

Botrylloides crystallinus n. sp.,
a new Botryllinae Adams & Adams, 1858
(Ascidacea) from Mediterranean Sea

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KEY WORDS

Styelidae,
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aestivation,
Mediterranean Sea,
new species.

ABSTRACT

Botrylloides crystallinus n. sp., collected on sublittoral hard substrata in North Mediterranean Sea is here described. The species presents a seasonal life cycle with a spring-summer regression of the zooids and their buds as known in the congeneric species *B. leachii* (Savigny, 1816), from which however differs in many unusual morphological traits.

RÉSUMÉ

Botrylloides crystallinus n. sp., une nouvelle espèce de Botryllinae Adams & Adams, 1858 (Ascidacea) de la mer Méditerranée.

MOTS CLÉS
Styelidae,
Botryllinae,
estivation,
mer Méditerranée,
espèce nouvelle.

Botrylloides crystallinus n. sp., collectée sur les substrats dur sublittoraux au nord de la Méditerranée, est décrite. L'espèce présente un cycle de vie saisonnier avec une régression printemps-été des zoïdes et de leurs bourgeons comme cela est connu pour une autre espèce congénérique *B. leachii* (Savigny, 1816), dont elle diffère cependant par de nombreux traits morphologiques inhabituels.

INTRODUCTION

The taxonomic description of botryllid ascidians is objectively difficult because of the deep morphological homogeneity among the putative species which requires a careful study of tiny characters that might be useful for their distinction.

For a long time these animals were described almost exclusively on the basis of the shape of systems and their coloration, and a high number of species were defined. Thanks to the publication of the *Faune de France* (Harant & Vernières 1933) and *The Tunicata* (Berrill 1950) all European botryllid species were reduced to only two species: *Botryllus schlosseri* (Pallas, 1766) and *Botrylloides leachii* (Savigny, 1816). But this drastic decrease may be the reason why the Mediterranean Sea, although extremely rich and diverse for many biotas, appears so poor in terms of species diversity for botryllid genera.

Only recently the taxonomy of Botryllinae Adams & Adams, 1858 excited new interest (Brunetti 2009, 2011; Brunetti & Mastrototaro 2012; Brunetti *et al.* 2017) and this is partly due to the development of molecular analysis (Bock *et al.* 2012).

MATERIAL AND METHODS

Colonies were collected by scuba diving on 9 May 2018 at Carro (France) (43°19'42"N, 5°2'42"E) where the species is present between 5 and 11 meters deep. Colonies of *Botrylloides crystallinus* n. sp. were also observed at the same period around Port-Cros (France) at 30 meters deep. The species had been previously observed in 2009, but not collected, in the area of Carry-le-Rouet where it was common up to 28 meters deep (Fig. 1A).

The colonies were fixed in the field just after sampling. Animals were anaesthetised combining the use of menthol and clove essential oil (CEO) whose active principle is Eugenol so far used in aquaculture (Chanseau *et al.* 2002). Samples were placed during the dive in re-sealable vials. Once back to the surface, they were pre-anaesthetised with menthol crystals, being protected from light and change in sea water temperature. After about 10-20 minutes the siphons of the zooids are usually opened and the anaesthesia with CEO was completed. However, as this substance is not soluble in water, three drops were vigorously shaken in a bottle with 100 ml of seawater to obtain an emulsion: this emulsion was then added to the vial, with the ratio of three drops for 100 ml. A complete anaesthesia, controlled by touching the oral syphon with a needle, was usually obtained in a few minutes. Subsequently, the formalin was added in the vial to obtain a 10% solution.

ABBREVIATION

Institution

MNHN Muséum national d'Histoire naturelle, Paris.

RESULTS

Family STYELIDAE Sluiter, 1895
Subfamily BOTRYLLINAE Adams & Adams, 1858
Genus *Botrylloides* Milne-Edwards, 1841

Botrylloides crystallinus n. sp. (Figs 2-5)

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TYPE LOCALITY. — France, Carro, North Tyrrhenyan Sea.

TYPE MATERIAL. — **Syntypes.** France • 4 colonies (Bc1, Bc2, Bc3 and BcA); Carro, North Tyrrhenyan Sea; 43°19'42"N, 5°2'42"E; 9.V.2018; depth 5-11 m; sea water temperature 16°C; MNHN-IT-2018-3, MNHN-IT-2018-4, MNHN-IT-2018-5, MNHN-IT-2018-6. [Part of the syntype named BcA (MNHN-IT-2018-6) was not anaesthetised and directly fixed in ethanol instead of formalin and sent to Prof. Carmela Gissi at the University of Bari who is working on the phylogeny of Botryllinae].

