Journal of the Marine Biological Association of the United Kingdom

cambridge.org/mbi

Asexual reproduction and regenerative responses of botryllid ascidians to salinity and temperature variations

Fatıma Nur Oğul Ünal, Mert Koray Ünal and Arzu Karahan

Marine Biology and Fisheries, Middle East Technical University, Institute of Marine Science, Erdemli, Mersin, Türkiye

Research Article

Cite this article: Oğul Ünal FN, Ünal MK and Karahan A (2025) Asexual reproduction and regenerative responses of botryllid ascidians to salinity and temperature variations. *Journal of the Marine Biological Association of the United Kingdom* **105**, **e109**, 1–9. https://doi.org/10.1017/S0025315425100611

Received: 20 January 2025
Revised: 31 July 2025
Accepted: 9 September 2025

Keywords:
blastogenic cycle, *Botrylloides niger*, *Botryllus humilis*, interactive stressors, whole-body regeneration

Corresponding author:
Fatıma Nur Oğul-Ünal;
Email: fogulnua@gmail.com

Abstract

The unique reproductive strategies of botryllid ascidians, which include both asexual and sexual mechanisms as well as an extensive capacity for regeneration, contribute to their fast population growth and wide-ranging ecological effects. These colonial organisms have unique ecological adaptations and responses to environmental factors, yet comprehensive comparative studies on their environmental preferences remain scarce. We conducted an experimental study to explore the asexual reproduction and regeneration response of *Botrylloides niger* and *Botryllus humilis* colonies to varying salinity (36.5–39.5 PSU) and temperature (26 ± 1–30 ± 1°C) levels. Experimental findings highlighted species-specific preferences and stress responses: *B. niger* demonstrated higher tolerance to elevated salinity (39.5 PSU) with optimal growth rates at 26 ± 1–30 ± 1°C, whereas *B. humilis* displayed a preference for lower salinity and tendencies towards vascular budding at higher temperatures (30 ± 1°C). These observations suggest potential niche differentiation and ecological success, particularly in Mediterranean conditions, implying possible coexistence without intense competition in similar habitats. This research offers insights into the adaptive mechanisms of these ascidians, shedding light on their ecological roles and potential implications in coastal ecosystems amid changing environmental scenarios.

Introduction

Ascidians (Phylum: *Chordata*, Subphylum: *Tunicata*, Class: *Ascidiacea*) are hermaphroditic, filter-feeding marine invertebrates with a global distribution, inhabiting oceanic regions from tropical to polar latitudes (Lambert, 2005). They occupy diverse benthic habitats, including sandy substrates, rocky shores, coral reefs, and kelp forests (Shenkar and Swalla, 2011). The capacity for both sexual and asexual reproduction contributes to their rapid population expansion (Blanchoud et al., 2018; Holland, 2016). In botryllid ascidians, sexual reproduction produces free-swimming larvae that metamorphose into zooids, and zooids expand to colonies through asexual reproduction by budding (Lauzon et al., 1992). A typical colonial ascidian is organized into a system that includes three co-existing generations – mature zooids and developing buds – spatially arranged and interconnected through a shared vascular network (Brown et al., 2009). This network ends peripherally in blind extensions known as ampullae (Figure 1). Buds originate either from the atrial epithelium of existing zooids (palleal or peribranchial budding; Berrill, 1941) or from haemocyte aggregates at the base of ampullae (vascular budding; Oka and Watanabe, 1957). The blastogenic cycle refers to the asexual reproductive process in botryllid ascidians that occurs via palleal budding, during which aging zooids are replaced by developing buds, and all old zooids are resorbed in a process known as 'take-over' (Lauzon et al., 2002; Manni et al., 2014). The blastogenetic cycle has been described using two main staging approaches. The first approach, introduced by Berrill (1951) and later revised by Manni et al. (2014), divides the cycle into 11 stages based on the development of budlets, primary buds, and zooids. The second approach, proposed by Mukai and Watanabe (1976), defines four main stages (A–D) and serves as the framework adopted in the present study. Stage A starts when the siphons of the zooids open. In stage B, heartbeats begin in the primary buds. Stage C involves the formation of organs in the secondary buds and the accumulation of pigment cells in the outer layer of the primary buds. Stage D, known as the take-over stage. During this stage, primary buds mature into new zooids, budlets become primary buds, and a new set of budlets are formed (Manni et al., 2014). Botryllid ascidians also represent the sole living chordate group capable of whole-body regeneration (WBR). Remarkably, they can regenerate a complete, functional individual from as few as 100–200 haemocytes within approximately 10 days (Brown et al., 2009; Karahan et al., 2022; Rinkevich et al., 1995; Voskoboynik et al., 2007).

Botryllid ascidians preferentially settle in coastal areas where the environment fluctuates rapidly. Species thriving in these dynamic environments, capable of enduring diverse

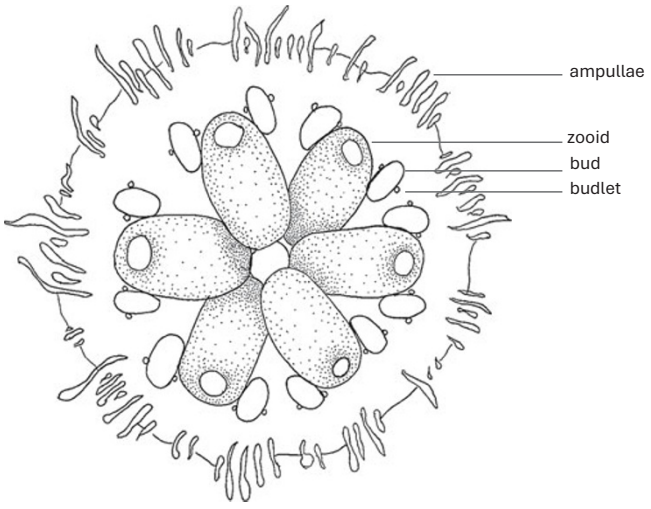

Figure 1. General schematic representation of a botryllid ascidian colony, illustrating the typical organization of a system with co-existing generations, including zooids, buds, budlets and ampullae. Illustrated by Bilge Yüceer Karakuş, used by permission.

stressors, often exhibit traits that facilitate their invasion into new ecosystems, potentially disrupting native communities, a matter of concern considering climate change reality (Lambert, 2001). The spatial distribution and settlement patterns of ascidians are influenced by various environmental and biological factors, such as light, substrate type, hydrodynamics, predation, and competition (Lambert, 2005). Understanding the relative influence of these factors on ascidian recruitment, dispersal, and survival is essential for interpreting their global distribution and assessing their invasive potential (Epelbaum et al., 2009; Lenz et al., 2011; Zhan et al., 2015). Among these variables, salinity and temperature are considered the most critical in shaping recruitment success (Goodbody, 2004; Millar, 1971). However, existing research has largely focused on solitary ascidian species and colonial groups such as *Didemnum* (Bae et al., 2023; Carver et al., 2003; Malfant et al., 2017; Therriault and Herborg, 2008). In contrast, current knowledge on the responses of botryllid ascidians to these factors remains comparatively limited, despite their global species diversity and ecological importance (Brunetti et al., 1980; Epelbaum et al., 2009; Malfant et al., 2017; McCarthy et al., 2007).

