Reproductive Cycles of the Ascidians *Microcosmus sabatieri* and *Halocynthia papillosa* in the Northwestern Mediterranean

M. A. Becerro[1] & X. Turon[2]

[1] Centre d'Estudis Avançats de Blanes, Camí de Santa Bàrbara s/n., E-17300 Blanes (Girona), Spain.
[2] Departament de Biologia Animal (Invertebrats), Facultat de Biologia, Avgda. Diagonal 645, E-08028 Barcelona, Spain.

Abstract. The reproductive cycles of *Microcosmus sabatieri* Roule and *Halocynthia papillosa* (Linnaeus) *(Ascidiacea, Pyuridae)* were investigated by means of histological observations of the gonads and gonad index measurements on monthly samples from Northeast Spanish coast populations during 1985. Both species are simultaneous hermaphrodites, and the spawning season lasts for two to three months – between September and November – after the period of highest water-temperatures in the area. The evolution of the gonads during the whole cycle is markedly different in both species. Spawning time is extended towards summer in *H. papillosa* and towards winter in *M. sabatieri*.

Problem

Ascidians are important members of the benthic assemblages in the Mediterranean Sea; they feature a variety of morphological and biological types resulting in a diverse ecological distribution. Typification requires studies of reproduction and population dynamics which, in turn, will help us to understand the processes underlying the pattern of the observed distribution. Moreover, the hermaphroditic nature of ascidians adds another point of interest to reproduction studies (Kessel, 1983; Franzen, 1983). Knowledge of the reproductive cycles of ascidians is, however, fragmentary. Studies carried out on this subject are reviewed in Millar (1971) and Berrill (1975), and recent data of the Mediterranean zone can be found in Tursis *et al.* (1974), Brunetti (1976), Brunetti *et al.* (1988), and Turon (1988).

In most colonial ascidians the cyclic presence of gonads and/or larvae allows an easy assessment of the reproductive condition. In many solitary species, however, gonads are present year round and evaluation of their maturing state

requires histological observation. Microscopical studies of the gonad condition are scarce (MILLAR, 1954; SABBADIN, 1958; DYBERN, 1965; SVANE & LUNDALV, 1981, 1982), and quantitative histological methods (estimation of oocyte diameters) have seldom been applied (SVANE & LUNDALV, 1982; SVANE, 1984). To our knowledge, the method of coupling histological data with gonad index measurements (GIESE & PEARSE, 1974) has not yet been used in ascidians.

In this paper, we analyze the reproductive cycles of the two most common solitary ascidian species on the rocky littoral of the Northeast coast of Spain, *Microcosmus sabatieri* ROULE, 1855, and *Halocynthia papillosa* (LINNAEUS, 1767) (family *Pyuridae*).

Material and Methods

This study was carried out in Tossa de Mar (NE Spain). Data on the sampling site and its ascidian fauna are given in TURON (1987). The reproductive cycles of the colonial species from this area have been the subject of another paper (TURON, 1988), whilst the ecological distribution is discussed in TURON (1990). *Microcosmus sabatieri* and *Halocynthia papillosa* are abundant at the sublittoral level, in sciaphilic assemblages under 10 m (Ros *et al.*, 1985). Specimens of both species were collected by monthly SCUBA diving samplings between February, 1985 and January, 1986. From six to ten large, uniformly-sized individuals of each species (length of contracted specimens: 7–9 cm for *H. papillosa*, 10–13 cm for *M. sabatieri*) were collected between 10 and 15 m depth for analysis of reproductive condition. Water temperature was recorded simultaneously at 10 m depth.

The organisms were dissected in the laboratory and the right gonads removed from the mantle and weighed (wet weight). Small portions of the central part of the right gonads were cut out and placed in BOUIN's fixative. This tissue was then embedded in paraffin, sectioned, and stained with hematoxylin-eosin.

The dry weight of the mantles (without tunic) and the gonads was obtained after drying to a constant weight (24 h at 105 °C). The dry weight of the gonad portions removed for histological study was inferred after calculation of the gonad wet weight/dry weight ratio. A gonad index was then calculated using the dry weight of the mantles and the right gonads. The left gonads were not included in order to increase the accuracy of the measurements: they are connected with the digestive system and a perfect separation is difficult.

