In-land long-term culture of colonial ascidians

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Highlights:

- First in-land recirculating aquaculture for colonial ascidians
- Over 3 years of continuous breeding
- Semi-automated setup with minimized maintenance
- Good biomass production for strain propagation
- 4 different culture conditions for optimized breeding for species of interest

1. Abstract

Tunicates are highly diverse marine invertebrate filter-feeders that are vertebrates' closest relatives. These organisms, despite a drastically different body plan during their adulthood, have a tissue complexity related to that of vertebrates. Ascidians, which compose most of the Tunicata, are benthic sessile hermaphrodites that reproduce sexually through a motile tadpole larval stage. Over half of the known ascidians species are able to reproduce asexually through

budding, typically leading to the formation of colonies where animals, called zooids, are interconnected by an external vascular system. In addition, colonial ascidians are established models for important biological processes including allorecognition, immunobiology, aging, angiogenesis and whole-body regeneration. However, the current paucity in breeding infrastructures limits the study of these animals to coastal regions.

To promote a wider scientific spreading and popularity of colonial ascidians, we have developed a flexible recirculating husbandry setup for their long-term in-lab culture. Our system is inspired both by the flow-through aquariums used by coastal ascidian labs, as well as by the recirculating in-lab systems used for zebrafish research. Our hybrid system thus combines colony breeding, water filtering and food culturing in a semi-automated system where specimens develop on hanging microscopy glass slides. Temperature, light/dark cycles, flow speed and feeding rates can be controlled independently in four different breeding environments to provide room for species-specific optimization as well as for running experiments. This setup is complemented with a quarantine for the acclimatization of wild isolates.

We here present our success in breeding *Botrylloides diegensis*, a species of colonial ascidians, for more than 3 years in recirculating artificial seawater over 600 km from their natural habitat. We show that colonies adapt well to in-lab culturing and that a specific strain can be isolated, propagated and used for research efficiently over prolonged periods of time. The flexible and modular structure of our system can be scaled and adapted to the needs of specific species as well as of particular laboratory spaces. Overall, we show that *Botrylloides diegensis* can be proficiently bred in-land and suggest that our results can be extended to other species of colonial ascidians to promote research on these fascinating animals.

2. Introduction

Tunicates are a group of over 3000 known extent highly diverse worldwide marine invertebrates, the majority of which are sub-tidal suspension-feeding hermaphrodites (Holland, 2016). Tunicata is separated into three classes (WoRMS Editorial Board, 2017): Appendicularia, Thaliacea and Ascidiacea. Appendicularia has 68 identified species of planktonic free-swimming organisms that possess chordate traits common to most tunicate larvae including a notochord, neural tube and pharyngeal slits (Holland, 2016). These social animals form communities where each individual is enclosed inside a special external mucous structure, termed house, which concentrates and funnels their food (Lambert, 2005). Thaliacea is composed of 77 species of planktonic pelagic animals forming free-floating compound colonies (Lambert, 2005). These organisms alternate between sexual reproduction to initiate new colonies, and asexual reproduction by budding to increase the size of the colony. With 2935 currently identified species, of which 1730 are colonial species (Shenkar and Swalla, 2011), Ascidiacea is by far the most diverse class of tunicates (WoRMS Editorial Board, 2017). Ascidians are sessile benthic organisms that are present in all marine habitats, from sandy beds to rocky shores and from coral reefs to kelp forests (Shenkar and Swalla, 2011). In addition, ascidians very efficiently colonize artificial substrata and have thus become a major source of biofouling, with a negative impact on economical sectors such as shellfish farming and marine transport (Aldred and Clare, 2014; Comeau et al., 2015).

