperate didemnids
30
31 329 (Bak et al. 1981; Ryland et al. 1984; Stocker 1991). In *D. fulgens*, these processes
32
33 330 were moderate, although they occurred all year round with a maximum of fissions
34
35 331 recorded in fall and fusions in spring. In other Mediterranean ascidians, clear seasonal
36
37 332 patterns in the occurrence of fission and fusion events were not observed (e.g.
38
39 333 *Cystodytes dellechiaiei*, López-Legentil et al. 2005). Fusion has been reported to be a
40
41 334 mechanism by which some ascidians species achieve the minimal colony size
42
43 335 necessary for reproduction (Ritzmann et al. 2009). Accordingly, fusions in *D. fulgens*
44
45 336 were more common in spring and early summer, coinciding with the reproduction
46
47 337 period of this species. Further, several studies have demonstrated that smaller colonies
48
49 338 of ascidians exhibit faster growth rates (Stocker 1991; Turon and Becerro 1992;
50
51 339 López-Legentil et al. 2005). In *D. fulgens*, fissions were more common when growth
52
53 340 rates were higher, indicating that most of the growth was conducted by smaller
54
55
56
57
58
59
60

colonies. Active growth of smaller colonies would also allow this species to rapidly colonize space left by regressing sessile invertebrates during summer. This strategy may confer an important competitive edge to *D. fulgens* over other species, as shown by the overall increase on total surface area observed during the study period and the observed proliferation of this species during the last decade.

Resource allocation to reproduction or growth depends on the requirements of an organism in a given environment and its energy budget within evolutionary and ecological constraints (Cohen 1971; Kozłowski 1992). Mounting evidence indicates that seasonal partitioning of available resources for either growth or reproduction is a common strategy among colonial ascidians in the Mediterranean Sea (López-Legentil et al. 2005; Pérez-Portela et al. 2007). These trade-offs in resource allocation may be linked to temperature changes, nutrient availability and/or ‘sexual exhaustion’ after larval brooding has drained all nutrient reserves (Berrill 1935; López-Legentil et al. 2007). Brooding strategies in colonial ascidians range from the production of a few large and complex larvae per zooid to many small and simple larvae (i.e. larvae with undifferentiated tissues). Reproductive investment per zooid has been shown to be significantly higher in species that produced few well-developed larvae, especially members of the family Didemnidae (Tarjuelo and Turon 2004). As *D. fulgens* produces a single larva per zooid, we hypothesize that investment in reproduction for this species is high and that little to no energy is available for other biological functions (i.e. growth) during its reproductive period. A temporal trade-off between growth and reproduction, where the latter acted as the major energy sink, was previously described for another colonial ascidian in the Mediterranean Sea (López-Legentil et al. 2007) and may be a common strategy among species with high reproductive investment.

366 In this study, we provided the first data on reproductive and growth cycles of a
367 proliferating didemnid ascidian in the Western Mediterranean. As reported for other
368 colonial ascidian species, the life cycle of *D. fulgens* presented a marked seasonality
369 and suggested resource partitioning between reproduction and growth. Seasonal
370 patterns in fission and fusion events were coincident with higher growth rates and the
371 onset of the reproductive periods, respectively, and may contribute to the optimization
372 of resource allocation and ecological success of *D. fulgens*. Overall, colonies
373 increased in surface area during the study period, continuing the expansion observed
374 for this species during the last decade that may displace other common species in the
375 rocky infralittoral. There is a lack of information on ascidian life cycles and their
376 response to current and future fluctuations in abiotic factors. Although primarily
377 descriptive, this information is critical to understand how further natural and
378 anthropological perturbations may affect the distribution and abundance of these
379 animals and the consequences of species expansions on benthic community diversity
380 in the Mediterranean Sea.

381
382 **Acknowledgements.** We thank Iosune Uriz, Sonia de Caralt and Janina González
383 (CEAB-CSIC) for providing some of the samples. This research was funded by the
384 Marie Curie International Reintegration Grant FP7-PEOPLE-2010-RG 277038 within
385 the 7th European Community Framework Program, the Spanish Government projects
386 CTM2010-17755 and CTM2010-22218, and the Catalan Government grant
387 2009SGR-484 for Consolidated Research Groups.