A whole section of each gonad was studied by light microscopy, and the diameter of each oocyte which appeared sectioned at the nucleolus level was measured. About one hundred oocytes per gonad were measured. When necessary, additional sections at other levels of the same gonad were measured to attain the required number of oocytes. The oocytes were placed into 50 μm size classes, a grouping suitable for constructing visually satisfactory histograms in view of the size range found.

The degree of maturity of the testes was also noted and classified according to five (subjective) categories of male follicle development (*i. e.*, absent, traces, low, medium, and high).

Statistical analyses were performed on the gonad index and oocyte diameter values. KOL-MOGOROV-SMIRNOV and BARTLETT tests were used to test normality and homoscedasticity of the data before applying parametric statistics (ZAR, 1984).

Results

1. Histology and development of the gonads

Both species are hermaphroditic. The gonads are elongated and U-shaped in *H. papillosa*, whereas in *M. sabatieri* they consist of several flattened lobes closely adhering to the mantle wall.

In *H. papillosa* the transverse sections were circular in outline. The male

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follicles, when present, appeared in the periphery of a central core of oocytes grouped around ciliated oviducts. The course of vitellogenesis was continuous throughout the year. Previtellogenic oocytes were found from June onwards, reaching large numbers in October and November. They matured gradually until by March the gonads appeared packed with fully vitellogenic oocytes. The development of the male follicles, in contrast, was sudden, mature sperm being visible from July onwards. The gonads in September were therefore full with mature oocytes and sperm (Figs. 1, 2). The release of gametes took place in September and October, and by November the gonads exhibited a spent condition (Fig. 3) with a few large oocytes, numerous young oocytes, and regressing testicular lobes. This was followed by the immature condition in which the new oocytes began their gradual development (Fig. 4).

In *M. sabatieri*, the female portions occupied the central and basal part of the flattened gonadal sections. The male follicles, when developed, filled the periphery of the gonadal lobes (sometimes extending towards the basal layer). Immature oocytes were found in the gonads year round. Spermatocytes appeared in some sections by the end of July. Sperm and oocyte maturation was rapid and synchronous during August and September (Fig. 5), reaching a maximum in October. From October to November the number of mature oocytes decreased. By January large oocytes were scattered among clusters of immature oocytes, and most of the male follicles appeared empty. From January to July, a large mass of clear, amorphous matrix substance (see Fig. 6) made up most of the gonads, with small, early vitellogenic oocytes clustered around ciliated oviducts.

No phagocytosis of residual gametes was observed in any species.

2. Quantitative measurements

The size-frequency histograms of the oocyte diameters, mean oocyte sizes, and the gonad index (GI) during the study period are depicted in Figs. 7 and 8. The development of the testes in the established categories is indicated. The recorded water temperatures are added for comparison.

In *H. papillosa* (Fig. 7) the decline of large size oocytes indicated spawning in September–October. Mean oocyte diameter increased gradually until September when large numbers of mature oocytes were present. Immature oocytes appeared in June, and by November almost all the oocytes in the gonad belonged to the size classes below 100μm.

The sperm maximum was also found in September–October. The GI reached values above 0.5 by the first half of September and decreased abruptly to a minimum below 0.1 by November, indicating gamete release.

