Behavioural Responses of the Colonial Sea Squirt *Botrylloides violaceus* Oka to Suspended Food Micro-Particles in Laboratory Cultures

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Abstract: Violet sea squirts are noteworthy model organisms, because they provide insights into various physiologic processes, including cell senescence, ageing, apoptosis and allorecognition. Consequently, their culture is critical to permit experimental studies. Most papers refer to short periods of rearing using various feeds, both living and conserved, missing a formal justification for their use or indications of their actual nutritional value. Here, we use two behavioural responses—the percentage of open siphons and the frequency of zooid contractions—as compared to the abundance of suspended microparticles during feeding tests, to identify feeds able to promote filter-feeding. The results will enable to formulate compound diets that maximise positive physiological responses. Our tests demonstrated that plant items, such as dry microalgae and cyanobacteria (*Arthrospira platensis*, commercially known as Spirulina), along with living planktonic Haptophyta (*Isochrysis galbana*), trigger clear positive reactions, represented by a higher frequency of zooid contractions and larger proportions of open siphons. These responses correspond to decreases in the concentrations of suspended microparticles during the experiment and indicate higher filter-feeding activity. In contrast, feeds commonly administered to colonies, such as milk powder, dried eggs and artificial plankton, triggered negative behavioural responses, and their intake was lower during the feeding trials.

Keywords: suspension feeds; tunicates; behavioural response; model organism; rearing; culture optimisation

1. Introduction

Invertebrate models closely related to vertebrates provide insights into various physiologic processes, including senescence [1], vascular regeneration [2], allorecognition and ageing [3]. Botryllid ascidians are small, sessile filter-feeding tunicates widely distributed in fouling communities at various latitudes [4,5]. The Japanese compound botryllid *Botrylloides violaceus* Oka belongs to the chordate subphylum Tunicata (Urochordata), a taxonomic group considered to be the closest living invertebrate relative of vertebrates [2,6]. It is locally common along the Mediterranean coasts, in low intertidal and subtidal habitats [7], where it may be abundant both on vertically and horizontally oriented substrates, as well as on algae [8,9]. Various species of *Botrylloides* have been adopted as spectacular models for the study of allorecognition [10] and indicators of environmental quality [11]. These sciaphilic species live on rocks, docks, ropes and floating buoys in shallow marine waters and estuaries [12]. In particular, *B. violaceus* is quite an interesting species for experimental purposes, also due to its viviparous strategy, with eggs developing in the colonial tunic, nourished by blood

circulation and larvae hatched with up to 30 *ampullae*. This reproductive feature makes *B. violaceus* an excellent model organism for various types of laboratory tests.

Increasing interest in ecological, biological, developmental and ecotoxicological studies (including ageing and invasion ecology) led to the employment of *Botrylloides* spp. as experimental model organisms since the 1980s, thanks to an easy and rapid development in the laboratory and the possibility to obtain quick results and the screening of malformations and abnormalities [12,13]. *Botrylloides* Milne Edwards (1841), along with *Botryllus* Gaertner, 1774, show a peculiar life cycle initially described by [11] and further recapitulated by [14]. Each colony of *B. violaceus* is composed of thousands of genetically identical modules named zooids, embedded into a gelatinous matrix named "tunic", which reproduce both asexually and sexually. A network of ramified vasculature connects all zooids within a colony that ends, at its margins, into pear-shaped vascular termini named "*ampullae*" [5]. A typical astogenic route, divided into four main phases, is named "blastogenesis" and characterises its life cycle [15]. Zooids are spread across the tunic matrix of mature colonies, surrounded by *ampullae* and continuously loaded with blood cells, visible also under a stereomicroscope at low magnification. When the colony is subjected to inappropriate environmental conditions, hibernation takes place, and zooids are resorbed [16], being replaced by a carpet of *lacunae*, dilated vasculature and pigmented cells. When environmental conditions turn to the preferred ones, new pallial buds emerge from the body of parental zooids, syphons open over the surface and the cycle ends after about seven days (according to temperature), concluding with massive apoptotic and phagocytic events affecting all parental zooids [15]. At the same time, primary buds develop into the next generation of adults, returning to a blastogenic stage.

The sexual reproduction proceeds through cross-fertilisation, leading to the birth of a tadpole-like larva. The planktonic larva swims a few hours before settling and starts metamorphosis towards a sessile oozooid, able to initiate a new colony. In contrast, for asexual reproduction, the colony develops through the above-mentioned budding [11]; the mature zooid supports on its body wall a primary bud that supports, in turn, a secondary bud (or "budlet"), consisting of three generations of clones at once and giving rise to a circle in which adults undergo resorption, buds replace adults and budlets replace buds. This process is called "take-over" and occurs every six days at temperatures around 18–19 °C [15]. Salinity, pH and feeding constraints influence the physiology and the life cycle of *Botrylloides* [17,18], besides temperature, which is the main factor inducing hibernation/aestivation in nature. Keeping these environmental variables within the preferred intervals makes consistent the availability of colonies throughout the year, and new larvae are frequently observed [12].