Within the Mediterranean context, the presence and potential spread of two botryllid species of interest were documented: *Botrylloides niger* and *Botryllus humilis*. The first record of *B. niger* in the Mediterranean dates to 1952 in Israel (Pérès, 1958), followed by reports in the Suez Canal (Halim and Messeih, 2016), Italy (Salonna et al., 2021), and Türkiye (Temiz et al., 2023). Similarly, the first record of *B. humilis* in the Mediterranean was in Israel as well (Brunetti, 2009 [=*Botrylloides anceps*]). Its recent introduction along the Turkish coastline is supported by the absence of prior records before 2012 (Karahan et al., 2023). Both species are considered non-native to the Mediterranean. The Caribbean (West Atlantic) is suggested as the native range of *B. niger*, supported by its high genetic diversity and continuous distribution in that region (Sheets et al., 2016). *B. humilis* was likely introduced from the Indo-Pacific through the Suez Canal (Brunetti, 2009 [= *B. anceps*]). Although Virgili et al., (2022) found no clear evidence of a competition between native species in Miseno Lake, it is known that *B. niger* has become dominant in fouling populations in both native and non-native areas (Nydam et al., 2021; Ramalhosa et al., 2021; Sheets et al., 2016). In comparison, *B. humilis* has been observed coexisting with other botryllid species along the Turkish coastline, yet no invasive behaviour has been documented (Karahan et al., 2023). While both species were initially reported from scattered locations within the Mediterranean, recent observations suggest a broader expansion of their distribution (Karahan et al., 2023; Sheets et al., 2016).

Given their recent occurrence and expansion in the region, it is critical to investigate how environmental conditions influence key biological processes such as asexual reproduction and regeneration in these species. Studying how environmental conditions influence asexual reproduction and regeneration is essential for understanding the survival and fitness strategies of colonial organisms such as ascidians. Asexual reproduction offers advantages like bypassing the costs of sexual reproduction and enabling indefinite propagation, especially in space-limited habitats (Crow, 1994; Jackson and Coates, 1986). It often shares cellular and molecular mechanisms with regeneration, suggesting a physiological link between the two processes (Agata et al., 2007; Martinez et al., 2005). However, these energetically demanding processes compete with other vital functions, leading to trade-offs under limited resources or other stressful conditions (Adamczuk, 2021; Sebestyén et al., 2018). Understanding how environmental factors shape these trade-offs is crucial for predicting population dynamics, resilience, and adaptation potential in changing ecosystems. This study aims to investigate how varying salinity and temperature regimes in the Mediterranean influence asexual reproduction and WBR in two colonial ascidians, *B. humilis* and *B. niger*. To this end, we exposed colonies to two salinity levels and three temperature conditions to examine their biological responses during the blastogenic cycle and WBR. By understanding how environmental factors affect these processes, we aim to gain insight into their roles in environmental adaptation and the potential invasiveness of these species.

Materials and methods

Aquaculture system

The aquaculture system (AS) was established at the Institute of Marine Sciences (IMS), Middle East Technical University (METU), located on the Turkish Mediterranean coast (Figure 2). Seawater is supplied to the system via a submersible pump from the IMS-METU harbour and initially stored in a 1000 L lightproof tank. It is then sequentially filtered through mechanical filters with pore sizes of 100, 50, 20, 10, and 1 μm into a secondary 500 L lightproof tank before being transferred to plastic cultivation tanks (Supplementary File Figure N1). Before each use, salinity and temperature are measured, and if there is a significant deviation from the conditions at the time of organism collection, manual adjustments are made. The aquaculture room is thermally insulated and maintained at a stable temperature of approximately 26°C year-round using an air conditioner, with a 12-hour light/12-hour dark photoperiod cycle. Colonies are cultivated in aerated plastic tanks, and feeding is performed every two days using approximately 1 mL of a plankton food mixture per litre of water. The colonies used in this study were collected from the Mezitli and Kızkalesi regions, located 30 and 15 km, respectively, from IMS-METU (Figure 2).

To evaluate the environmental similarity between the (AS) and the natural habitat (NH), physicochemical parameters and bacterial composition of seawater were assessed. Seawater samples were collected from NH and multiple points of AS in June and

https://doi.org/10.1017/S0025315425100611 Published online by Cambridge University Press

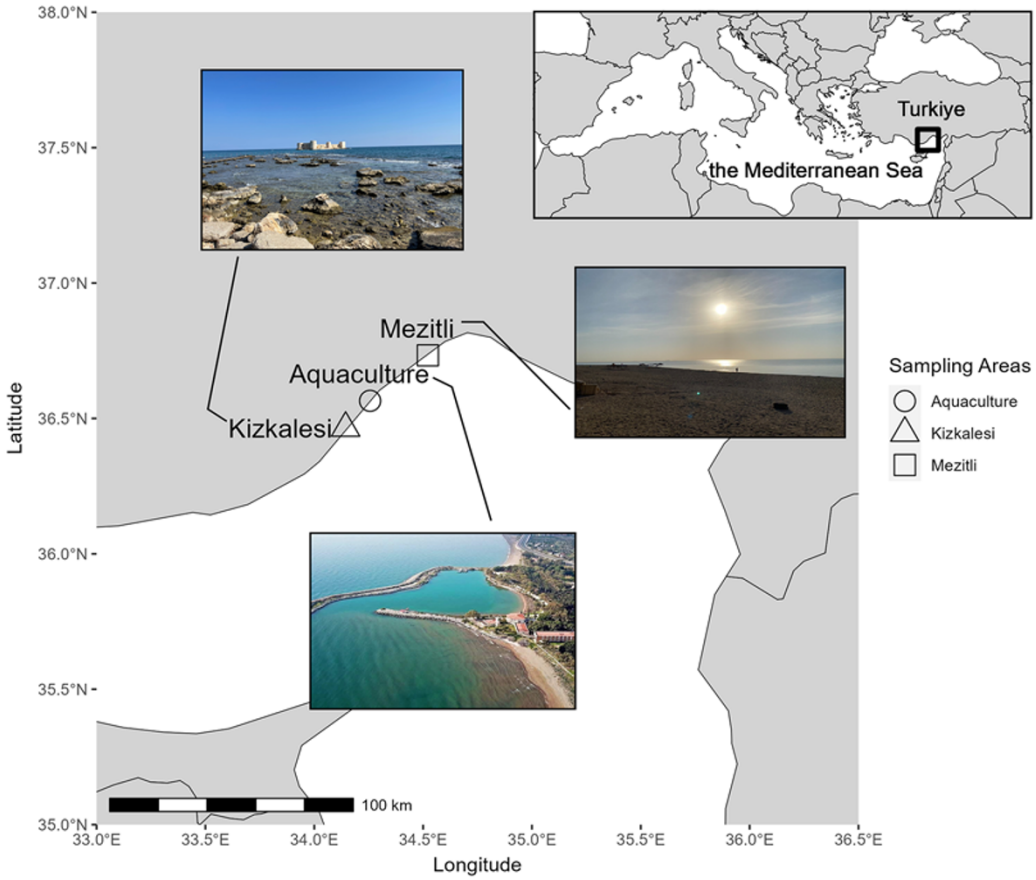

Figure 2. IMS-METU aquaculture system and sampling sites in the Northeast Mediterranean coast.