In *M. sabatieri* (Fig. 8), spawning activity, as shown by the decline in the number of oocytes of the larger size classes and the decrease of the GI, lasted from October–November through January. The gamete release decreased from November onwards, although some large-sized oocytes were present in the gonad until December-January. The maximum oocyte size is similar to that found in *H. papillosa* (300μm). The mean oocyte diameters remained fairly constant (< 100μm) from January to August. In August, September, and
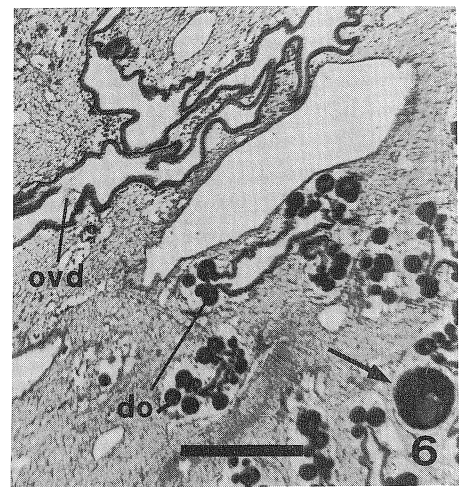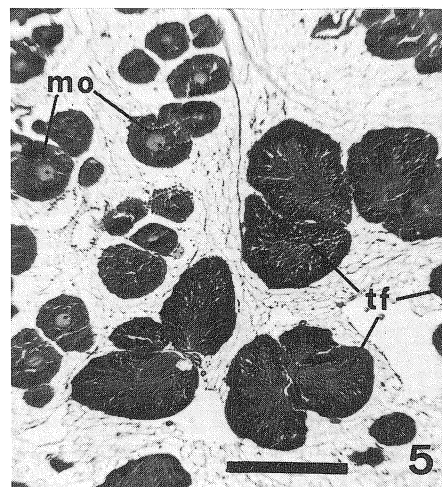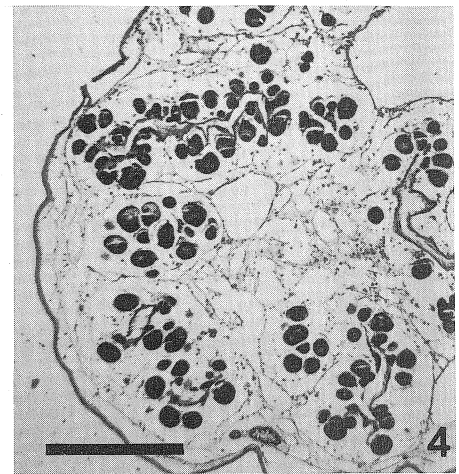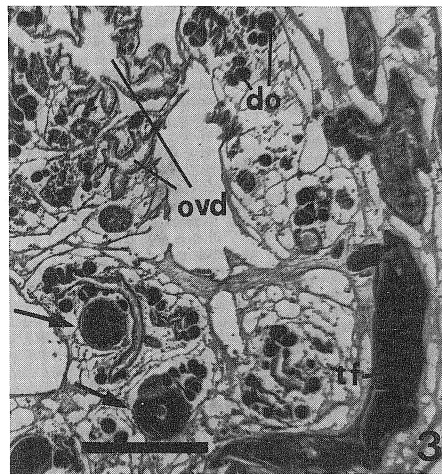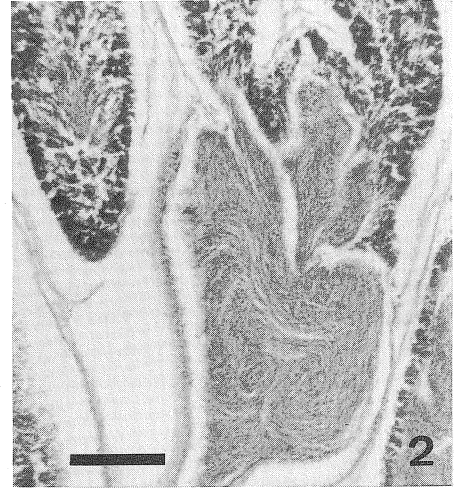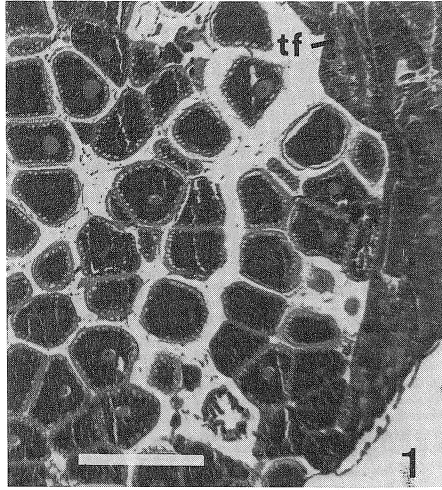
October, oocytes of the larger size classes (up to 300μm) appeared. The GI showed a similar pattern, indicating a maximum of maturity (GI about 0.3) in October, while during a long period (February–August) the GI values were under 0.1.