Tunicate husbandries were developed [17,19] to outline an appropriate diet able to increase the growth rates of colonies while avoiding larval and juvenile mortality by pooling live phytoplankton and the compound feed (e.g., Liquifry Marine®, Invertfood® and Marine Invertebrate Diet®). In particular, the efficacy of a mixture of food types on the development and growth rates of *B. leachii* has been demonstrated [17]. They are known to be mucus filter-feeders or suspension feeders, able to extract particles of two to three μm, or even larger, up to a limit set by the size of their oesophagus [20]. In *Botrylloides*, as in *Botryllus*, the inhalant siphons are enlarged and conical, and buds developing adjacent to one another orientate into two facing rows to form a typical system called "*leachii*-type" [21]. Here, the narrow ends of the atrial (exhalant) cones open independently or in common (according to the species) into a cloacal chamber of each system [11]. The internal structure of zooids is dominated by the branchial sac, or "pharynx", which is dedicated to the removal of food microparticles from the water column. Seawater is drawn through the oral siphons, pushed through gill slits by body contractions and, finally, expelled, along with wastes, through an exhalant siphon shared by all zooids [22]. Their feeding ecology, in terms of quality and abundance of food, influences the blastogenesis, the sex ratios and the number of eggs produced [14]. A few data are available on the effect of live and conserved feeds on their growth and reproduction in culture conditions. Single species of microalgae are often administered for experimental rearing, with scarce attention to their ability to promote physiologic answers by the animals, limiting the possibility to obtain continuous cultures for experimental purposes [23]. Here,

we investigated the behavioural responses of *B. violaceus* [18] to the administration of various feeds and live phytoplankton to set a good "recipe" for rearing and experimental purposes. We monitored the behavioural responses of colonies fed on a range of possible food resources to detect the influences of individual feeds and set an effective diet able to maximise their ingestion rates in the laboratory cultures.

2. Materials and Methods

2.1. Design of Experiment

We recorded the contractions and the opening of siphons by collecting photos of the colonies before and after each feeding trial. In addition, feed particles were counted before feeding assays and immediately after. Seven feed components (Table 1) and a control feed (milk powder) were tested, as previously experimented on *Ciona robusta* [24]. In previous investigations, the best results were obtained by adopting a mixed diet containing both formulated and live particles, but the possible individual effects of each feed component are still unknown. Here, we aim at testing whether every single feed component of a mixed diet is effectively filtered by *Botrylloides violaceus* and the reactions of zooids to the food microparticles suspended in the seawater. The results will permit to formulate an effective diet by dosing various feed components according to their demonstrated efficiency. Thus, the experiment was made of seven treatments and one control, each tested over four replicates.

Table 1. Feed components tested, their contents and percent composition (*Nutritional values of *I. galbana* from Lavens and Sorgeloos, 1996). All listed components are relatively inexpensive and quite easy to obtain on the market. Most feeds were provided by SHG (Super High Group s.r.l., Ovada, Italy). Each feed component was tested in four replicates of about 30 individuals. In Feed component column, manufacturers are reported in brackets. HUFA is the abbreviation for highly unsatured fatty acids.

Feed Component	Content	% Proteins	% Lipids	% Carbohydrates	% Fibres	% Ashes	% Moisture
Milk Powder (Ristora)	Dried skimmed milk	34.00	1.20	56.00	-	-	-
SnowReef® (SHG)	Yeast (*Saccharomices cerevisiae*) and torula yeast, microalgae, squid pulp, fish oil at high content of HUFA	42.00	7.00	-	-	7.00	-
Freeze-dried egg	Yolk and albumen	12.56	9.51	0.72	-	-	-
PhytoReef® (SHG)	Principal component: lyophilised *Chlorella* sp.	58.00	11.00	-	19.00	-	< 10.00
AlgaMac® Protein Plus® (PTAqua)	Dried microalgae	42.90	21.00	17.50	-	12.40	6.00
*Isochrysis galbana**	Live axenic culture of microalgae	29.00	23.00	12.90	-	-	-
Dry Spirulina® (SHG)	100% freeze-dried microalgae	60.00	4.00	-	7.00	7.00	-
Dried Yeast (Selex)	Dried brewer yeast	19.00	19.00	72.00	2.50	-	-

2.2. Animal Identification and Rearing

Specimens were collected in the harbour of Ischia (Bay of Naples, South Tyrrhenian Sea) in October and November 2019. Colonies were gently detached from floating buoys and ropes using a razorblade and a paddle. They were promptly transferred to the laboratory and reared in two aerated 15-L tanks with open flowthrough seawater. The identification was based on morphological characters [18,22,25], since we observed quite small cylindrical zooids without finger-like tentacles with a formula 6L + 6M + 6S. After five days of acclimation, colonies were transferred to a thermostatic chamber (18 °C; 12:12-h light:dark cycle) in 15-L aerated tanks equipped with a layer of 5-cm calcareous sand and under-gravel filter. A lid covered the tank to limit evaporation. Constant water temperature and salinity conditions were imposed to avoid possible torpor or death of colonies due to environmental stresses [5]. Water was replaced every 3 days to avoid the settlement of microalgae and biofilms. In fact, films that settle on the surface of colonies obstruct the inhaling oral siphon, hinder normal feeding and may trigger premature deaths.