December 2022 to measure temperature, salinity, nutrient levels, chlorophyll-a, and particulate organic carbon. Additionally, bacterial communities in AS and NH were compared using 16S rRNA gene sequencing of seawater samples collected in December 2022, when colonial ascidian diversity in the NH was highest. Detailed methods and the results are available in the Supplementary File SECTION II.

Blastogenic cycle and WBR staging

The collected colonies were acclimatized to the system and subcloned (i.e., physically divided into smaller, genetically identical fragments) following Karahan et al. (2022). The subclones were then observed daily under 26ºC and 38 PSU to monitor both the blastogenic cycle and WBR.

The blastogenic cycle was staged according to criteria established for *B. schlosseri* by Manni et al. (2014). However, due to the relatively rapid cycle in both species, the stages were designated as A, B, C, and D, following the simplified system of Mukai and Watanabe, (1976) described in the introduction. WBR was initiated when colonies reached blastogenic stage A, as described for *Botrylloides leachii* by Zondag et al. (2016) and for *B. humilis* (=*B. anceps*) by Karahan et al. (2022). Colonies were monitored daily after ablation until the first filter-feeding zooid formed. Representative images and data for blastogenic cycle through palleal budding and WBR are provided in the Supplementary File Figures S1–S4 and Tables S1–S3 for each species.

Experimental design

The temperature and salinity levels used in the experimental set-up were selected to reflect the seasonal ranges observed at Mediterranean sampling sites where large and healthy colonies were found (26–31.1°C and 37.5–40 PSU), as well as the conditions under which colonies showed successful survival and maintenance in the laboratory (26–32°C and 35–40.5 PSU). Prior to the main experiment, an initial preliminary experiment was conducted using a range of salinity levels (35.5–38.6 PSU) and temperatures (27–32°C), with three subclones per condition (resulting in 18 combinations in total), to assess species preferences (Supplementary File Tables S4 and S5).

Based on the results of the preliminary experiment, the main experimental set-up was then designed with two salinity levels (36.5 and 39.5 PSU; S36 and S40 in the experimental group, respectively) and three temperature conditions (26 ± 1°C, 28 ± 1°C, and 30 ± 1°C; T26, T28, and T30 in the experimental group, respectively), resulting in six distinct treatment combinations (Table 1). We did not designate any specific group as a control in our experimental design, as we assumed the organism's adaptation capacity to environmental conditions exists along a spectrum, and no 'optimum' condition had been previously established for this species. Temperature was adjusted with 25-watt thermostats for each experimental subgroup. Filtered seawater had a natural salinity of ~38 PSU (September–December 2023), and the high salinity subgroup was adjusted to 39.5 PSU by adding commercial reef salt, while the low salinity subgroup was adjusted to 36.5 PSU by

https://doi.org/10.1017/S0025315425100611 Published online by Cambridge University Press

Table 1. Experimental conditions and group codes. Temperature for each subgroup fluctuated during the experiment, and a minimum–maximum observed value was given as interval. Experiment groups codes: S: salinity, T: temperature

Experiment groups	S36-T26	S36-T28	S36-T30	S40-T26	S40-T28	S40-T30
Salinity (PSU)		36.5			39.5	
Temperature (ºC)	26 ± 1	28 ± 1	30 ± 1	26 ± 1	28 ± 1	30 ± 1

adding double-distilled, UV-treated water. There was no significant difference in pH values before and after salinity manipulation (pH ~ 8.1).

For the main experiment, 10–12 subclones of the same colony that were approximately the same size were selected for each of the six experimental conditions for both species. All the colonies were stabilized under the same conditions (26 ºC, 38 PSU) before the start of the blastogenic cycle and WBR experiments. For the blastogenic cycle, after the first observation (day 0), the colonies were placed in their respective experimental conditions while keeping other conditions stable and observed daily for 11 days. For the blastogenic cycle, the growth rate was calculated by dividing the final zooid numbers by the initial zooid numbers of each colony. For the WBR, all the generations were removed from colonies, as explained in Zondag et al. (2016) at blastogenic stage A, then placed in their respective experimental conditions without feeding. Once a colony died (when haemolymph circulation ceased) or completed WBR by producing filter-feeding zooids, it was removed from the experiment to avoid any potential interference, such as changes in the water quality or biological processes, which could affect the remaining colonies. Daily observations on each group were conducted via a stereo microscope (SX16 Olympus stereo microscope equipped with an Olympus DP26 camera).

To evaluate the individual effects of temperature and salinity on colony growth and regeneration in *B. niger*, as well as the interaction between these two factors, a two-way analysis of variance (ANOVA) was performed. This statistical test allowed us to assess whether each factor significantly influenced the biological responses and whether their interaction had a combined effect. To further explore how changes in one environmental variable affected colony growth and regeneration under fixed conditions of the other, multiple comparisons were carried out using Tukey's Honestly Significant Difference (HSD) post-hoc test using two-way ANOVA results. In this approach, one factor (either temperature or salinity) was held constant at a specific level, while the other was varied, allowing us to identify statistically significant differences among subgroups with different temperature or salinity levels. These comparisons helped clarify which specific conditions drove the observed variation in growth and regeneration responses. Neither two-way ANOVA nor Tukey's HSD post-hoc test was applied to the *B. humilis* experimental data, as the numerical results did not consistently correspond to the same biological process across experimental groups.

Results

Blastogenic cycle and WBR response to salinity and temperature

Botrylloides niger

B. niger colonies were monitored to assess the increase in zooid numbers during the blastogenic cycle under all experimental conditions. No colony exhibited regression or abnormalities in the blastogenic cycle relative to the palleal budding stages determined prior to the experiment (Supplementary File Figure S1 and Table S1). For daily blastogenic staging observations and initial and final zooid counts, see Supplementary Dataset A. Among the six experimental groups, the highest growth rate was recorded in the S40-T28 group (mean = 5.6), followed by S40-T26 (mean = 5). The lowest growth rate was observed in the S40-T30 group (mean = 2.2) (Figure 3 and Supplementary File Figure S5). Two-way ANOVA revealed a statistically significant result pointing an effect of temperature on growth rate ($P = 0.0068$), while neither salinity nor the interaction between temperature and salinity showed statistically significant effects ($P > 0.05$). To further investigate the independent effects of each factor, multiple comparison completed across subgroups using Tukey's HSD post-hoc test. The results revealed that the effect of temperature on the growth rate was dependent on salinity levels. Specifically, within the 39.5 PSU group, temperature had a significant impact: the growth rate at 30 ± 1°C was significantly lower from both 26 ± 1 °C (diff = −2.9, *P*-adj = 0.035) and 28 ± 1°C (diff = −3.4, *P*-adj = 0.007) (Table 2). In contrast, no statistically significant differences were detected among temperature treatments within the 36.5 PSU group (*P*-adj > 0.05 for all comparisons, Table 2), suggesting that colonies may be more sensitive to temperature changes under high salinity conditions during the blastogenic cycle. At 39.5 PSU, there is no significant difference on growth rate between 26 ± 1°C and 28 ± 1°C temperature treatments.