DISTRIBUTION. — Mediterranean French coasts (Fig. 1A), from the east of the Rhone delta up to the Italian coasts.

ETYMOLOGY. — Crystalline, name based on the transparent aspect of the colonies, from Latin *crystallus*.

DIAGNOSIS. — Globular colonies with a thick tunic. Zooids with spoon-shaped oral tentacles (Fig. 3G). Internal longitudinal branchial vessels extending anteriorly up to the pre-pharyngeal ring and getting in touch with it (Fig. 3A, B, H, I); stomach with nine folds and with a triangular space between a tiny typhlosole and the last fold (Fig. 4D); pyloric caecum long about half of the stomach length; rotation of the intestine in the second curve of intestinal loop (Fig. 4A).

DESCRIPTION

Colonies

Globular, usually up to 3 cm in diameter, or massive with several rounded lobes and reaching 7-8 cm thick. Adhering to solid substrata by an attaching surface without marginal expanded ampullae. Zooids, arranged in *leachii*-type systems (Brunetti 2009), and perpendicularly ordered at the surface of the colony with their buds (Fig. 2C) that lie, immersed in the tunic, below the filtering zooid level (see below in "Zooids" subsection). Tunic always very thick, up to several times the height of the filtering zooids, and crossed by a network of very thin colonial blood vessels connecting zooids and buds. Tunic soft and sticky, making the extraction of zooids and buds difficult. Colonial vessels ending with very small spherical ampullae. Larger ampullae present only at colony surface. Tunic crystalline, transparent and white to diaphanous pale yellow; thin white lines highlighting the tunic from the base of oral siphon to the rim of dorsal lip and atrial aperture, drawing on the surface of the colony a thin branched pattern of lines running through the zooids up to the rim of the common cloacal opening (Fig. 2A). White longitudinal lines also present in branchial sac: two large ones emphasizing the endostyle and the dorsal lamina, and six thin ones highlighting the internal longitudinal vessels. White pigment present

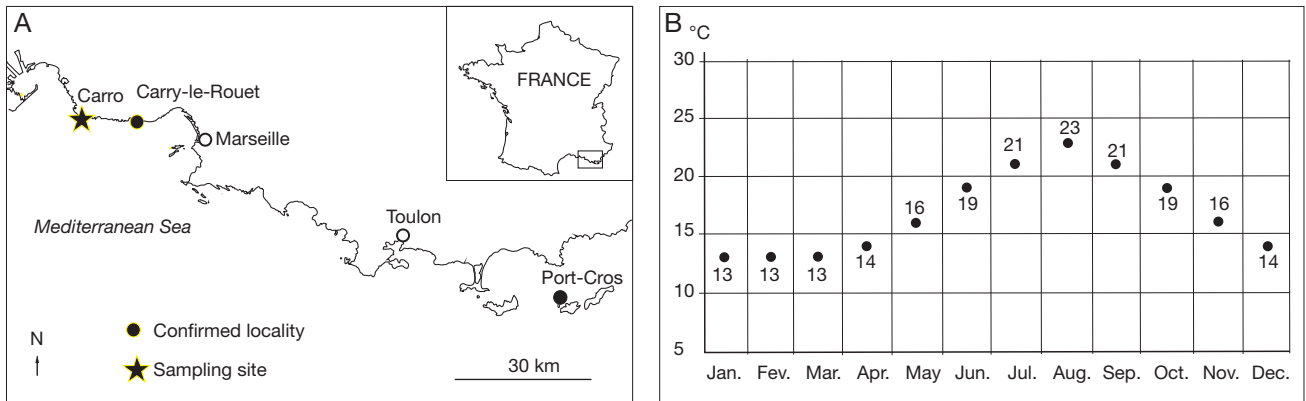


FIG. 1. — **A**, Map of the sampling sites; **B**, range of monthly Marseille seawater temperatures derived from the website <https://seatemperature.net/current/france/marseille-provence-alpes-cote-d-azur-france-sea-temperature> (website visited in march 2019, showing data collected from 2016 to 2018 from open sources, using the NOAA satellite, river and lake surfaces satellite map).