2.3. Preparation of Feeds

Candidate feed components were established, taking into account previous investigations on the culture of tunicates [24], modified according to the smaller size of *Botrylloides* zooids and branchial sacs [18]. For this reason, some common feeds were tested to formulate a compound diet (Table 1), and their performances were compared to those of a very simple and common feed, represented by milk powder. In fact, milk powder and its equivalents (as Liquifry® meal; [26]) are often used as a quick solution for the short time of the rearing of tunicates. Feeds were individually tested on colonies reared in dishes filled with 300 mL of filtered seawater (0.22-μm Millipore, Burlington, MA, USA) during 2-h trials. Each feed was dosed to reach a final concentration of 2 μg mL^{-1} seawater. To this end, feeds were weighed and previously suspended in filtered (0.22-μm Millipore) seawater prior to being dosed to obtain 0.6 mg dry weight (d.w.) in each test dish. The cell densities of live cultures of microalgae were assessed by employing a cell counting chamber, and the proportions of cultures corresponding to the same dry weight of other feeds (0.6 mg) were collected and adjusted to 10 mL using filtered seawater. Further, these aliquots were added to each test dish, containing 290 mL of filtered seawater. Dried eggs were prepared using boiled eggs that were freeze-dried in a Modu-Lyo (5-Paskal, Trezzano sul Naviglio, Italy) high-vacuum system, then ground in a tissue grinder potter and sieved through a 10-μ polystyrene filter (Merck KGaA, Darmstadt, Germany) to assure the size consistency of particles in a range of 2–7μ when still dry. This range corresponds to that characterising all other feed items herein described (see Table 1).

2.4. Feeding Tests

Botrylloides violaceus colonies were finally transferred to the test dishes and covered with transparent lids to avoid evaporation. Four replicate tests were performed on individual colonies of about 30 individuals for each of the 8 food items (7 tested and 1 control, as above specified), obtaining a total of 32 replicate colonies bioassayed in individual 500-mL dishes. All tests were conducted in walk-in thermostatic chambers kept at the same temperature and light irradiance of collected cultures. Colonies selected for the tests were approximately at the same developmental stage, with mature and active filtering zooids. Temperature, salinity and pH were recorded at the beginning and the end of tests. Feeds were individually tested (see Table 1), and their administration started simultaneously. All animals were checked prior to start bioassays to assure their viability. However, a large variability was recorded at the start of each test in the percentage of open siphons and the frequency of contractions, due to natural variability of the individual metabolisms. For this reason, the effects of the feeds were evaluated as a percentage of variation (the percentage of open siphons referred to the start of the test; the percentage of contractions per minute referred to the start of the test) with respect to the measures recorded prior to starting the feeding trials. Similarly, the decrease of suspended feed particles referred to their abundance prior to starting each feeding test.

Feeding activities and the reactions to feeds were recorded before (t_0) and at the end of the tests (t_1), two hours after the administration of feeds. In particular, the percentage of open oral siphons (%OS) was evaluated under a Leica MZ16 macroscope (Leica Microsystems s.r.l., Buccinasco, Italy); the percentage of zoids contracting each minute (C/min) was counted under a stereomicroscope Leica MZ6; the portion of feed microparticles that decreased between the start and the end of the experiments was evaluated by counting particles in a Neubauer chamber under an inverted microscope Leica DMLB and referring to the number of particles per μL. These behavioural physiology indicators are all indicators of feeding activities and preferences, being related to the responses of sensory cells to stimulation [27]. To record these indicators, for each replicate under each treatment, zooids were observed at t_0 and again at t_1. The number of open siphons and the number of contractions (as a percentage of total individuals in the colonies) were recorded each minute for three times (for a total of 3 min at the start of the experiment and 3 min at the end of the experiment). One minute of continuous observation was considered enough to detect active contractions, and siphons stably closed or open, during the whole time of observation, were easily detected. At the start of each 1-min record, a photo was also obtained for

further comparisons. The data recorded were averaged over three 1-min replicates to obtain the mean differences in feeding activities between t_0 and t_1.

2.5. Statistical Analyses

The significance of differences in the percentages of open siphons, contractions and microparticle concentrations between each feed and the control feed (milk powder) were tested by means of *t*-tests performed using GraphPad Prism 8.0.0 for Mac OS (GraphPad Software, San Diego, CA, USA). Feeds were then ranked according to their efficiency to trigger animals' reactions. The significance of differences in open siphons, contractions and particle concentrations between t_0 and t_1 for all feeds was evaluated by means of a paired *t*-test (two-tailed) using the same software. Simple linear regressions between all measured indicators (percentage of open siphons, percentage of contractions and feed particle concentrations) were also computed. R-squared indices of the regression were evaluated, and slopes were compared to assess the significance of regressions and the feeding efficiency trends exhibited by each feed, according to the chosen descriptors.

3. Results

The main descriptors of water quality were kept within acceptable ranges for the reared species. In particular, the temperature was kept at 17.8 °C (± 0.3 SD), salinity was 36.8 g L^{-1} (± 0.9 SD) and pH was 8.13 (± 0.04 SD). Tested feeds triggered large differences in the percentage of contractions referring to the start of the feeding tests. Such algal feeds as AlgaMac®, live *Isochrysis galbana* and dry Spirulina triggered the most evident reactions (Figure 1A), with percent differences in the number of contractions as high as 4.9% ($\pm 1.6\%$ SD), 5.6% ($\pm 1.5\%$ SD) and 8.7% ($\pm 1.2\%$ SD). All these values significantly differed ($p < 0.05$) from those obtained under the milk powder control treatment. As well, dried yeast triggered a percentage of contractions as high as 6.05% ($\pm 2.6\%$ SD) significantly different ($p < 0.05$) from the milk powder control treatment (Figure 1A). SnowReef®, dried egg and PhytoReef® triggered reactions not significantly different from those prompted by the milk powder control treatment.