Across all experimental conditions, *B. niger* colonies successfully completed WBR, with slight variation in regeneration duration and no substantial difference in the number of newly formed zooids (Supplementary File Figure S3 and Table S3). The shortest regeneration duration was recorded in S36-T28, S36-T30, and S40-T30 (minimum = 4 days; means = 5.2, 4.6, and 4.6 days, respectively), while the slowest regeneration occurred in S40-T26 (maximum = 9 days; mean = 8.2 days), excluding outliers (Supplementary File Figure S5). Two-way ANOVA indicated that both temperature and salinity effect is significant on regeneration with different *P* values ($P < 0.001$ and $P = 0.0256$ respectively), but no significant interaction between the two ($P > 0.05$), indicating independent contributions of each factor. Temperature-driven differences in regeneration duration were statistically significant at both 36.5 and 39.5 PSU (Table 2). At 36.5 PSU, regeneration was significantly faster at 28 ± 1°C (diff = −2.4 days, *P*-adj = 0.002) and 30 ± 1°C (diff = −3 days, *P*-adj < 0.001) compared to 26 ± 1°C, while no difference was detected between 28 ± 1°C and 30 ± 1°C (Table 2). Similarly, at 39.5 PSU, 30 ± 1°C significantly accelerated regeneration relative to both 26 ± 1°C (diff = −3.6 days, *P*-adj < 0.001) and 28 ± 1°C (diff = −2.4 days, *P*-adj = 0.002), but the latter two did not differ significantly (Table 2). These results indicate that temperature is a key driver of regeneration speed, with acceleration becoming evident from 28°C and peaking at 30°C under both salinity conditions.

When comparing salinity levels at fixed temperatures, a significant difference was only detected at 28 ± 1°C, where regeneration was slower under 39.5 PSU compared to 36.5 PSU (difference = 1.8 days, $P = 0.040$). No significant differences were found

https://doi.org/10.1017/S0025315425100611 Published online by Cambridge University Press

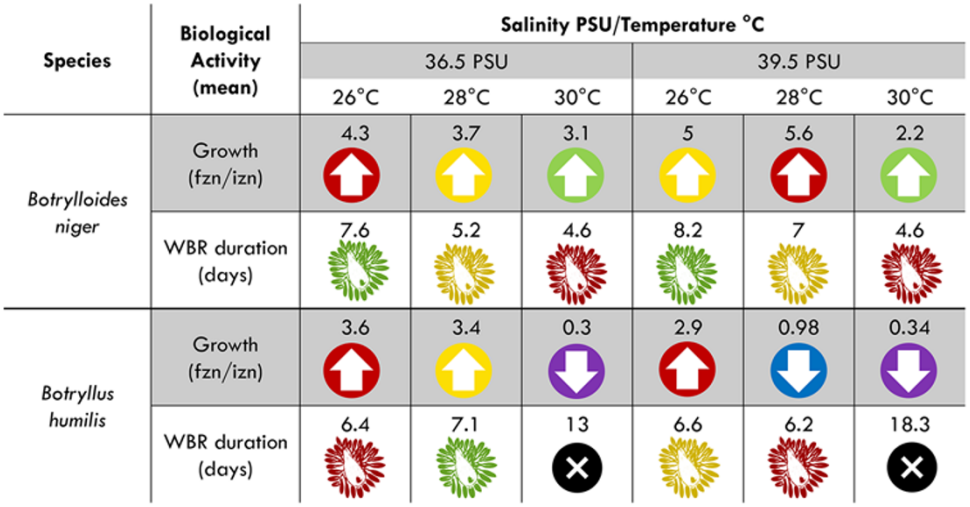

Figure 3. Summary of colony response under different experimental conditions, provided along with mean values of growth (final zooid numbers-fzn/initial zooid numbers-izn) and mean days of whole-body regeneration (WBR) duration. Upward arrows indicate growth (increase in zooid numbers) by palleal budding (blastogenic cycle) downward arrows indicates regression (decrease in zooid numbers). Zooids indicate completion of WBR with creation of filter feeding zooids, cross indicates unsuccessful at WBR completion and death. Red: Highest value for growth and fastest WBR completion. Yellow: Intermediate value. Green: Lowest value for growth and slowest WBR completion. Purple arrow: Maximum value for regression by vascular budding. Blue arrow: Intermediate value for regression.

Table 2. Multiple comparisons of group mean using Tukey's Honest Significant Difference (HSD) post-hoc test following two-way ANOVA, separately for growth rate and whole-body regeneration (WBR) duration under different salinity and temperature conditions. The table shows the estimated differences (diff) between group means, and the adjusted *P*-values (*P*-adj) accounting for multiple comparisons. Only comparisons with statistical significance (*P*-adj < 0.05) are shown, non-significant comparisons are omitted for clarity. A negative diff indicates that the first group has a lower mean value than the second group. Results for growth rate and WBR duration were analysed independently; they are presented in the same table for convenience, not for direct comparison. Growth rate: final zooid numbers-fzn/initial zooid numbers-izn

		Growth rate (fzn/izn)		WBR duration (days)	
Subgroups	Contrasts	Diff	*P*-adj	Diff	*P*-adj
Within 36.5 PSU	28 ± 1–26 ± 1°C			−2.4	0.002
	30 ± 1–26 ± 1°C			−3	0.000
	30 ± 1–28 ± 1°C				
Within 39.5 PSU	28 ± 1–26 ± 1°C				
	30 ± 1–26 ± 1°C	−2.9	0.035	−3.6	0.000
	30 ± 1–28 ± 1°C	−3.4	0.007	−2.4	0.002
At 26 ± 1°C	39.5–36.5 PSU				
At 28 ± 1°C				1.8	0.040
At 30 ± 1°C					

between salinity conditions at 26 ± 1°C and 30 ± 1°C (Table 2). This result indicates that the response to salinity is most pronounced at 28 ± 1°C during the WBR, and 36.5 PSU salinity is more favourable for regenerating colonies comparing to 39.5 PSU.