However, ascidians also represent great opportunities for research as well as for the industry. First, Tunicata's phylogenetic position as vertebrates' closest relatives (Delsuc et al., 2006) renders them key models for evolutionary research, and to study the emergence of chordate-specific processes in particular. Second, colonial ascidians (Fig. 1A) are the only

known chordates capable of undergoing whole-body regeneration whereby a fully functional adult is restored from a minute fragment of vasculature in as little as 10 days (Blanchoud et al., 2018a). Third, their recently established application as bioindicators of micro-pollution (Navon et al., 2020; Tzafriri-Milo et al., 2019; Vered et al., 2019) combined with their ubiquitous localization makes these animals a perfect tool for environmental bio-surveillance. Fourth, some colonial ascidians have emerged as model organisms for an increasing number of research fields, including immunobiology, allorecognition, angiogenesis and aging (Blanchoud et al., 2018a). In addition, ascidians are a particularly promising source of marine natural products (MNP) with already over 1000 isolated molecules (Chen et al., 2019) and numerous reported applications including antibacterial, anti-fungal, anti-viral, anti-inflammatory and antitumoral (Palanisamy et al., 2017). There is thus a tremendous scientific interest in the study of ascidians. In our lab, we are particularly interested in *Botrylloides diegensis* because it is the only know colonial tunicate that combines a high quality genome assembly (Blanchoud et al., 2018b), a constant WBR ability throughout its developmental cycle (Blanchoud et al., 2018a) and an identified population of stem-cells responsible for its capacity to undergo WBR (Kassmer et al., 2020). Consequently, we set out to develop a suitable husbandry setup in our research institute, located over 5 h of travel from the closest sea.

Ascidian research is a particularly active community with numerous groups working worldwide using three main culturing methods. One most straightforward technique is onsite processing (Roberts et al., 2007; Sheets et al., 2016; Zondag et al., 2016; Ueki et al., 2018; Hiebert et al., 2019) (Fig. 1B). No long-term culture is involved, animals are sampled in their wild environment and directly processed for downstream analysis. This approach is thus well suited for studying *in situ* physiological aspects of Tunicates but it requires a reasonably close-by

sampling site and can be influenced by the potential seasonal availability of some species in some regions of the globe (Coma et al., 2000). A second prominent approach is the culture in a flow-through system (Hirose et al., 1995; Taketa et al., 2015; Blanchoud et al., 2017; Scelzo et al., 2019) (Fig. 1C), typically with filtered natural seawater from the site of sampling, which provides close to natural environmental conditions including in terms of feeding diet. This method allows to isolate and maintain strains on the long-term in confined and hazards-free conditions. It does require either a close proximity to a source of seawater or a consequent organization to provide for the necessary amounts of seawater. A third common approach is the culture in a closed system with periodic water changes (Brown et al., 2009; Epelbaum, 2009; Rinkevich et al., 2010) (Fig. 1D). This setup is most typically used with water from the sampling site for short-term culture of animals, although it has been used for extensively long studies too (Rinkevich and Shapira, 1998), as well as with artificial sea water (Nagar and Shenkar, 2016). The main limitations of this approach is the maintenance it requires on the long term, and the amount of water it requires when culturing a large number of colonies simultaneously. Consequently, all the labs that have the opportunity to do so combine these three approaches to benefit from their specific advantages. Yet, it consistently requires a routine access to either wild specimens or natural seawater and thus restricts their application to coastal locations. The only exception to this spatial limitation that we are aware of is that of Joly et al. who developed a recirculating system for breeding transgenic lines of the solitary ascidian *Ciona intestinalis* (Joly et al., 2007). Consequently, there currently is to our knowledge no recirculating husbandry system for the culture of colonial ascidians, which spatially limits their study and thus hinders their popularity as model organisms.