Differences in the percentage of open siphons followed a similar trend (Figure 1B), with dried yeast peaking at 31.3% (± 5.4 SD), followed by dry Spirulina, live *I. galbana* and AlgaMac® (20.0% $\pm$ 2.8% SD, 17.2% $\pm$ 3.8% SD and 11.1% $\pm$ 2.4% SD, respectively). Other feeds did not differ significantly from the performances of the milk powder control feed. In parallel, dried yeast, dry Spirulina and live *I. galbana* exhibited the highest differences in microparticle concentrations, with respect to the start of the experiments, followed by AlgaMac® and PhytoReef®, demonstrating that their suspensions were actively ingested by *B. violaceus* (Figure 1C). In contrast, dried egg and SnowReef® did not show significant differences from milk powder, and their particle concentrations decreased in the liquid suspension at rates as low as 3.8% ($\pm 0.02\%$ SD) and 3.06% ($\pm 0.02\%$ SD), indicating low ingestion rates (Figure 1C).

Most indicators were strictly related to each other (Figure 2). Differences in the number of particles and percentage of contractions exhibited a direct linear relationship ($R^2 = 0.64$; $p < 0.0001$) with a straight slope of 14.43, indicating that contractions are well-correlated to the decrease in the concentration of feeds (Figure 2A). The differences in open siphons (Figure 2B) are also correlated to the decrease in the number of suspended particles ($R^2 = 0.56$; $p < 0.0001$), although the slope is lower (0.38). As a consequence, both indicators of animals' reactions (differences in open siphons and in the number of contractions) are directly and linearly related ($R^2 = 0.57$; $p < 0.0001$), indicating that an increase of activity is observed according to the rhythms of particle ingestions (slope 26.49; Figure 2C) and that different feeds are chosen according to the mentioned ranking values. On the whole, the behavioural reactions of cultured specimens were easily determined based on simple observations under optical microscopy (Figure 3).
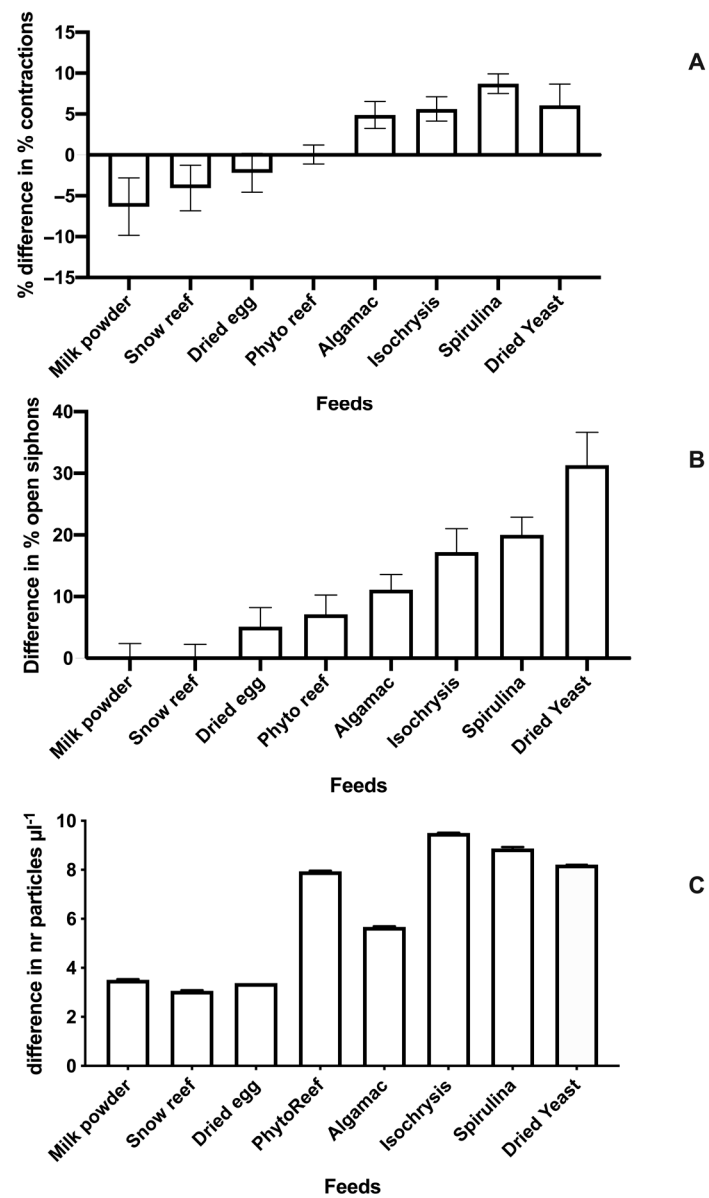
Figure 1. Effects of tested feeds and behavioural responses of *Botrylloides violaceus*, mean values for 4 replicates and error bars. (**A**) Percent difference in contractions between the start of the experiment (t_0) and the end (t_1), (**B**) percent difference in open siphons between the start of the experiment (t_0) and the end (t_1) and (**C**) difference in the number of particles between the start of the experiment (t_0) and the end (t_1).