Botryllus humilis

B. humilis showed various responses to salinity and temperature changes along the blastogenic cycle and the WBR. The highest growth rates are observed in the S36-T26 (mean = 3.57) group followed by S36-T28 (mean = 3.39) and S40-T26 (mean = 2.85) (Figure 3). Regression (decrease in zooid numbers) was observed in three of the six experimental group, with the lowest growth rate observed at S36-T30 (mean = 0.28) followed by S40-T30 (mean = 0.35) and S40-T28 (mean = 0.98). Daily observations on experimental colonies and their zooid number information can be found in the Supplementary Dataset B. Two of the regressed groups, S36-T30 and S40-T30, exhibited disrupted colony synchrony starting from the first day in the experimental environment, compared to the palleal budding stages observed prior to the experiment (Supplementary File Figure S2 and Table S2). The ampullae appeared thickened, and the overall morphology of the colony did not resemble any of the blastogenic stages described in the Supplementary File Figure S2. No palleal buds developing on zooids were detected throughout the entire observation period, and thus blastogenic staging based on palleal budding could not be performed. Despite the absence of clear palleal buds, filter-feeding, regressing, and developing zooid structures were observed within the same colonies (Figure 4A), suggesting that new zooids continued to emerge during the experiment. These zooids were scattered across the colony among the ampullae, lacked any apparent budding structures, and were not arranged in a clear system-like pattern. They also regressed asynchronously

https://doi.org/10.1017/S0025315425100611 Published online by Cambridge University Press

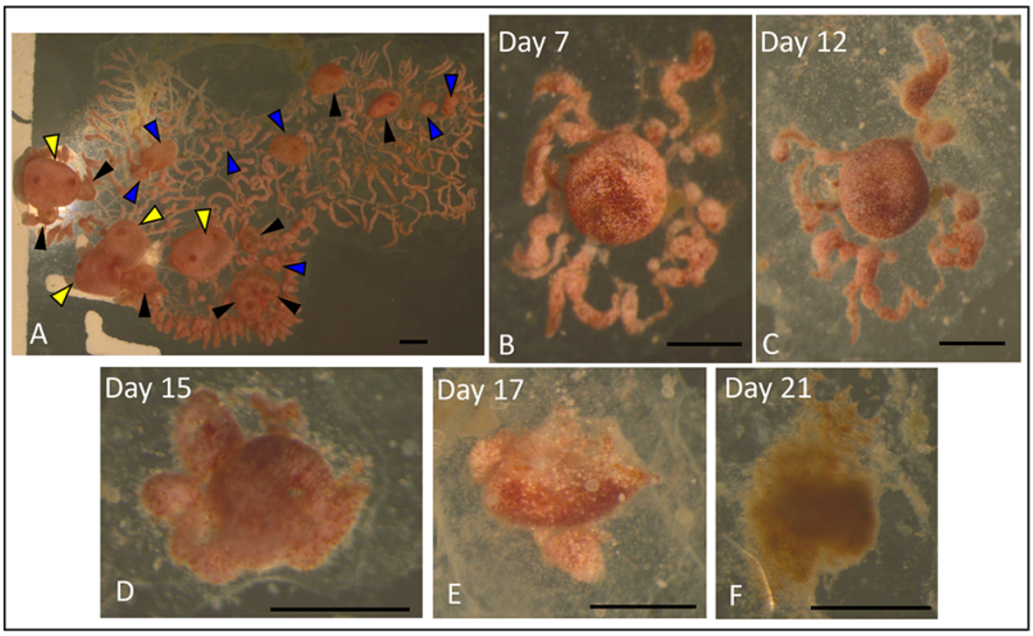

Figure 4. (A) Representative image of a *B. Humilis* colony maintained at high temperature (30 ± 1°C), showing asynchronous formation and regression of zooids under both salinity conditions (36.5 and 39.5 PSU). Functional zooids (yellow arrows), regressing zooids (black arrows), and newly forming zooids (blue arrows), which lack clear palleal bud appearance, are irregularly dispersed among the ampullae. (B–F) Whole-body regeneration (WBR) colony shrinkage process after seventh day for *B. Humilis* colonies under 39.5 PSU salinity and 30 ± 1°C temperature (S40_T30). Scale bar = 500 µm.

(Figure 4A). Furthermore, the number of filtering zooids did not follow a consistent increase or decrease over time. However, by day 12, the total number of zooids in both groups showed an overall decline compared to the initial count at day 0 (Supplementary File Figure S5). This combination of disrupted colonial synchrony, lack of detectable palleal budding, and continued emergence of new zooids led us to interpret this process as indicative of vascular budding. Overall, according to our study *B. humilis* colonies prefer low salinity over high salinity for growth, and they prefer vascular budding in high temperatures (30 ± 1°C) for asexual reproduction (Supplementary File Figure S5).

Four of the experimental groups of WBR colonies successfully completed their regeneration by creating filter-feeding zooids with no substantial difference in the number of zooids created (Supplementary File Figure S5 and Table S3). All the colonies within the group S36_T30 died immediately after reaching stage 4 (mean = 13 days). On the other hand, 2 out of 11 subclones under group S40_T30 completed their WBR within 5 and 11 days respectively. The remaining 9 colonies reached stage 4 and stayed there with continuous blood circulation. The colonies' remaining ampullae shrunk to disappearance and the colonies died within 21 days (Figures 4B–F). No substantial difference was observed between the groups in terms of the duration required to complete WBR, although the fastest WBR completion was observed in the S40_T28 group (mean = 6.2 days) (Supplementary File Figure S5 and Figure 2).

Discussion

Biological response

Botrylloides niger

According to the literature, there is no experimental information on the temperature preferences and responses of *B. niger* to changes in temperature, and very limited information on its responses to changes in salinity. In a study conducted in Panama, five *B. niger* colonies were experimentally exposed to five different salinity levels ranging from 20 to 35 PSU. Colonies exposed to lower salinities (20 and 24 PSU) underwent fewer asexual phases, and all colonies at 20 PSU died by the third day (Dijkstra and Simkanin, 2016). In the present study, we exposed 10 *B. niger* colonies to a total of 6 different temperature-salinity conditions and observed them during both the blastogenic cycle and WBR. According to our results, *B. niger* colonies thrive best at 28 ± 1°C with highest growth rate observation and, to a slightly lesser extent, at 26 ± 1°C, when salinity is 39.5 PSU. These results are consistent with the seasonal changes of Mediterranean coastal water and on-site observations. From December to April (average daily temperature of 2019 = 17.72°C), due to high precipitation (average daily precipitation 2019 = 4.95 kg/m^2) salinity decreases as well. However, May to November data fits with our experimental results and on-site observations. During this season, we observe bigger *B. niger* colonies in the area, the seawater temperature rises (average daily temperature of 2019 = 26.74°C) and due to evaporation and low precipitation (average daily precipitation of 2019 = 0.34 kg/m^2), salinity also increases (between 38 and 41 PSU, IMS Data). When the temperature is mild in April to December due to rainfall (ends in April and starts in December), the chance of finding *B. niger* colonies in the field (coastal areas) decreases (pers. observ.). This indicates that the re-recruitment of the *B. niger* on the site is dependent on the salinity increase. The salinity dependency was also confirmed by a statistically significant response to temperature changes at 39.5 PSU. In parallel, high salinity (39.5 PSU) is more favourable than low salinity (36.5 PSU) for *B. niger* during asexual reproduction due to higher growth rates. The experimental study of Dijkstra and Simkanin (2016) shows that high salinity is also favourable for local *B. niger* colonies within the local annual range (Bocas del Toro, Panama).