Two-way analyses of variance for unbalanced designs were performed using as factors species (two levels) and month (twelve levels). The results of this analysis on the mean oocyte diameters are presented in Table 1. Both main factors and the interaction term featured a significant F value. In the presence of a significant interaction, no general conclusion can be drawn from each factor separately. Instead, the significance of one factor must be studied at fixed values of the other factor (UNDERWOOD, 1981). In our case, since a significant sample (month) effect is expected due to the observed seasonal pattern, it is more interesting to substantiate differences between species than between months. Thus, separate t-tests were performed between the species at each month. These analyses showed significant differences in mean oocyte diameter between species in February, March, April, May, June, July, August, and November.

In the analysis of variance of the GI values the dry body weight was included as a covariate to test whether gonad indices were independent of body size (GRAND & TYLER, 1983 a). In a first step, the interaction of body weight with the other factors was tested and proved not significant; the assumption of homogeneity of slopes was thus not rejected and the model for a two-way analysis of covariance was tested with the results presented in Table 2. There were significant F-ratios for both species and month. The covariate was not

Fig. 1. *Halocynthia papillosa*, high activity gonadal condition for a specimen sampled in September. The gonad is full with large oocytes and peripheral testicular follicles (tf). Scale bar: 400 μm.

Fig. 2. *Halocynthia papillosa*, high magnification of the male portion of a gonad from a specimen of September. Scale bar: 100 μm.

Fig. 3. *Halocynthia papillosa*, spent condition (November). The oviducts (ovd) appear empty, and new developing oocytes (do) can be seen. Some residual oocytes of large size (arrows) and testicular follicles (tf) are still present. Scale bar: 400 μm.

Fig. 4. *Halocynthia papillosa*, developing oocytes in a gonad of December; no sperm follicles are visible. Scale bar: 400 μm.

Fig. 5. *Microcosmus sabatieri*, gonadal tissue. High gonadal activity condition at the end of September. The mantle wall is located towards the bottom and right of the figure. A layer of testicular follicles (tf) lies below the clusters of mature oocytes (mo). Scale bar: 400 μm.

Fig. 6. *Microcosmus sabatieri*, spent condition of a gonad from a specimen of January. Matrix material occupies most of the section. Empty oviducts (ovd) and newly developing oocytes (do) are visible. Arrow points at a residual, large-sized oocyte. Scale bar: 400 μm.
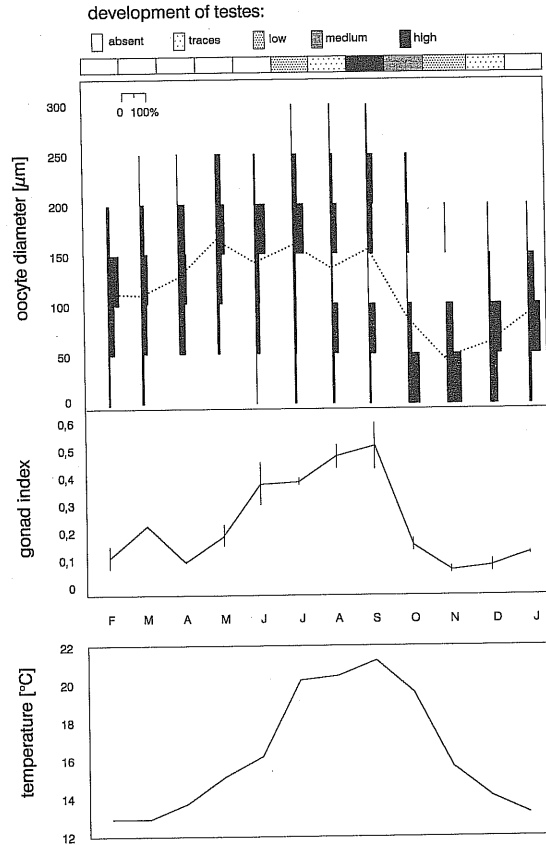
Fig. 7. *Halocynthia papillosa*, diagram showing the state of testes, oocyte size-frequency distribution, mean oocyte diameter (dashed line), and gonad index (vertical bars indicate standard deviations). Values are monthly means. The water temperature at 10 m depth is indicated.