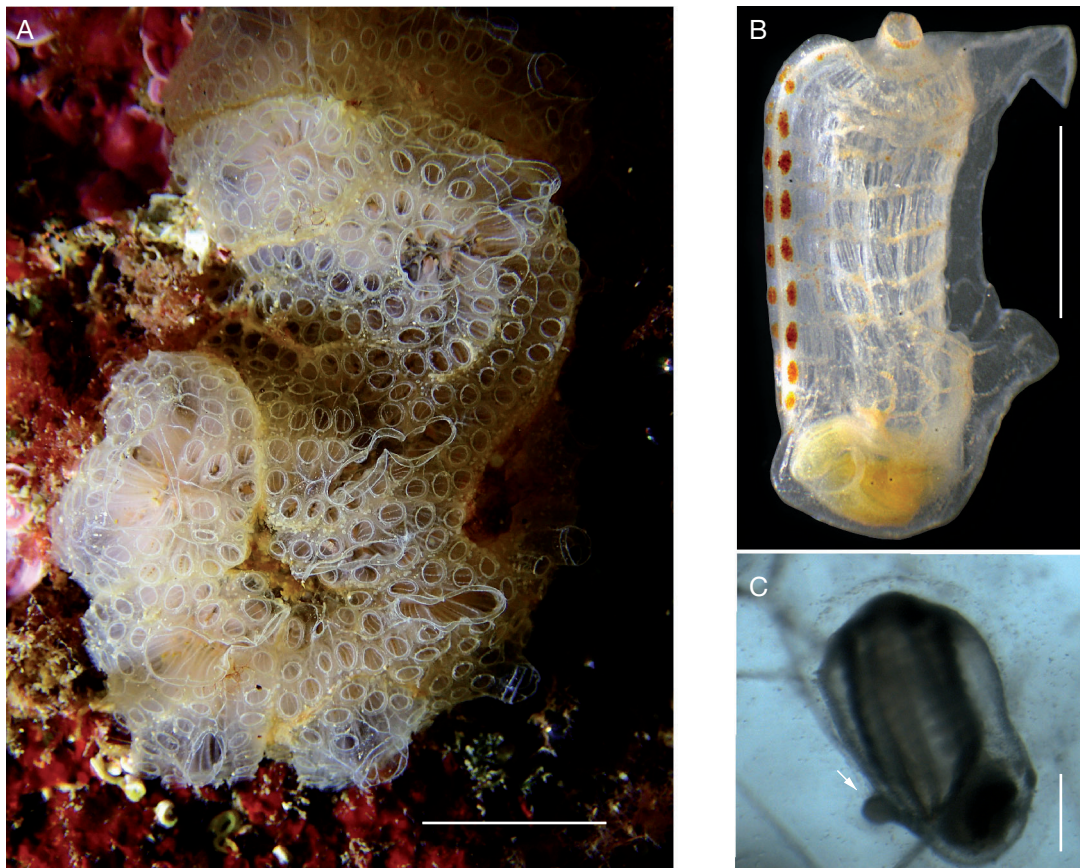


FIG. 2. — *Botrylloides crystallinus* n. sp.: **A**, colony in the field; **B**, single zooid (fixed but not stained); **C**, first order bud in stage 8 with its budlet (arrowhead) in stage 3 (staging of Berrill, 1941). Scale bars: A, 0.5 cm; B, 2 cm; C, 100 μ m.

in all these structures. In fixed animals, orange pigmentation present in cell islands close to the endostyle (see below), and around stomach and the first curve of intestinal loop (Fig. 2B).

Zooids

Up to 3–4 mm long. Body wall with a faintly visible network of very fine muscles, making circular bands only at siphon apertures. Size, distribution and number of the tentacles strongly

variable in zooids of the same colony (Fig. 3B–E). In general, tentacles not very long; longest tentacles spoon-shaped when observed at high magnification, with the concavity toward the outside (Fig. 3G). Contrary to some other Botryllinae species (e.g. *B. schlosseri*, see Brunetti *et al.* 2017), vascular lacunae at tentacle bases without masses of haematic cells. Branchial sac with usually 12 rows of stigmata, the second one dorsally incomplete (Fig. 3A) and the last one (often difficult to see)