388 389 References

390

391 Agell G, Turon X, De Caralt S, López-Legentil S, Uriz MJ & 2004. Molecular and
392 organism biomarkers of copper pollution in the ascidian *Pseudodistoma*
393 *crucigaster*. Marine Pollution Bulletin 48:759-767.

394 Azumi K, Amano S, Sabau SV, Kamimura A, Satoh N & Koyanagi R. 2008.
395 Application of ascidian DNA microarray analysis for risk assessment of
396 marine chemical pollutants. In: Interdisciplinary studies on environmental
397 chemistry - Biological responses to chemical pollutants. Murakami Y,
398 Nakayama K, Kitamura S-I, Iwata H, Tanabe S, eds. pp. 107-110. Terrapub.

399 Bak RPM, Sybesma J & van Duyl FC. 1981. The ecology of the tropical compound
400 ascidian *Trididemnum solidum*. II. Abundance, growth and survival. Marine
401 Ecology Progress Series 6:43-52.

402 Balata D, Nesti U, Piazzzi L & Cinelli F. 2007. Patterns of spatial variability of
403 seagrass epiphytes in the north-west Mediterranean Sea. Marine Biology
404 151:2025-2035.

405 Bates WR. 2005. Environmental factors affecting reproduction and development in
406 ascidians and other protochordates. Canadian Journal of Zoology 83:51-61.

407 Berrill NJ. 1935. Studies in tunicate development. Part III. Differential retardation and
408 acceleration. Philosophical Transactions of the Royal Society of London.
409 Series B, Biological Sciences 225:255-326.

410 Berrill NJ. 1951. Regeneration and budding in tunicates. Biological Reviews 26:456-
411 476.

412 Bishop DD & Sommerfeldt AD. 1999. Not like *Botryllus*: Indiscriminate post-
413 metamorphic fusion in a compound ascidian. Proceedings of the Royal Society
414 of London 266:241-248.

- 1
2
3 415 Burighel P, Brunetti R & Zaniolo G. 1976. Hibernation of the colonial ascidian
4
5 416 *Botrylloides leachi* (Savigny): Histological observations. Bolletino di zoologia
6
7 417 43:293-301.
8
9
10 418 Carman MR. 2007. Water quality, nitrogen pollution, and ascidian diversity in coastal
11
12 419 waters of southern Massachusetts, USA. Journal of Experimental Biology and
13
14 420 Ecology 342:175-178.
15
16 421 Carman MR, Morris JA, Karney RC & Grunden DW. 2010. An initial assessment of
17
18 422 native and invasive tunicates in shellfish aquaculture of the North American
19
20 423 east coast. Journal of Applied Ichthyology 26:8-11.
21
22
23 424 Cohen D. 1971. Maximizing final yield when growth is limited by time or by limiting
24
25 425 resources. Journal of Theoretical Biology 33:299-307.
26
27 426 Coma R & Ribes M. 2003. Seasonal energetic constraints in Mediterranean benthic
28
29 427 suspension feeders: effects at different levels of ecological organization. Oikos
30
31 428 101:205-215.
32
33
34 429 Coma R, Ribes M, Gili J-M & Zabala M. 2000. Seasonality in coastal benthic
35
36 430 ecosystems. Trends in Ecology and Evolution 15:448-453.
37
38 431 de Caralt S, López-Legentil S, Tarjuelo I, Uriz JM & Turon X. 2002. Contrasting
39
40 432 biological traits of *Clavelina lepadiformis* (Ascidiacea) populations from
41
42 433 inside and outside harbours in the western Mediterranean. Marine Ecology
43
44 434 Progress Series 244:125–137.
45
46
47 435 Dias GM, Delboni CG & Duarte L. 2008. Effects of competition on sexual and clonal
48
49 436 reproduction of a tunicate: the importance of competitor identity. Marine
50
51 437 Ecology Progress Series 362:149-156.
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

438 Dijkstra J, Sherman H & Harris LG. 2007. The role of colonial ascidians in altering
439 biodiversity in marine fouling communities. *Journal of Experimental Biology*
440 and *Ecology* 342:169-171.

441 Dijkstra JA & Harris LG. 2009. Maintenance of diversity altered by a shift in
442 dominant species: implications for species coexistence. *Marine Ecology*
443 *Progress Series* 387:71-80.