https://doi.org/10.1017/S0025315425100611 Published online by Cambridge University Press

Under high salinity conditions (39.5 PSU), the colony growth rate was higher than at 36.5 PSU and showed stronger response to temperature changes. Colonies grew better at moderate temperatures (26 ± 1°C and 28 ± 1°C), while growth decreased at 30 ± 1°C. On the other hand, regeneration was faster at higher temperatures in both salinity levels. Especially at 28 ± 1°C, regeneration was significantly quicker under low salinity (36.5 PSU) than under high salinity. These results suggest that different biological processes in *B. niger* react differently when two stress factors are combined where WBR initiation is accepted as a stress factor. As described by Fredersdorf et al. (2009), organisms become more sensitive to additional stress when the general condition is already not ideal. This was also observed in regenerating colonies, which were more affected by temperature than colonies growing asexually. According to the salinity experiment conducted on *B. niger* and *Botryllus planus (Van Name, 1902)*, an immediate response was observed in the neural gland (Dijkstra and Simkanin, 2016). Studies conducted on ascidian species suggest that the neural gland act as an osmoregulatory organ (Deyts et al., 2006; Ruppert, 1990). In the present study, regenerating colonies, which lack zooids and neural glands, may be unable to perceive changes in salinity and, therefore, may not adjust their responses accordingly. This could explain why the optimal salinity appears to shift from 39.5 PSU during asexual reproduction to 36.5 PSU during regeneration.

Botryllus humilis

While Karahan et al. (2023) provided habitat features such as sampling temperature and salinity records, no additional ecological information has been provided to date. The present study is the first to report the effects of salinity and temperature on colony growth. According to our results, the *B. humilis* colonies prefer low salinity over high salinity in contrast with *B. niger*, while choosing vascular budding under high temperatures (30 ± 1°C) (Figures 3 and 4A). Vascular budding occurs in botryllid ascidians either simultaneously with palleal budding with induced partial regeneration (Oka and Watanabe, 1957; Okuyama and Saito, 2001), or independently of palleal budding when the colony emerges from hibernation or WBR (Burighel et al., 1976). Rinkevich et al. (1995) reported that vascular budding represents a response of regeneration following damage, which also carries an ecological advantage. Voskoboynik et al. (2007) reported that vascular budding in *B. schlosseri* is only induced by the removal of zooids and buds at a specific blastogenic stage, suggesting that the process of reproduction through vascular budding is a feasible but less preferable pathway. Considering this information, this is the first study to observe vascular budding without the removal of zooids and buds. This may indicate that the high temperature harms zooids and buds therefore initiate an indirect regeneration process via vascular budding as a survival response. The zooids and buds are reported as the source of necessary energy and nutritive material for vascular budding (Manni et al., 2007; Sabbadin, 1956, 1958). According to our study, colonies from which zooids and buds were removed (i.e., colonies induced to undergo WBR) were unable to complete WBR under high-temperature conditions, unlike those in low-temperature groups. It seems that the colonies could not compensate for the energy lost by zooids and buds for vascular budding under additional stress.

Niche occupation and adaptation

When we compare the two species, *B. humilis* prefers lower salinity and *B. niger* prefers higher salinity. These observations complement the seasonal variations in the Mediterranean's coastal waters for *B. niger*. Although the origin of *B. niger* is thought to be the Atlantic Ocean (Sheets et al., 2016), Temiz et al. (2023) questioned this assumption based on the high morphological diversity in the Mediterranean. Besides, Sheets et al. (2016) suggested that the widespread and environmentally diverse distribution of *B. niger* indicates a high tolerance to varying conditions. It is possible that the morphological diversity observed in Mediterranean populations reflects phenotypic plasticity associated with high environmental tolerance. The first record of *B. humilis* along the Turkish coast of the Mediterranean dates to 2018, based on regular observations extending back to 2012, indicating its relatively recent arrival in the area (Karahan et al., 2023). This is in line with the suggestion of the origin of *B. humilis* as the Indo Pacific by Brunetti (2009 [=*B. anceps*]), suggesting a recent east-to-west expansion across the Mediterranean. The divergent responses of the two species to temperature and salinity may reflect their ecological niches in natural environments. *B. humilis* employs a vascular budding response, while *B. niger* employs hibernation (Temiz et al., 2023), which may help species to survive in more severe conditions that we did not cover in this study. These distinct responses signify niche differentiation, suggesting both species can coexist without intense competition, a notion supported by our site observation that both species' colonies co-exist on the same rock (Supplementary File Figure S6). Although there are no current records of these two species being invasive or forming fouling communities along the Turkish coasts (Karahan et al., 2023; Temiz et al., 2023), their potential impacts on native species should be monitored through regular observations. In particular, the high tolerance of *B. niger* and its dominance in fouling communities in other regions (Nydam et al., 2021; Ramalhosa et al., 2021; Sheets et al., 2016) highlight the need for close attention to this species.

This is the first experimental study on species *B. niger* and *B. humilis* in which the effects of two conditions are observed. The study provides significant insights into the niche occupation and ecological success of both species by analysing their physiological responses during blastogenic cycle and WBR to salinity and temperature variations. The observed growth rates and regeneration under different conditions reveal that *B. niger* preferences towards high salinity and moderate temperatures, while *B. humilis* prefers low salinity and displays vascular budding under high temperatures. Notably, our study is the first to document vascular budding in *B. humilis* without the removal of zooids and buds, highlighting a novel aspect of its biology. Our low-budget seawater filtration system effectively maintained viable ascidian colonies, demonstrating its feasibility for aquaculture. These findings advance our understanding of the biology and adaptability of ascidians to environmental changes, providing valuable information for their management in marine ecosystems.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0025315425100611.

Acknowledgements. The authors are grateful to Esra Öztürk and Begüm Ece Tohumcu for their help during sampling; Hurşit Kip, Mehmet Solmaz, and Öcal Işık for their technical assistance during aquaculture settlement; and Bilge Yüceer Karakuş (https://www.instagram.com/fungiandsnail) for the artwork used in Figure 1.

Author contributions. F.N.O.Ü. contributed to conceptualization, methodology, investigation, visualization, funding acquisition, and writing (original draft). M.K.Ü contributed to conceptualization and methodology. A.K. contributed to conceptualization, supervision, funding acquisition, and writing (review and editing).

Financial support. This research was supported by the Middle East Technical University BAP projects (HDESP-701-2021-10817, TEZ-D-701-2021-10653, and ADEP-701-2023-11278). The aquaculture facility was constructed by the DEKOSIM (BAP-08-11-DPT2012K120880) project. Funding sources are not involved in study design; in the collection, analysis, and interpretation of data; and in the decision to submit the article for publication.