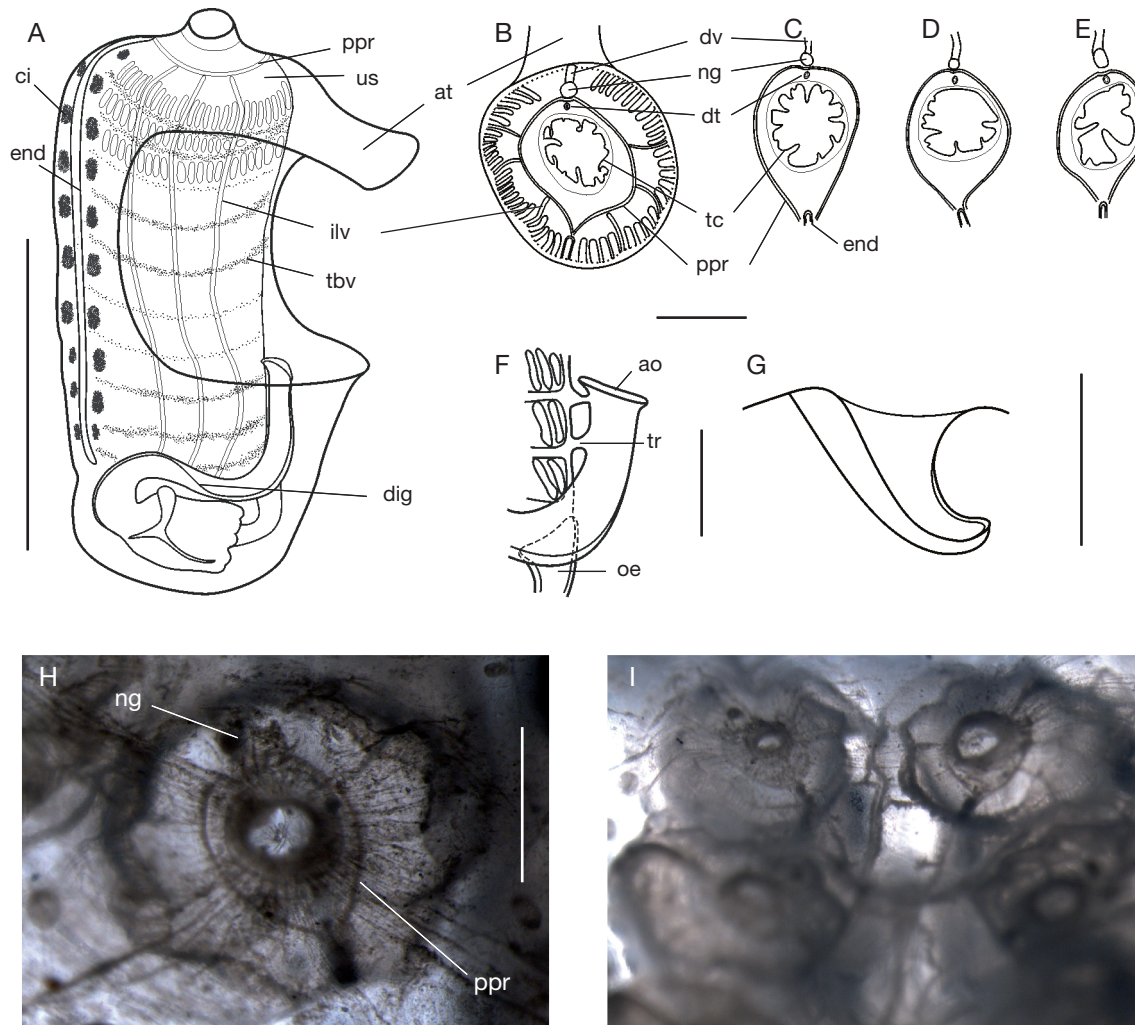


FIG. 3. — *Botrylloides crystallinus* n. sp.: **A**, zooid; **A**, **B**, **H**, **I**, the same see from above; **B-E**, tentacular crown in different zooids of the same system; **F**, rectus with trabeculae; **G**, tentacle. Abbreviations: **ao**, anal opening; **at**, atrial tongue; **ci**, cell island; **dig**, dorsal intestinal groove; **dt**, dorsal tubercle; **dv**, dorsal vessel; **ilv**, internal longitudinal vessels; **ng**, neural gland; **oe**, oesophagus; **ppr**, pre-pharyngeal ring; **tbv**, transversal branchial vessels; **tc**, tentacular crown; **tr**, trabecula; **us**, unperforate space. Scale bars: **A**, 2 cm; **B-F**, 300 µm; **G**, 10 µm; **H**, 500 µm; **I**, 1 mm.

with small stigmata. Branchial sac cylindrical (Fig. 2A), with about 18 stigmata in the first half row and about 14 in the 11th half row. Ventral “cell islands” (as defined by Manni *et al.* 2014) present on both sides of the endostyle, at the level of the rows of stigmata (Figs 2B; 3A). Each internal longitudinal vessel rising apically to form a lamina; its diameter just a little larger than the diameter of interstigmatic vessels. Internal longitudinal vessels developing anteriorly to the first stigmata row, reaching and touching, but not fusing with, the pre-pharyngeal ring (Fig. 3A, B). Therefore, each branchial sector of the first stigmata row is protruding centripetally in the body between the two delimiting vessels, and the row appears wavy, a feature visible both in living and fixed specimens (Fig. 3H, I). Dorsal and ventral sectors equal in width and wider than lateral sectors. Branchial formula at about half of pharynx length usually DL 5.4.4.5 E. Few, thin muscle fibres along the transversal branchial vessels. Atrial opening exposing 6-7 rows of branchial stigmata (Figs 2B; 3A), and with a dorsal languet more or less developed according to the position of the zooid in the system.