444 Erwin PM, López-Legentil S & Schuhmann PW. 2010. The pharmaceutical value of
445 marine biodiversity for anti-cancer drug discovery. *Ecological Economics*
446 70:445-451.

447 Fiore CL & Cox Jutte P. 2010. Characterization of macrofaunal assemblages
448 associated with sponges and tunicates collected off the southeastern United
449 States. *Invertebrate Biology* 129:105-120.

450 Fukuda T & Hirose E. 2008. Differences in associated crustacean fauna and
451 seasonality of sexual reproduction between two color morphs of the
452 photosymbiotic ascidian *Didemnum molle* (Asciacea: Didemnidae). *BioOne*
453 62:309-316.

454 Hernández-Zanuy A, García-Cagide A, Esquivel M & Blanco A. 2000. Reproducción
455 y desarrollo de *Ecteinascidia turbinata* (Ascideacea: Perophoridae) en Cuba
456 *Revista de Biología Tropical* 48.

457 Hewitt CL, Gollasch S & Minchin D. 2009. The vessel as a vector - Biofouling,
458 ballast water and sediments. In: *Biological invasions in marine ecosystems*.
459 Rilov GR, Crooks JA, eds. pp. 117-131. Springer-Verlag, Berlin Heidelberg.

460 Kozlowski J. 1992. Optimal allocation of resources to growth and reproduction:
461 Implications for age and size at maturity. *Trends in Ecology and Evolution*
462 7:15-19.

- 1
2
3 463 Kremer LP, Rocha RM & Roper JJ. 2010. An experimental test of colonization ability
4
5 464 in the potentially invasive *Didemnum perlucidum* (Tunicata, Ascidiacea).
6
7 465 Biological Invasions 12:1581-1590.
8
9
10 466 Lafargue F. 1976. Révision taxonomique des Didemnidae des côtes de France
11
12 467 (Ascidies composées). Les espèces de Banyuls-Sur-Mer. Genre *Didemnum*.
13
14 468 Deuxième partie: Larves a deux ventouses. Annales de l'Institut
15
16 469 Océanographique 52:259-281.
17
18
19 470 Lafargue F & Wahl M. 1987. The didemnid ascidian fauna of France. Annales de
20
21 471 l'Institut Océanographique 63:1-46.
22
23 472 Lambert CC. 2005. Historical introduction, overview, and reproductive biology of the
24
25 473 protochordates. Canadian Journal of Zoology 83:1-7.
26
27
28 474 Lambert G. 2009. Adventures of a sea squirt sleuth: unraveling the identity of
29
30 475 *Didemnum vexillum*, a global ascidian invader. Aquatic Invasions 4:5-28.
31
32 476 López-Legentil S, Bontemps-Subielos N, Turon X & Banaigs B. 2007. Secondary
33
34 477 metabolite and inorganic contents in *Cystodytes* sp. (Ascidiacea): temporal
35
36 478 patterns and association with reproduction and growth. Marine Biology
37
38 479 151:293-299.
39
40
41 480 López-Legentil S, Ruchty M, Domenech A & Turon X. 2005. Life cycles and growth
42
43 481 rates of two morphotypes of *Cystodytes* (Ascidiacea) in the western
44
45 482 Mediterranean. Marine Ecology Progress Series 296:219-228.
46
47
48 483 Marshall DJ, Cook CN & Emlet RB. 2006. Offspring size effects mediate competitive
49
50 484 interactions in a colonial marine invertebrate. Ecology 87:214-225.
51
52 485 Millar RH. 1971. The biology of ascidians. Advances in Marine Biology 9:1-100.
53
54 486 Molin E, Gabriele M & Brunetti R. 2003. Further news on hard substrate communities
55
56 487 of the northern Adriatic Sea with data on growth and reproduction in *Polycitor*
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

488 *adriaticus* (Von Drasche, 1883). Bollettino del Museo Civico di Storia
489 Naturale di Venezia 54:19-28.

490 Newlon III AW, Yund PO & Stewart-Savage J. 2003. Phenotypic plasticity of
491 reproductive effort in a colonial ascidian, *Botryllus schlosseri*. Journal of
492 Experimental Zoology 297:180-188.