Conflict of interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data accessibility statement. The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

References

Adamczuk M (2021) Demographic consequences of mitigating strategies in planktonic invertebrates facing global browning of freshwater ecosystem. *Aquatic Sciences* **83**(2), 31. https://doi.org/10.1007/s00027-021-00787-7

Agata K, Saito Y and Nakajima E (2007) Unifying principles of regeneration I: Epimorphosis versus morphallaxis. *Development, Growth & Differentiation* **49**(2), 73–78. https://doi.org/10.1111/j.1440-169X.2007.00919.x

Bae S, Lee S, Kim JM, Yi C-H, Choe K and Choi KH (2023) The effects of initiation season on succession patterns of benthic fouling organisms in coastal waters from two regions. *Regional Studies in Marine Science* **66**, 103150. https://doi.org/10.1016/j.rsma.2023.103150

Berrill NJ (1941) The development of the bud in *Botryllus*. *The Biological Bulletin* **80**(2), 169–184. https://doi.org/10.2307/1537595

Berrill NJ (1951) Regeneration and budding in tunicates. *Biological Reviews* **26**(4), 456–475. https://doi.org/10.1111/j.1469-185X.1951.tb01207.x

Blanchoud S, Rinkevich B and Wilson MJ (2018) Whole-body regeneration in the colonial tunicate *Botrylloides leachii*. In Kloc M and Kubiak J (eds), *Marine Organisms as Model Systems in Biology and Medicine. Results and Problems in Cell Differentiation*. Cham: Springer, Vol. 65, 337–355. https://doi.org/10.1007/978-3-319-92486-1_16

Brown FD, Keeling EL, Le AD and Swalla BJ (2009) Whole body regeneration in a colonial ascidian, *Botrylloides violaceus*. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution* **312**(8), 885–900. https://doi.org/10.1002/jez.b.21303

Brunetti R (2009) Botryllid species (*Tunicata, Ascidiacea*) from the Mediterranean coast of Israel, with some considerations on the systematics of *Botryllinae*. *Zootaxa* **2289**(1), 18–32. https://doi.org/10.11646/zootaxa.2289.1.2

Brunetti R, Beghi L, Bressan M and Marin MG (1980) Combined effects of temperature and salinity on colonies of *Botryllus schlosseri* and *Botrylloides leachi* (*Ascidiacea*) from the Venetian Lagoon. *Marine Ecology Progress Series* **2**, 303–314. https://doi.org/10.3354/meps002303

Burighel P, Brunetti R and Zaniolo G (1976) Hibernation of the colonial ascidian *Botrylloides leachi* (Savigny): Histological observations. *Italian Journal of Zoology* **43**(3), 293–301. https://doi.org/10.1080/11250007609430146

Carver CE, Chisholm A and Mallet AL (2003) Strategies to mitigate the impact of *Ciona intestinalis* (L.) biofouling on shellfish production. *Journal of Shellfish Research* **22**(3), 621–632.

Crow JF (1994) Advantages of sexual reproduction. *Developmental Genetics* **15**(3), 205–213. https://doi.org/10.1002/dvg.1020150303

Deyts C, Casañe D, Vernier P, Bourrat F and Joly J-S (2006) Morphological and gene expression similarities suggest that the ascidian neural gland may be osmoregulatory and homologous to vertebrate peri-ventricular organs. *European Journal of Neuroscience* **24**(8), 2299–2308. https://doi.org/10.1111/j.1460-9568.2006.05073.x

Dijkstra JA and Simkanin C (2016) Intraspecific response of colonial ascidians to variable salinity stress in an era of global change. *Marine Ecology Progress Series* **551**, 215–225. https://doi.org/10.3354/meps11719

Epelbaum A, Herborg L-M, Therriault TW, Pearce CM and Pearce CM (2009) Temperature and salinity effects on growth, survival, reproduction, and potential distribution of two non-indigenous botryllid ascidians in British Columbia. *Journal of Experimental Marine Biology and Ecology* **369**(1), 43–52. https://doi.org/10.1016/j.jembe.2008.10.028

Fredersdorf J, Müller R, Becker S, Wiencke C and Bischof K (2009) Interactive effects of radiation, temperature and salinity on different life history stages of the Arctic kelp *Alaria esculenta* (*Phaeophyceae*). *Oecologia* **160**(3), 483–492. https://doi.org/10.1007/s00442-009-1326-9

Goodbody I (2004) *Diversity and Distribution of Ascidians (Tunicata) at Twin Cay, Belize*. Washington, DC, USA: National Museum of Natural History Smithsonian Institution September.

Halim Y and Messeih MA (2016) Aliens in Egyptian waters. A checklist of ascidians of the Suez Canal and the adjacent Mediterranean waters. *Egyptian Journal of Aquatic Research* **42**(4), 449–457. https://doi.org/10.1016/j.ejar.2016.08.004

Holland LZ (2016) Tunicates. *Current Biology* **26**(4), R146–R152. https://doi.org/10.1016/j.cub.2015.12.024

Jackson JBC and Coates AG (1986) Life cycles and evolution of clonal (modular) animals. *Philosophical Transactions of the Royal Society of London. Biological Sciences* **313**(1159), 7–22. https://doi.org/10.1098/rstb.1986.0022

Karahan A, Öztürk E, Temiz B and Blanchoud S (2022) Studying tunicata WBR using *Botrylloides anceps*. In Blanchoud S, and Galliot B (eds), *Methods in Molecular Biology Whole-Body Regeneration: Methods and Protocols*, Vol. 2425, New York: Springer, pp. 311–332.

Karahan A, Temiz B, Öztürk E, Douek J and Rinkevich B (2023) Seven ascidian (Tunicata) species from the Northeastern Mediterranean. *Mediterranean Marine Science* **24**(3), 545–557. https://doi.org/10.12681/mms.32937

Lambert G (2001) A global overview of ascidian introductions and their possible impact on the endemic fauna. In Sawada, H, Yokosawa, H, and Lambert, CC (eds.), *The Biology of Ascidians*. Tokyo: Springer, pp. 249–257.

Lambert G (2005) Ecology and natural history of the protochordates. *Canadian Journal of Zoology* **83**(1), 34–50. https://doi.org/10.1139/z04-156

Lauzon RJ, Ishizuka KJ and Weissman IL (1992) A cyclical, developmentally regulated death phenomenon in a colonial urochordate. *Developmental Dynamics* **194**(1), 71–83. https://doi.org/10.1002/aja.1001940109

Lauzon RJ, Ishizuka KJ and Weissman IL (2002) Cyclical generation and degeneration of organs in a colonial urochordate involves crosstalk between old and new: A model for development and regeneration. *Developmental Biology* **249**(2), 333–348. https://doi.org/10.1006/dbio.2002.0772

Lenz M, da Gama BAP, Gerner NV, Gobin J, Gröner F, Harry A, Jenkins SR, Kraufvelin P, Mummelthei C, Sareyka J, de Xavier EA and Wahl M (2011) Non-native marine invertebrates are more tolerant towards environmental stress than taxonomically related native species: Results from a globally replicated study. *Environmental Research* **111**(7), 943–952. https://doi.org/10.1016/j.envres.2011.05.001

Malfant M, Coudret J, Le Merdy R and Viard F (2017) Effects of temperature and salinity on juveniles of two ascidians, one native and one invasive, and their hybrids. *Journal of Experimental Marine Biology and Ecology* **497**, 180–187. https://doi.org/10.1016/j.jembe.2017.09.019