Stomach arranged almost completely posteriorly to branchial sac; axis of well-relaxed zooid inclined at an angle of about 135° compared to anterior-posterior axis of the zooid (Figs 2B; 3A). Almost cylindrical in shape with cardiac end slightly larger than pyloric end; with 9 slightly spiralized folds (Fig. 4D-F); a broad smooth triangular space present between the typhlosole and the last fold (Fig. 4D) (note that gastric folds, observed from the cardiac end, are numbered clockwise from the typhlosole). A pyloric caecum rising from posterior part of a tiny typhlosole; about half as long as stomach length (see Fig. 4D, F), slightly tilted back and with a slightly swollen tip. Intestinal loop moderately curved. Rectus running along the dorsal edge of the branchial sac; the two structures connected through two trabeculae, at level of the transversal vessel between stigmata rows 9 and 10 (Fig. 3F). A smooth edge anus opening at stigmata row 8: three rows of stigmata anteriorly to oesophageal opening, located at the level of stigmata last row (Fig. 3A, F). Anal opening not lobed, (Fig. 4A, B) but sometimes looking outward (Fig. 4C). Intestine with two

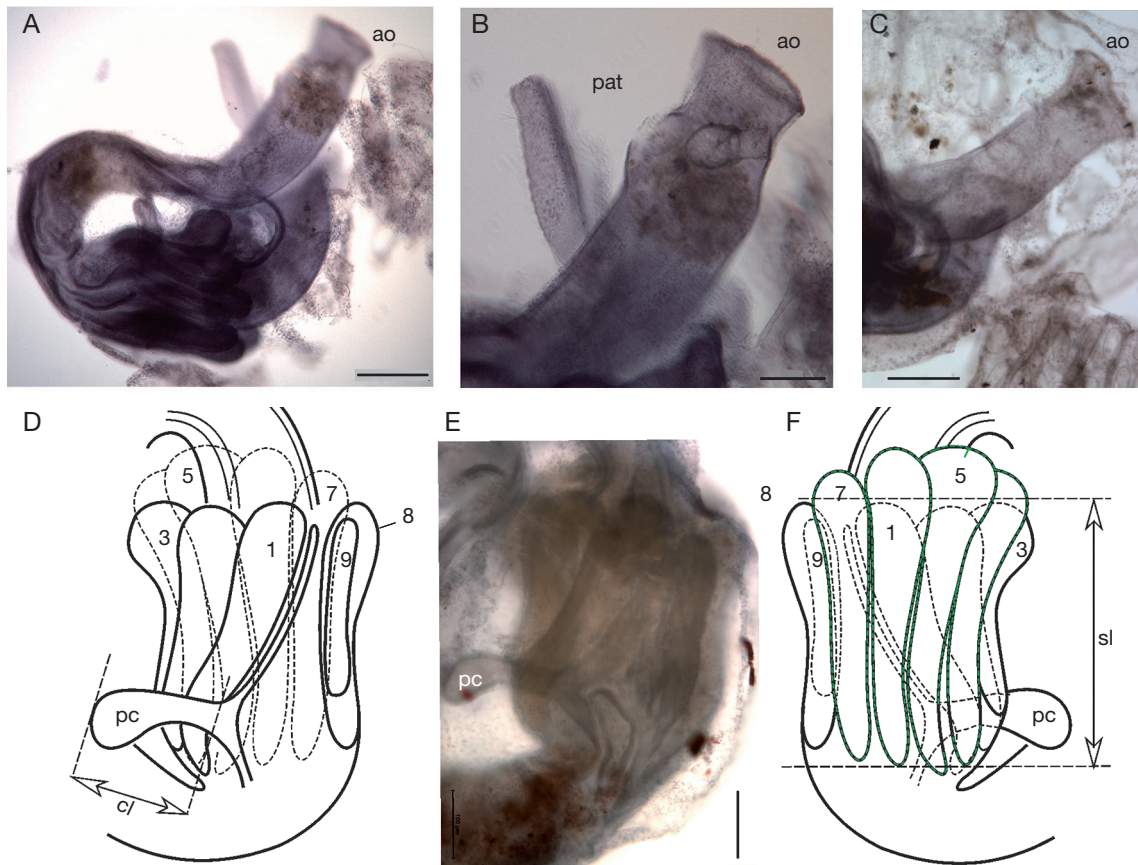


FIG. 4. — *Botrylloides crystallinus* n. sp.: **A**, intestinal loop with its folding (arrow's head); **B**, rectus of the same sample of **A** on a different focal plane; **C**, other intestinal loop with the anal opening spread outward; **D-F**, parietal and mesial sides of the stomach. Abbreviations: **ao**, anal opening; **cl**, coecum length; **pat**, point of attachment of one trabecula; **pc**, pyloric caecum; **sl**, stomach length. Scale bars: A-C, 20 μ m; D-F, 400 μ m.

grooves along its ventral and dorsal sides, the first the most evident. Two grooves also present along the two sides of the oesophagus. Second curve of intestinal loop accompanied by a rotation of intestine (Fig. 4A), as in *Botrylloides israeliensis* Brunetti, 2009: as a consequence, in gut loop terminal tract, dorsal side of intestine distanced from