493 Pérez-Portela R, Palacín C, Duran S & Turon X. 2007. Biological traits of three
494 closely related species of *Pycnoclavella* (Ascidacea) in the Western
495 Mediterranean. Marine Biology 152:1031-1038.

496 Rinkevich B. 2005. Natural chimerism in colonial urochordates. Journal of
497 Experimental Biology and Ecology 322:93-109.

498 Rinkevich B & Saito Y. 1992. Self-nonsel self recognition in the colonial protochordate
499 *Botryllus schlosseri* from Mutsu Bay, Japan. Zoological Science 9:983-988.

500 Ritzmann NF, da Rocha RM & Roper JJ. 2009. Sexual and asexual reproduction in
501 *Didemnum rodriguezi* (Ascidacea, Didemnidae). Iheringia, Série Zoologia
502 99:106-110.

503 Rius M, Turon X, Dias GM & Marshall DJ. 2010. Propagule size effects across
504 multiple life-history stages in a marine invertebrate. Functional Ecology
505 24:685-693.

506 Rodriguez LF & Ibarra-Obando SE. 2008. Cover and colonization of commercial
507 oyster (*Crassostrea gigas*) shells by fouling organisms in San Quintin Bay,
508 Mexico. BioOne 27:337-343.

509 Ryland JS, Wigley RA & Muirhead A. 1984. Ecology and colonial dynamics of some
510 Pacific reef flat Didemnidae (Ascidacea). Zoological Journal of the Linnean
511 Society 80:261-282.

- 1
2
3 512 Saito Y, Hirose E & Watanabe H. 1994. Allorecognition in compound ascidians.
4
5 513 International Journal of Developmental Biology 38:237-247.
6
7 514 Shenkar N, Bronstein O & Loya Y. 2008. Population dynamics of a coral reef
8
9 515 ascidian in a deteriorating environment. Marine Ecology Progress Series
10
11 516 367:163-171.
12
13 517 Shenkar N & Loya Y. 2008. The solitary ascidian *Herdmania momus*: native (Red
14
15 518 Sea) versus non-indigenous (Mediterranean) populations. Biological
16
17 519 Invasions:1-9.
18
19 520 Smale DA & Childs S. 2012. The occurrence of a widespread marine invader,
20
21 521 *Didemnum perlucidum* (Tunicata, Ascidiacea) in Western Australia.
22
23 522 Biological Invasions 14:1325-1330.
24
25 523 Stefaniak L, Lambert G, Gittenberger A, Zhang H, Lin S & Whitlatch RB. 2009.
26
27 524 Genetic conspecificity of the worldwide populations of *Didemnum vexillum*
28
29 525 Kott, 2002. Aquatic Invasions 4:29-44.
30
31 526 Stefaniak L, Zhang H, Gittenberger A, Smith K, Holsinger K, Lin S & Whitlatch RB.
32
33 527 2012. Determining the native region of the putatively invasive ascidian
34
35 528 *Didemnum vexillum* Kott, 2002. Journal of Experimental Marine Biology and
36
37 529 Ecology 422-423.
38
39 530 Stocker LJ. 1991. Effects of size and shape of colony in rates of fission, fusion,
40
41 531 growth and mortality in a subtidal invertebrate. Journal of Experimental
42
43 532 Biology and Ecology 149:161-175.
44
45 533 Stocker LJ & Underwood AJ. 1991. The relationship between the presence of
46
47 534 neighbours and rates of sexual and asexual reproduction in a colonial
48
49 535 invertebrate. Journal of Experimental Biology and Ecology 149:191-205.
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

536 Svane IB & Young CM. 1989. The ecology and behaviour of ascidian larvae.
537 Oceanography and Marine Biology: Annual Reviews 27:45-90.
538 Tarjuelo I & Turon X. 2004. Resource allocation in ascidians: reproductive
539 investment vs. other life-history traits. Invertebrate Biology 123:168-180.
540 Turner SJ, Thrush SF, Cummings VJ, Hewitt JE, Wilkinson MR, Williamson RB &
541 Lee DJ. 1997. Changes in epifaunal assemblages in response to marina
542 operations and boating activities. Marine Environmental Research 43:181-199.
543 Turon X. 1988. The ascidians of Tossa de Mar (NE Spain). II.- Biological cycles of
544 the colonial species. Cahiers de Biologie Marine 29:407-418.
545 Turon X. 1992. Periods of non-feeding in *Polysyncraton lacazei* (Ascidacea:
546 Didemnidae): a rejuvenative process? Marine Biology 112:647-655.
547 Turon X & Becerro MA. 1992. Growth and survival of several ascidian species from
548 the northwestern Mediterranean. Marine Ecology Progress Series 82:235-247.
549 Westerman EL, Dijkstra JA & Harris LG. 2009. High natural fusion rates in a
550 botryllid ascidian. Marine Biology 156:2613-2619.
551