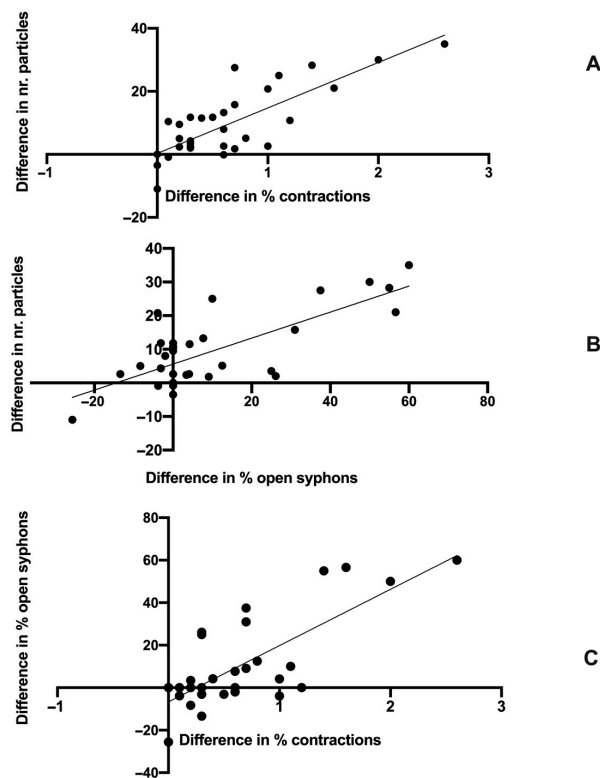
Figure 2. Linear relationships between behavioural responses and suspended feed microparticles according to the tested feeds. (**A**) Relationship between the number of suspended particles and percent contractions of *B. violaceus* ($R^2 = 0.64$; slope 14.43), (**B**) relationship between the number of particles and open siphons ($R^2 = 0.56$; slope 0.38) and (**C**) relationship between the open siphons and number of contractions ($R^2 = 0.57$; slope 24.49).
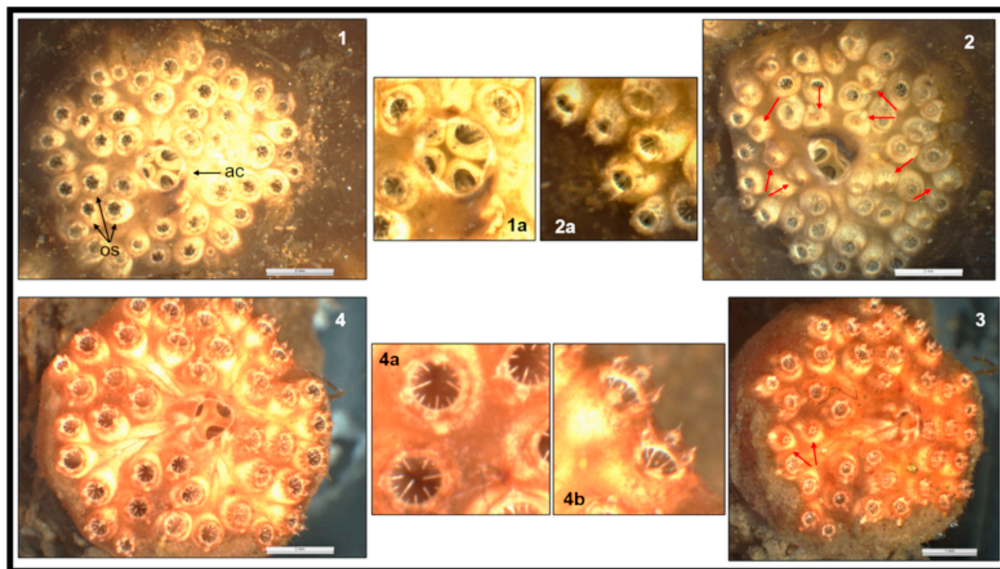
Figure 3. Colour morphs of *Botrylloides violaceus*: (**1,4**) Two different colonies formed by double-facing rows of zooids, with open inhaling siphons actively filtering. (**2,3**) The same colonies of (**1**) and (**4**) showing some closed inhaling siphons, not actively filtering (red arrows), and common cloacal channel slightly closed. (**1a**) Details of the common atrial channel of the colony in (**1**) the view on the branchial sac. (**2a,4b**) Details of the oral siphons rims of the colonies in (**2**) and in (**4**), respectively, in which tentacles are well-visible. (**4a**) Frontal view of oral siphons and rims of the colony in (**4**). **os** = oral siphons. **ac** = common atrial channel.

4. Discussion

Among ascidians, several species are worldwide invaders of various marine habitats [8], and *Botrylloides violaceus* has been particularly successful [9]. In fact, it invaded most of the North Pacific, North Atlantic and the Mediterranean Sea [28]. *B. violaceus* first appeared in New England and in the Gulf of Maine, possibly through multiple introductions [8]. Its spread was facilitated by increasing water temperatures due to global changes, combined with a selection of cold-tolerant genotypes [8,29], but its adaptability to a range of particulate foods may represent an additional advantage for its spread in fouling communities of the Mediterranean. The feeding rates of *B. violaceus* were within the range reported for other urochordates [27] during our feeding tests.