the dorsal edge of branchial sac; ventral side of intestine by contrast close to the branchial sac dorsal edge, and connected to it by two trabeculae, as described above. Buds connected through blood vessels to parental zooids, not leaning but outdistanced from them (Fig. 2C); this disposition probably takes place during the change of generation when the zooids regress and are substituted by first order buds.

Gonads

No gonads observed in zooids, but oocytes present posteriorly and closed to testis primordia in first order buds of same colonies.

ECOLOGY

The species, locally common along the French Mediterranean coasts (Fig. 1A), lives in shaded areas fixed on vertical side of rocks and overhangs, from 5 to more than 30 meters deep. All samples were collected the 9 May 2018 (water

temperature 16°C); many of them were in full activity with filtering zooids but others presented some zooids in regression (Fig. 5A). In spring when temperature rises to summer values (Fig. 1B), the number of regressing zooids increased, the regression moved from the base of the colony to its top (Fig. 5B, C) until all zooids are regressed (Fig. 5D). The regression concerns also the buds and the colony is reduced to a collection of tiny round bodies (Fig. 5E); during the following July (mean water temperature 21°C) almost all colonies (not collected) appear as gelatinous masses with only tiny whitish ampullae at their surface (Fig. 5F). Finally, during scuba diving of August 10 and 17, after a period of high temperature with values up to 28°C, no colonies were detected.

Our observations, although not covering a full annual period, suggest that the species presents a seasonal cycle with a preference for the lower temperature of winter. During the summer, when water temperature is constantly above 20°C (Fig. 1B), probably there is a high mortality that would explain the ostensible absence of the species.

REMARKS

Beside its phenotypic appearance, the species is characterised by the anterior terminal part of the internal longitudinal