552 **Figure legends**

553

554 **Figure 1.** Annual reproductive cycle in *Didemnum fulgens* and ambient seawater
555 temperature over the monitoring period. The percentage of zooids in different
556 reproductive stages (mean of 10 zooids per colony, 5 colonies per month) are shown
557 for each month, with stars indicating the presence of larvae in the colonies. Empty
558 columns correspond to months without data and temperature data (dashed line) are
559 superimposed.

560

561 **Figure 2.** Time course of maturity index (MI) evolution in *Didemnum fulgens*. Error
562 bars represent ± 1 standard error and temperature data (dashed line) are superimposed.

563

564 **Figure 3.** Cross-correlations analysis between the maturity index and temperature,
565 with time lag in months. Curved lines indicate significant positive (upper) and
566 negative (lower) correlation values.

567

568 **Figure 4.** *In situ* photographs of a *Didemnum fulgens* colony monitored for growth in
569 the Western Mediterranean. (A) Resting form in July 2011, and (B) the same colony
570 in January 2012. Scale bar = 2 cm.

571

572 **Figure 5.** Mean cumulative growth of *Didemnum fulgens* over time in the surveyed
573 area. Error bars represent ± 1 standard error.

574

575 **Figure 6.** Monthly growth rates of *Didemnum fulgens* and seawater temperature over
576 time. Error bars represent ± 1 standard error.

577

578 **Figure 7.** Number of fusion (dark gray bars) and fission (light gray bars) events
579 recorded during the monitoring period, with monthly growth rates (line)
580 superimposed. Error bars represent ± 1 standard error.

581

582 **Figure 8.** Cross-correlations analyses between (A) growth rate and temperature, and
583 (B) maturity index and growth rate, with time lag in months. Curved lines indicate
584 significant positive (upper) and negative (lower) correlation values.

585

Annual reproductive cycle in *Didemnum fulgens* and ambient seawater temperature over the monitoring period. The percentage of zooids in different reproductive stages (mean of 10 zooids per colony, 5 colonies per month) are shown for each month, with stars indicating the presence of larvae in the colonies. Empty columns correspond to months without data and temperature data (dashed line) are superimposed.

352x264mm (72 x 72 DPI)

Time course of maturity index (MI) evolution in *Didemnum fulgens*. Error bars represent ± 1 standard error and temperature data (dashed line) are superimposed.
96x64mm (300 x 300 DPI)

Cross-correlations analysis between the maturity index and temperature, with time lag in months. Curved lines indicate significant positive (upper) and negative (lower) correlation values.

264x382mm (72 x 72 DPI)

In situ photographs of a *Didemnum fulgens* colony monitored for growth in the Western Mediterranean. (A) Resting form in July 2011, and (B) the same colony in January 2012. Scale bar = 2 cm. 140x60mm (300 x 300 DPI)

Mean cumulative growth of *Didemnum fulgens* over time in the surveyed area. Error bars represent ± 1 standard error.
152x132mm (300 x 300 DPI)

Monthly growth rates of *Didemnum fulgens* and seawater temperature over time. Error bars represent ± 1 standard error.
152x122mm (300 x 300 DPI)

Number of fusion (dark gray bars) and fission (light gray bars) events recorded during the monitoring period, with monthly growth rates (line) superimposed. Error bars represent ± 1 standard error.
352x264mm (72 x 72 DPI)

Cross-correlations analyses between (A) growth rate and temperature, and (B) maturity index and growth rate, with time lag in months. Curved lines indicate significant positive (upper) and negative (lower) correlation values.
264x382mm (72 x 72 DPI)