This species is relatively insensible to sedimentation, and consequently, it may be found also on horizontal substrates, while other colonial ascidians prefer vertical substrates, where sedimentation is limited [30]. Its adaptability was confirmed by our feeding experiments, because it was able to filter items that were quite different in terms of composition (animal or plant origins), the size of microparticles and shapes (e.g., live algae vs. dry organic particles). Conceivable stressing factors, such as alterations of pH and temperature, were never revealed during the experiments. In particular, the pH was monitored and kept quite stable in test vessels during the two-hour experimental time, because it is known that some ascidians sequester acids in their tunics, which can repel predators [31]. Evidently, this mechanism did not affect the pH of the culture medium. Since there was no interactions among the feed components, the responses of the animals were directly triggered by chemoreception and tactile stimulation by individual feeds [27]. Thus, our results will be useful to identify formulations able to stimulate filter-feeding activities in colonial ascidians, according to the behavioural reactions triggered by their sensory organs. In particular, the coronal organ was described in *B. violaceus* as a key sensory structure present in the tentacles and velum of the oral siphon [32], able to detect various features of the feed particles, and this organ is of great interest, because it is made of peculiar axonless sensory ciliated cells directly connected with neurons. The coronal organ may thus represent an early expression of the genes that gave rise to the acustico–lateralis system of chordates [33].

The positive reactions to the presence of algal feeds (AlgaMac®, *I. galbana* and Spirulina) indicate that the above-mentioned chemoreceptors actively select algal items. In fact, the particle size distributions of these algal items do not differ significantly from such feed components as milk powder and dried eggs, but tested colonies markedly preferred diatoms and planktonic algae. In addition, the above-mentioned three algal feed items triggered consistent reactions in terms of animal contractions and open siphons, indicating that both behavioural reactions may be useful to select the preferred feeds. This evidence was confirmed by the rates of consumption of the feed particles, because both *I. galbana* and Spirulina produced a prompt decrease in the number of suspended particles during the feeding experiments, followed by AlgaMac®.

Contrasting evidence is represented by PhytoReef®. In fact, this feed mainly contains plant items, and it showed very high values of the depletion of suspended feed particles, indicating that cultured animals actively filtered it. However, it triggered lower rates of contractions and open siphons. Thus, it may represent a convenient feed in terms of size particles, and it is actively retained into the guts, but it did not stimulate the behavioural reactions of atrial siphons through the contact with the cilia and coronal organ [34]. An alternative explication could take into account the evidence that both PhytoReef® and SnowReef® contain large amounts of the alga *Chlorella* sp., known to synthesise metabolites used as feeding inhibitors [35,36]. Thus, the negative reaction of animals could be due to the presence of deterrents, although suspended microparticles are actively filtered in the case of PhytoReef®.

In agreement with this view are the rates of consumption of other feeds containing "animal" items, such as milk powder, dried eggs and SnowReef® food. All three exhibited low rates of ingestion (even if their particle size distributions are comparable with those of other feeds tested) triggered low rates of open siphons and produced a negative response in terms of the contractions of zooids. We may conclude that their presence in the suspension prompts the closure of siphons and reduction

of the filter-feeding behaviour as an active response to "nonpreferred" feeds. It has been highlighted by previous studies [37] that high concentrations of food particles and sediments may trigger rapid contractions and squirting in colonial ascidians. This behaviour might aid the removal of particles and waste from the peribranchial mucus to facilitate respiration. However, squirting was never observed during our experiments, suggesting that all feeds selected for our trials were dosed at a convenient concentration and exhibited a convenient size range of particles, not altering the respiratory capacity of zooids.

Complementary evidence is represented by dried yeast. In fact, although it is not a plant item, it produced highly positive responses in terms of open siphons, good behavioural responses in terms of contractions and high rates of ingestion. Consequently, it should be considered as a preferred feed, although it is not a plant item. However, this result may be in-line with the known preferences of tunicates in nature, since they normally select both suspended microalgae and bacteria [23]. We should consider that a complete study of the diet of *B. violaceus* has never been accomplished in nature, by investigations on the gut contents or other, direct methods, and our knowledge of its feeding preferences are due to direct tests in the laboratory and evidences from other species of tunicates. In this view, a compound diet containing live algae such as *I. galbana* and other protein-rich algal items, such as Spirulina and AlgaMac®, coupled with yeasts as a source of suspended microorganisms may represent an effective method to obtain continuous cultures of colonial tunicates. The choice of live algal feeds ought to take into account possible interactions with deterrent compounds and secondary metabolites able to influence the physiology of invertebrates [36,38]. In fact, some feeds containing both plant and bacterial items, with adequate particle size distributions, triggered negative behavioural responses, probably due to the presence of allelochemicals [35,39]. Consequently, the addition of compound feeds such as PhytoReef® could be considered, with some precautions. However, feeds commonly adopted for the short rearing of colonial tunicates, such as dried milk and dried eggs, should be avoided, because they reduce the intensity of the feeding behaviour. This study also demonstrates that feeding techniques for experimental model animals should take advantage of the information obtained from behavioural and physiologic responses of organisms [39] and avoid the adoption of generic feeds.