Table 1. Two-way ANOVA of the mean oocyte diameters with species and month as main factors.

source	SS	df	MS	F ratio	P
species	0.28 E + 03	1	0.28 E + 03	0.66 E + 02	< 0.001
month	0.33 E + 03	11	0.299 E + 02	7.093	< 0.001
sp*m	0.26 E + 03	11	0.24 E + 02	5.660	< 0.001
error	0.16 E + 03	138	4.222		

Table 2. Two-way ANCOVA of the GI values with species and month as main factors. Data were arcsine transformed. The covariate is dry body weight.

source	SS	df	MS	F ratio	P
species	0.115	1	0.115	0.10 E + 02	0.002
month	0.662	11	0.060	5.283	< 0.001
sp*m	0.653	11	0.059	5.210	< 0.001
covariate	0.88 E–4	1	0.88 E–04	0.77 E – 02	0.930
error	0.752	137	0.011		

Fig. 8. *Microcosmus sabatieri*, diagram showing the state of testes, oocyte size-frequency distribution, mean oocyte diameter (dashed line), and gonad index (vertical bars indicate standard deviations). Values are monthly means. The water temperature at 10 m depth is indicated.
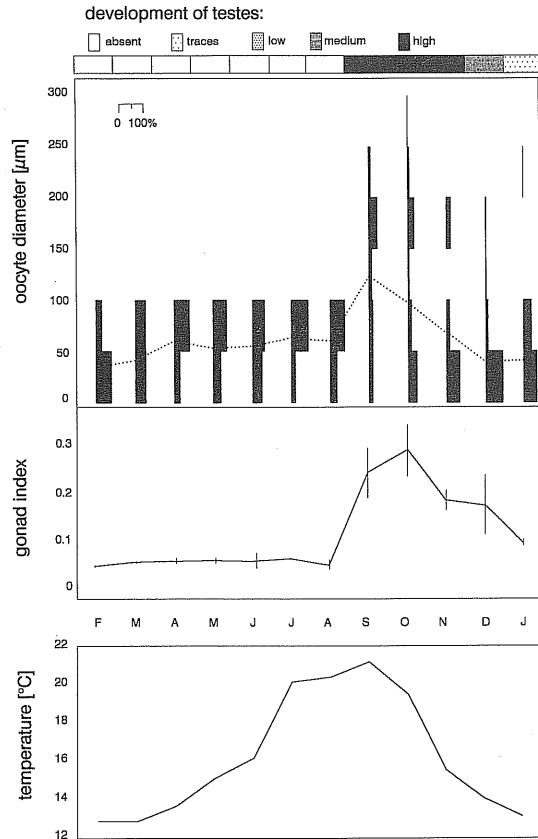
significant (P = 0.93), therefore a dependence of GI values on body weight was not demonstrated, at least in the range of body sizes studied (which was narrow due to the selection criteria). As in the previous case, the significant interaction term hindered separate interpretation of the factors. T-test analyses at each month showed significant differences of GI values between species in March, May, June, July, August, October, and November.

Discussion

Monthly variations in gonad histology, gonad index, and oocyte diameters indicate that both species in the studied area have reproductive cycles that include a single spawning season per year. The maximum gamete release took place between September and November.

Sperm and oocyte maturation took place synchronously, indicating that both species are simultaneous hermaphrodites; this condition is found in some solitary ascidians (SVANE & LUNDALV, 1981, 1982), while other species show a protandrous condition (SABBADIN, 1958).

Significant differences between both species in mean oocyte diameter from February to August reflect the gradual increase of oocyte sizes in *H. papillosa* during these months, whilst in *M. sabatieri* all the oocytes belonged to the size classes up to 100µm until September. The significant differences in GI values from March to August (with the exception of April) correspond to the higher GI values displayed by *H. papillosa* (see below) and to the gradual buildup of the gonad of this species during these months, while in *M. sabatieri* the GI did not rise until September. There were also significant differences between species during the spawning period (November for the oocyte diameter, October and November for the GI). This correlates with a lack of spawning synchrony: the graphs show an approximately one month time lag in spawning time between both species. The reproductive season of *M. sabatieri* is somewhat displaced towards winter, whereas that of *H. papillosa* starts earlier, by the second half of September, and ends by early November. In addition, gamete release appears to be gradual in *M. sabatieri* and more rapid in *H. papillosa*.