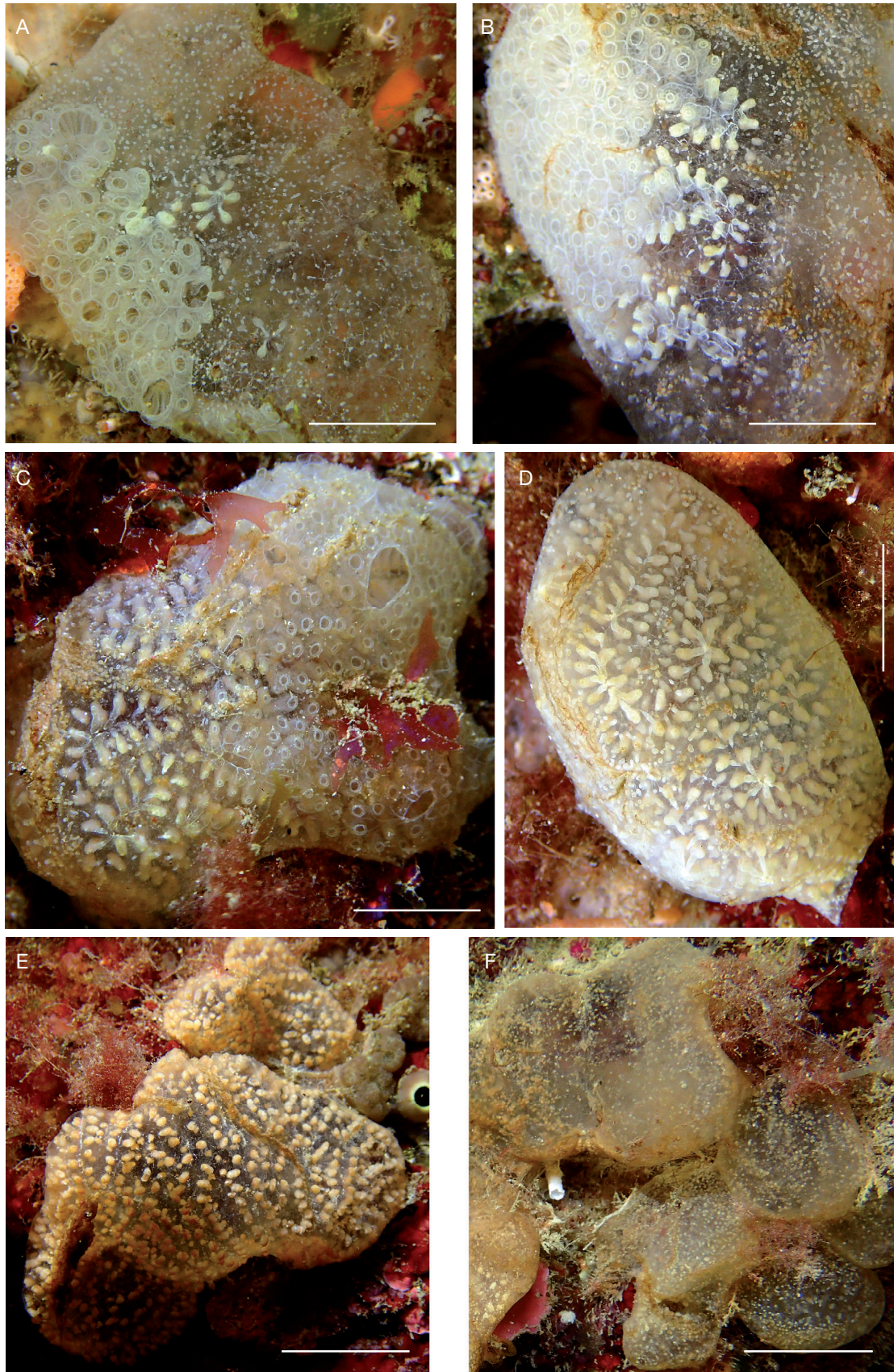


FIG. 5. — *Botrylloides crystallinus* n. sp.: steps in aestivation: **A-C**, early phases: zooid regression moves from the base of the colony to its top; **D**, colony top where all zooids and buds were in regression; the disposition in systems is still visible; **E**, advanced phase: regressed zooids are reduced to little bodies; **F**, final phase: colonies appear as gelatinous masses with only tiny whitish ampullae at their surface. Scale bars: 0.5 cm.

TABLE 1. — Comparison among the main morphological traits of *Botrylloides* Milne Edwards, 1841 species present in the Mediterranean Sea: *B. crystallinus* n. sp., *B. anceps* (Herdman, 1891), *B. giganteus* (Pérès, 1949), *B. israeliensis* Brunetti, 2009, *B. leachii* (Savigny, 1816), *B. violaceus* Oka, 1927. Special features: *B. crystallinus* n. sp.: aestivation, *B. anceps*: colony surface sandy, *B. israeliensis*: the haematic cells usually forming the branchial cell islands accumulate in the ventral portion of transversal vessels, intestine folded; *B. leachi*: hibernation; *B. violaceus*: viviparous, egg developing in the colonial tunic nourished by blood circulation, larva with up to 30 ampullae. Abbreviations and symbols: c., circa; Ds, dorsal; Lt, lateral; L, large; M, medium size; S, small; Vn, ventral; * the term is used as proposed in the “Annotated glossary” by Kott (1985: 15); ** ninth fold rudimentary and sometimes absent.