References

1. Chadwick-Furman, N.E.; Weissman, I.L. Life histories and senescence of *Botryllus schlosseri* (Chordata, Ascidiacea) in Monterey Bay. *Biol. Bull.* **1995**, *189*, 36–41. [CrossRef] [PubMed]
2. Voskoboynik, A.; Weissman, I.L. *Botryllus schlosseri*, an emerging model for the study of ageing, stem cells, and mechanisms of regeneration. *Invertebr. Reprod. Dev.* **2015**, *59*, 33–38. [CrossRef] [PubMed]
3. Austad, S. Recent advances in vertebrate ageing research. *Ageing Cell* **2009**, *9*, 297–303. [CrossRef] [PubMed]
4. Kott, P. The Australian ascidians. I. Phlebobranchia and Stolidobranchia. *Mem. Queensl. Mus.* **1985**, *23*, 1–440.

5.   Rinkevich, B.; Rabinowitz, C. In vitro culture of blood cells from the colonial protochordate *Botryllus schlosseri*. *In Vitro Cell. Dev. Biol.* **1993**, *29*, 79–85. [CrossRef]

6.   Delsuc, F.; Brinkmann, H.; Chourrout, D.; Hervé, P. Tunicates and not cephalochordates are the closest living relatives of vertebrates. *Nature* **2006**, *439*, 965–968. [CrossRef]

7.   Zupo, V.; Buia, M.C.; Gambi, M.C.; Lorenti, M.; Procaccini, G. Temporal variations in the spatial distribution of shoot density in a *Posidonia oceanica* meadow and patterns of genetic diversity. *Mar. Ecol.* **2006**, *27*, 328–338. [CrossRef]

8.   Grosholz, E. Small spatial-scale differentiation among populations of an introduced colonial invertebrate. *Oecologia* **2001**, *129*, 58–64. [CrossRef]

9.   Simoncini, M.; Miller, R.J. Feeding preference of *Strongylocentrotus droebachiensis* (Echinoidea) for a dominant native ascidian, *Aplidium glabrum*, relative to the invasive ascidian *Botrylloides violaceus*. *J. Exp. Mar. Biol. Ecol.* **2007**, *342*, 93–98. [CrossRef]

10.   Hirose, E. Colonial allorecognition, haemolytic rejection, and viviparity in Botryllid ascidians. *Zool. Sci.* **2003**, *20*, 387–394. [CrossRef]

11.   Berrill, N.J. The development and growth of Ciona. *J. Mar. Biol. Assoc.* **1947**, *26*, 616–625. [CrossRef] [PubMed]

12.   Epelbaum, A.; Therriault, T.W.; Paulson, A.; Pearce, C.M. Botryllid tunicates: Culture techniques and experimental procedures. *Aquat. Invasions* **2009**, *4*, 111–120. [CrossRef]

13.   Zega, G.; Pennati, R.; Candiani, S.; Pestarino, M.; De Bernardi, F. Solitary ascidians embryos (Chordata, Tunicata) as model organisms for testing coastal pollutant toxicity. *Invertebr. Surviv. J.* **2009**, *6*, 29–34.

14.   Hyams, Y.; Paza, G.; Rabinowitza, C.; Rinkevich, B. Insights into the unique torpor of *Botrylloides leachi*, a colonial urochordate. *Dev. Biol.* **2007**, *428*, 101–117. [CrossRef] [PubMed]

15.   Manni, L.; Gasparini, F.; Hotta, K.; Ishizuka, K.J.; Ricci, L.; Tiozzo, S.; Voskoboynik, A.; Dauga, D. Ontology for the asexual development and anatomy of the colonial chordate *Botryllus schlosseri*. *PLoS ONE* **2014**, *9*, e96434. [CrossRef]

16.   Ballarin, L.; Burighel, P.; Cima, F. A tale of death and life: Natural apoptosis in the colonial ascidian *Botryllus schlosseri* (Urochordata, Ascidiacea). *Curr. Pharm. Des.* **2008**, *14*, 138–147. [CrossRef]

17.   Rinkevich, B.; Shapira, M. An improved diet for inland broodstock and the establishment of an inbred line from a colonial sea squirt (Ascidiacea). *Aquat. Living Resourc.* **1998**, *11*, 163–171. [CrossRef]

18.   Brunetti, R.; Mastrototaro, F. *Botrylloides pizoni*, a new species of Botryllinae (Ascidiacea) from the Mediterranean Sea. *Zootaxa* **2012**, *3258*, 28–36. [CrossRef]

19.   Brunetti, R.; Beghi, L.; Bressan, M.; Marin, M.G. Combined effects of temperature and salinity on colonies of *Botryllus schlosseri* and *Botrylloides leachi* (Ascidiacea) from the Venetian lagoon. *Mar. Ecol. Prog. Ser.* **1980**, *2*, 303–314. [CrossRef]

20.   Bone, Q.; Carré, C.; Chang, P. Tunicate feeding filters. *J. Mar. Biol. Assoc.* **2003**, *83*, 907–919. [CrossRef]

21.   Brunetti, R. Botryllid species (Tunicata, Ascidiacea) from the Mediterranean coast of Israel, with some consideration on the systematic of Botryllinae. *Zootaxa* **2009**, *2289*, 18–32. [CrossRef]

22.   Sawada, H.; Yokosawa, H.; Lambert, C.C. (Eds.) *The Biology of Ascidians*; Springer: Tokyo, Japan, 2001; p. 470.

23.   Kloc, M.; Kubiak, J.K. *Marine Organisms as Model Systems in Biology and Medicine*; Springer: Houston, TX, USA, 2018; p. 65.