The gonad indices of *H. papillosa* were markedly higher than those observed in *M. sabatieri* (Figs. 7 and 8). However, the relative volume occupied by the gonads is higher in *M. sabatieri* (the right gonad occupies an average of 9.7 % of the total mantle volume) than in *H. papillosa* (average 6.3 %). This implies a higher gonad density in *H. papillosa* than in *M. sabatieri*. Since no accumulation of nutritional tissue has been observed histologically, the different gonad index may indicate a higher investment in reproduction in *H. papillosa* – an investment which is obtained throughout the year, as the oocyte diameters and GI increase gradually until the spawning season. On the other hand, *M. sabatieri* seems to allocate proportionally less energy to gamete production, and does so over a short period, just before spawning. This indicates that the reproductive strategie of these two taxonomically and ecologically related forms are different. Of course, these considerations on energy allocation require confirmation by studies on the calorific content of the gonads and the number of gametes released.

Another aspect is to examine the extent to which gonad indices and mean oocyte diameters gave coincident information (GRANT & TYLER, 1983 b). Histological preparation of gonad sections, together with oocyte measurements, are time-consuming activities that may be omitted if the data show a close relationship with other types of data of easier calculation, as the gonad indices. In our case, there is a good agreement between the gonad index used and the mean oocyte diameter in each month (*M. sabatieri*: PEARSON's r = 0.73, d. f. = 10, P < 0.01; *H. papillosa*: r = 0.71, P < 0.01). This implies that, for species of similar characteristics (as can be expected for other members of the family), analyses of gonad indices alone may provide a quick and easy estimation of the reproductive cycle. Temperature has been claimed as the main factor regulating the reproduction of marine invertebrates (ORTON, 1920), although the influence of other parameters cannot be ruled out (GIESE & PEARSE, 1974). In the case of ascidians, food availability (YAMAGUCHI, 1975), turbidity (MILLAR, 1974), or depth (SVANE, 1984) may be important factors. In particular, phytoplankton abundance can be relevant in our case, since strong seasonal variations in nutrients occur in the area, with minimal values observed during summer (BALLESTEROS, 1984).

Seasonal breeding has also been reported for colonial ascidian species in this area (TURON, 1988), which may not be surprising since temperatures in the Mediterranean fluctuate seasonally (ZABALA & BALLESTEROS, 1989). As in many invertebrates (GIESE & PEARSE, 1974), ascidians occurring in seas with fluctuating temperatures usually have sharply distinguished breeding and non-breeding periods (MILLAR, 1974), although exceptions to this rule have also been reported (LAMBERT, 1968; SVANE & LUNDALV, 1981).

A clear relationship between the reproductive periods and the latitudinal distribution of ascidians has been demonstrated (MILLAR, 1958; DYBERN, 1965; TURON, 1988). The species studied in this paper have a very narrow latitudinal range of distribution, restricted to the Mediterranean Sea according to PÉRÈS (1958), but reaching Portuguese Atlantic waters according to SALDANHA (1974). *H. papillosa* has also been reported from the French Atlantic coast and the Canary Islands (HARANT & VERNIERES, 1933; RIOS, 1985). It would be instructive to investigate the breeding seasons in these distributionally extreme zones. To our knowledge, the sole reference about reproductive periods of the two species studied here is LO BIANCO's (1909) statement that *Halocynthia papillosa* is mature and spawns in the Gulf of Naples (Southern Italy) in November. Water temperature in this area of the Mediterranean Sea is higher than on the Northeast Spanish coast, which may account for the displacement of the reproductive period towards colder months.

Summary

The parameters studied in *H. papillosa* and *M. sabatieri* indicate a single spawning season per year in the area studied. Both species are simultaneous hermaphrodites, and gamete release occurred after the period of highest seawater temperatures, with a maximum in September–October for *H. papillosa* and in October–November for *M. sabatieri*. Moreover, the release is noticeably more rapid in *H. papillosa*.

Mean oocyte diameter and gonad index show a good correlation. The values of the latter were almost twice as high in *H. papillosa* than in *M. sabatieri*. Since no development of nutritional tissue has been observed in histological sections, this can reasonably be interpreted as a higher investment in gamete production in *H. papillosa*.

Rapid buildup of the gonad, gradual release of gametes, and low gonad indices occur in *M. sabatieri*, whereas gradual buildup, rapid release, and higher gonad indices are displayed by *H. papillosa*. This indicates different reproductive behavior in these species, a conclusion which is confirmed by statistical analyses.

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