Manni L, Gasparini F, Hotta K, Ishizuka KJ, Ricci L, Tiozzo S, Voskoboynik A and Dauga D (2014) Ontology for the asexual development and anatomy of the colonial chordate *Botryllus schlosseri*. *Plos One* **9**(5), e96434. https://doi.org/10.1371/journal.pone.0096434

Manni L, Zaniolo G, Cima F, Burighel P and Ballarin L (2007) *Botryllus schlosseri*: A model ascidian for the study of asexual reproduction. *Developmental Dynamics* **236**(2), 335–352. https://doi.org/10.1002/dvdy.21037

Martinez VG, Menger GJ, III and Zoran MJ (2005) Regeneration and asexual reproduction share common molecular changes: Upregulation of a neural glycoepitope during morphallaxis in Lumbriculus. *Mechanisms of Development* **122**(5), 721–732. https://doi.org/10.1016/j.mod.2004.12.003

McCarthy A, Osman RW and Whitlatch RB (2007) Effects of temperature on growth rates of colonial ascidians: A comparison of *Didemnum sp.* to *Botryllus schlosseri* and *Botrylloides violaceus*. *Journal of Experimental Marine Biology and Ecology* **342**(1), 172–174. https://doi.org/10.1016/j.jembe.2006.10.036

Millar RH (1971) The biology of ascidians. *Advances in Marine Biology* **9**, 1–100. https://doi.org/10.1016/s0065-2881(08)60341-7

Mukai H and Watanabe H (1976) Relation between sexual and asexual reproduction in the compound ascidian, *Botryllus primigenus*. Science reports of the Faculty of Education. *Gunma University* **25**, 61–79.

Nydam ML, Lemmon AR, Cherry JR, Kortyna ML, Clancy DL, Hernandez C and Cohen CS (2021) Phylogenomic and morphological relationships among the botryllid ascidians (subphylum *Tunicata*, class *Ascidiacea*, family *Styelidae*). *Scientific Reports* **11**, 8351. https://doi.org/10.1038/s41598-021-87255-2

Oka H and Watanabe H (1957) Vascular budding, a new type of budding in *Botryllus*. *The Biological Bulletin* **112**(2), 225–240. https://doi.org/10.2307/1539200

Okuyama M and Saito Y (2001) Studies on Japanese botryllid ascidians. I. A new species of the genus *Botryllus* from the Izu Islands. *Zoological Science* **18**(2), 261–267. https://doi.org/10.2108/zsj.18.261

Pérès JM (1958) Ascidies recoltées sur les côtes Méditerranéennes d'Israel. *Bulletin of the Research Council of Israel B* **7**, 143–150.

Ramalhosa P, Gestoso I, Rocha RM, Lambert G and Canning-Clode J (2021) Ascidian biodiversity in the shallow waters of the Madeira Archipelago: fouling studies on artificial substrates and new records. *Regional Studies in Marine Science* **43**, 101672. https://doi.org/10.1016/j.rsma.2021.101672

Rinkevich B, Shlemberg Z and Fishelson L (1995) Whole-body protochordate regeneration from totipotent blood cells. *Proceedings of the National Academy of Sciences of the United States of America* **92**(17), 7695–7699. https://doi.org/10.1073/pnas.92.17.7695

Ruppert EE (1990) Structure, ultrastructure and function of the neural gland complex of Ascidia interrupta (Chordata, *Ascidiacea*): clarification of hypotheses regarding the evolution of the vertebrate anterior pituitary. *Acta Zoologica* **71**(3), 135–149. https://doi.org/10.1111/j.1463-6395.1990.tb01189.x

Sabbadin A (1956) Situs inversus viscerum provocato sperimentalmente in *Botryllus schlosseri* (Pallas) (*Ascidiacea*). *Rendiconti Accademia Nazionale Lincei* **20**, 659–666.

Sabbadin A (1958) Analisi sperimentale dello sviluppo delle colonie di *Botryllus schlosseri* (Pallas) (Ascidiacea). *Archivio Italiano di Anatomia e di Embriologia* **63**, 178–221.

Salonna M, Gasparini F, Huchon D, Montesanto F, Haddas-Sasson M, Ekins M and Gissi C (2021) An elongated COI fragment to discriminate botryllid species and as an improved ascidian DNA barcode. *Scientific Reports* **11**(1), 4078. https://doi.org/10.1038/s41598-021-83127-x

Sebestyén F, Barta Z and Tökölyi J (2018) Reproductive mode, stem cells and regeneration in a freshwater cnidarian with post reproductive senescence. *Functional Ecology* **32**(11), 2497–2508. https://doi.org/10.1111/1365-2435.13189

Sheets EA, Cohen CS, Ruiz GM and Da Rocha RM (2016) Investigating the widespread introduction of a tropical marine fouling species. *Ecology and Evolution* **6**(8), 2453–2471. https://doi.org/10.1002/ece3.2065

Shenkar N and Swalla BJ (2011) Global Diversity of Ascidiacea. *Plos One* **6**(6), e20657. https://doi.org/10.1371/journal.pone.0020657

Temiz B, Öztürk E, Blanchoud S and Karahan A (2023) Phylogeographic and morphological analysis of *Botrylloides niger* Herdman, 1886 from the Northeastern Mediterranean Sea. *Diversity* **15**(3), 367. https://doi.org/10.3390/d15030367

Therriault TW and Herborg L-M (2008) Predicting the potential distribution of the vase tunicate *Ciona intestinalis* in Canadian waters: Informing a risk assessment. *ICES Journal of Marine Science* **65**(5), 788–794. https://doi.org/10.1093/icesjms/fsn054

Virgili R, Tanduo V, Katsanevakis S, Terlizzi F, Villani G, Fontana A and Crocetta F (2022) The Miseno Lake (central-western Mediterranean Sea): An overlooked reservoir of non-indigenous and cryptogenic ascidians in a marine reserve. *Frontiers in Marine Science* **9**, 866906. https://doi.org/10.3389/fmars.2022.866906

Voskoboynik A, Simon-Blecher N, Soen Y, Rinkevich B, De TAW, Ishizuka KJ and Weissman IL (2007) Striving for normality: Whole body regeneration through a series of abnormal generations. *The FASEB Journal* **21**(7), 1335–1344. https://doi.org/10.1096/fj.06-7337com

Zhan A, Briski E, Bock DG, Ghabooli S and MacIsaac HJ (2015) Ascidians as models for studying invasion success. *Marine Biology* **162**(12), 2449–2470. https://doi.org/10.1007/s00227-015-2734-5

Zondag L, Rutherford K, Gemmell NJ and Wilson MJ (2016) Uncovering the pathways underlying whole body regeneration in a chordate model, *Botrylloides leachi* using de novo transcriptome analysis. *BMC Genomics* **17**(1), 114. https://doi.org/10.1186/s12864-016-2435-6

https://doi.org/10.1017/S0025315425100611 Published online by Cambridge University Press