24.   Liu, L.; Xiang, J.; Dong, B.; Natarajan, P.; Yu, K.; Cai, N. *Ciona intestinalis* as an emerging model organism: Its regeneration under controlled conditions and methodology for egg dechorionation. *J. Zhejiang Univ. Sci.* **2006**, *7*, 467–474. [CrossRef] [PubMed]

25.   Bay-Nouailhat, A.; Bay-Nouailhat, W.; Gasparini, F.; Brunetti, R. *Botrylloides crystallinus* n. sp., a new Botryllinae Adams & Adams, 1858 (Ascidiacea) from Mediterranean Sea. *Zoosystema* **2020**, *42*, 131–138.

26.   Degasperi, V.; Gasparini, F.; Shimeld, S.M.; Sinigaglia, C.; Burighel, P.; Manni, L. Muscle differentiation in a colonial ascidian: Organisation, gene expression and evolutionary considerations. *BMC Dev. Biol.* **2009**, *9*, 48–58. [CrossRef] [PubMed]

27.   Mackie, G.O.; Burighel, P.; Caicci, F.; Manni, L. Innervation of ascidian siphons and their responses to stimulation. *Can. J. Zool.* **2006**, *84*, 1146–1162. [CrossRef]

28.   Lambert, G. New records of ascidians from the NE Pacific: A new species of *Trididemnum*, range extension and redescription of *Aplidiopsis pannosum* (Ritter, 1899) including its larva, and several nonindigenous species. *Zoosystema* **2003**, *25*, 665–679.

29. Stachowicz, J.J.; Terwin, J.R.; Whitlatch, R.B.; Osman, R.W. Linking climate change and biological invasions: Ocean warming facilitates nonindigenous species invasions. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 15497–15500. [CrossRef]

30. Young, C.M. Selection of predator-free settlement sites by larval ascidians. *Ophelia* **1989**, *30*, 131–140. [CrossRef]

31. Pisut, D.P.; Pawlik, J.R. Antipredatory chemical defenses of ascidians: Secondary metabolites or inorganic acids. *J. Exp. Mar. Biol. Ecol.* **2002**, *270*, 203–214. [CrossRef]

32. Burighel, P.; Lane, N.J.; Gasparini, F.; Tiozzo, S.; Zaniolo, G.; Candia Carnevali, M.D.; Manni, L. Novel secondary sensory cell organ in Ascidians: In search of the ancestor of the vertebrate lateral line. *J. Comp. Neurol.* **2003**, *461*, 236–249. [CrossRef]

33. Mackie, G.O.; Burighel, P. The nervous system in adult tunicates: Current research directions. *Can. J. Zool.* **2005**, *83*, 151–183. [CrossRef]

34. Manni, L.; Mackie, G.O.; Caicci, F.; Zaniolo, G.; Burighel, P. Coronal organ of ascidians and the evolutionary significance of secondary sensory cells in chordates. *J. Comp. Neurol.* **2006**, *495*, 363–373. [CrossRef] [PubMed]

35. Bagwell, C.E.; Abernathy, A.; Barnwell, R.; Milliken, C.E.; Noble, P.A.; Dale, T.; Beauchesne, K.R.; Moeller, P.D.R. Discovery of bioactive metabolites in biofuel microalgae that offer protection against predatory bacteria. Front. *Microbiology* **2016**, *7*, 516–525.

36. Varrella, S.; Romano, G.; Ruocco, N.; Ianora, A.; Bentley, M.G.; Costantini, M. First morphological and molecular evidence of the negative impact of diatom-derived hydroxyacids on the sea urchin *Paracentrotus lividus*. *Toxicol. Sci.* **2016**, *151*, 419–433. [CrossRef] [PubMed]

37. Armsworthy, S.L.; MacDonald, B.A.; Ward, J.A. Feeding activity, absorption efficiency and suspension feeding processes in the ascidian, *Halocynthia pyriformis* (Stolidobranchia: Ascidiacea): Responses to variations in diet quantity and quality. *J. Exp. Mar. Biol. Ecol.* **2001**, *260*, 41–69. [CrossRef]

38. Nappo, M.; Berkov, S.; Codina, C.; Avila, C.; Messina, P.; Zupo, V.; Bastida, J. Metabolite profiling of the benthic diatom *Cocconeis scutellum* by GC-MS. *J. Appl. Phycol.* **2009**, *21*, 295–306. [CrossRef]

39. Raniello, R.; Iannicelli, M.M.; Nappo, M.; Avila, C.; Zupo, V. Production of *Cocconeis neothumensis* (Bacillariophyceae) biomass in batch cultures and bioreactors for biotechnological applications: Light and nutrient requirements. *J. Appl. Phycol.* **2007**, *19*, 383–391. [CrossRef]