		<i>B. crystallinus</i> n. sp.	<i>B. anceps</i>	<i>B. giganteus</i>	<i>B. israeliensis</i>	<i>B. leachii</i>	<i>B. violaceus</i>
Zooid	height shape	c. 3 mm cylindrical	c. 1.5 mm conical	c. 4 mm cylindrical	c. 3 mm conical	c. 1.5 mm cylindrical	c. 1.3 mm cylindrical
Tentacles	number shape haematic cells mass at the base of L ones	strong variability spoon shaped absent	2L+6M-S finger like present	up to 30 finger like absent	4L + 4M finger like absent	4L + 4M + 8S finger like present	4L + 4M + 4S finger like absent
Atrial lip	2 pigmented spots	absent	present	absent	absent	absent	absent
Stigmata	number rows second row	12 incomplete	9 incomplete	17 complete	8 complete	9 incomplete	10-14 incomplete
Branchial	ventral cell islands sectors width	present Ds = Vn > Lt	present Ds = Vn > Lt	absent Ds = Vn > Lt	absent Ds = Vn > Lt	present Ds = Vn > Lt	present Ds = Vn > Lt
Intestinal loop shape		curved*	moderately curved	curved	deeply curved	moderately curved	moderately curved
Stomach	shape number of folds cardiac swellings unfolded space	c. cylindrical 9 absent present	globular 10 absent absent	bell shaped 11 moderate present	globular 8-9** absent present	bell shaped 9 present present	trumpet shaped 10 absent absent
Pyloric Caecum	length as for stomach shape	c. 1/2 club shaped	c. 1/1 club shaped	c. 1/4 finger like	c. 1/1 club shaped	c. 1/4 finger like	c. 1/2 finger like
Anus opening	nb of stigmata rows above the oesophagus opening	3	1	5-6	1-2	1-2	2
Ovary	position as for testis	posterior	anterior	posterior	not seen	posterior	posterior
Egg	number per side	not seen	1	1	not seen	1	1
Brood pouch	-	not seen	absent	present	not seen	present	present

branchial vessels and the undulating surface of branchial wall at the level of the first row of stigmata. This characteristic was never reported until now in Botryllidae species, but a similar condition seems to be present in some species of the genus *Symplegma* Herdman, 1886 (Styelidae, Polyzoinae) although it was not quoted among its generic characters (Monniot & Monniot 1972). So in *S. reptans* (Oka, 1927) Kott (1985: 259, fig. 127) quotes “[...]internal longitudinal vessels extend the whole length of the branchial sac.” and in the figure 127 these vessels extend an unperforated space coming up to the pre-pharyngeal ring; and in Monniot (2018) the photos in fig. 10 regarding *S. brakenhielmi* (Michaelsen, 1904) clearly show the vessels going over the first stigmata row and ending in an unperforated space anterior to it, close to the pre-pharyngeal ring.

Also, the folding of the intestinal loop is an unusual character mentioned only in *Botrylloides israeliensis* Brunetti (2009), from which however *B. crystallinus* n. sp. differs in many other characters such as the shape of the stomach the number of stigmata rows, and the incompleteness of the second row.

Among the European species (Brunetti & Mastrototaro 2017) only *B. leachii*, *B. giganteus* (Pérès, 1949) and *B. diegensis* Ritter & Forsyth, 1917 present some resemblance with the here described species, mainly in stomach shape with a smooth rhomboid area between the typhlosole and the last fold. Only *B. leachii* has the same number of stomach folds,

but its stomach has a more campanulate shape and overall a clear swelling of the cardiac ends of folds and a pyloric caecum shorter than half of the stomach length. Moreover the *B. leachii* has smaller zooids. Finally, in *B. leachii* the tunic is strongly thinner than *B. crystallinus* n. sp. A comparison among the main morphological traits of these species are reported in Table 1.

The seasonal cycle of *B. crystallinus* n. sp., with a regression stage during summer, recalls the seasonal cycle described in *Botrylloides leachii* (Brunetti 1976) in the lagoon of Venice. Total or partial regression phase was reported for many colonial ascidians (Millar 1971); this is usually named aestivation or hibernation depending on whether it takes place during winter or summer, which may suggest that the cause of the phenomenon is the change in temperature. When the regression concerns only a particular organ of the zooid it may be interpreted as a renewal of organs damaged by an intense metabolic activity (Turon 1992). In botryllids an aestivation with a total regression, was first described by Bancroft (1903) on colonies of *Botrylloides gascoi* Della Valle, 1877 (presently junior synonym of *B. leachii*) which at the end of June (in Naples) completely degenerated zooids and buds living only the test vascular system. The same phenomenon was described for *B. leachii* (Brunetti 1976) in the lagoon of Venice where the difference from winter and summer temperature is stronger

(Brunetti & Canzonier 1973): when sea water temperature drops below 10°C the zooids and their buds undergo a general regression and the colonies, devoid of filtering zooids, appear as a carpet of vascular ampullae mainly crowded of macrophages. In spring, with temperature increase, some zooids originate by vascular budding (Burighel *et al.* 1976) giving rise to new systems. At present material do not allow to know if a similar process happens also in the here studied species, that is if the observed regression of zooids and their buds is followed by the development of new buds which rebuild the colony.

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