Here, we present a husbandry system designed for the in-land breeding of sessile colonial tunicates (Fig. 2). We have been using this setup successfully for over 3 years with the continuous presence of some of our strains, combined with a periodic addition of new wild isolates. Our recirculating system runs on artificial seawater and uses commercial algae pastes as feeding diets. Our aquarium is designed to permit up to 4 different culture conditions in parallel for optimizing the breeding of the species of interest. In this study, we have been growing both *Botrylloides diegensis* as well as *Botryllus schlosseri* on microscopy glass slides. We present our developed aquaculture system, the maintenance setup used for the colonies, our long-term results in the clonal expansion of colonial ascidians and an example of a comparative breeding experiment. Our data shows that we are able on the long-term to abolish any spatial constrains with respect to the natural environment of colonial ascidians. We believe that this setup will enable research on colonial tunicates to be undertaken in novel places and will thus promote as well as popularize these fascinating animals as model organisms.

3. Materials and Methods

3.1 Aquarium structure

The complete list of material and equipment used to assemble our system is provided in Table 1. Briefly, the structure of the system is a SS 316 40x40 and 30x30 mm powder coated stainless steel stand (LxWxH 2.0 x 0.9 x 2.2 m), custom manufactured PE/PP 40 L specimen tanks, a custom manufactured PE/PP 200 L filtration sump as well as four custom manufactured PE/PP 10 L food culturing vessels. The system is equipped with the following material: filter and feed pumps (FP 350-2000 and FP 150, Sera Gmbh, DE); fountain pump (1800, Eheim, DE); diaphragm pump (Air400, AniOne, DE), air blower (Medo LA60B, Nitto Kohki,

DE), bi-stop ball valve (Bi-Stop, Plasson, IL), cooling unit (Titan 4000, Aqua Medic, DE), heaters (titanium D-D Schego 600W, Complete Aqualics, UK), motor driven skimmer (EVO 1000, AquaMedic Gmbh, DE) with its circulation pump (AquaMedic Gmbh, DE), circulation pump (Titan-Tec 10000, Messner Gmbh, DE), UV steriliser (Vecton2, TMC Aquarium, US); Lights (AquaBeam 1500 Ocean blues with AquaBar control, TMC Aquarium, US); Mortar mixer (TC-MC 1400E, Einhell AG, DE). The construction of our prototype was outsourced to a company specialized in aquatic research equipment (Zebcare, Fleuren & Nooijen BV, NL).

A quarantine area composed of two flow-through 30 L tanks (Semadeni AG), aerated by one pump each (Wavemaker ECO SW2, Jecod) and illuminated by a led rack each (Marine hybrid powerLED+, Eheim) was added to the main system by our departmental workshop. Our quarantine tanks are placed on a heavy-duty plastic stand (Cambro). Staining plastic racks (Semadei AG) were used from hosting the animals on their glass slides.

The entire setup was equipped with a range of sensors to obtain a fully automatized control on the environment of the aquarium using the Apex (Neptune Systems) controller. In particular, salinity, pH, day/night cycles, temperature and changes of water are performed automatically and reproducibly.

3.2 Artificial sea water production and filtration

All kits for water quality as well as syn-biotic sea salt were purchased from Tropic Marin. Seawater refractometer was purchased from Red Sea, Optic USB base station and Hobo Pendant temperature/light data logger were purchased from Onset Computer Corporation. Filtration is performed by a nitrate filter (Sulphur BioDenitrator, Korallin), ceramic beads (Siporax, Sera), nitrifying bacteria (bio reefclear, Sera), stirred plastic media (K+Media, Evolution

Aqua), an algae scrubber (Medium Turf Scrubber, IceCap), resin and phosphate absorption cubes (Brightwell Aquatics). Live stones we bought cured with Coralline algae from Aqua-Home (CH). Concentrated marine biota was made by sterile filtering 10 L of gravity filtered natural seawater using 0.22 µm Nalgene filters and resuspending the content of the filters in 50 mL of gravity filtered natural seawater. Other chemicals and small laboratory equipment were purchased from Fisher BioReagents.

3.3 Animal breeding and handling

Numerous colonial ascidians (*Botrylloides diegensis* in particular) were collected in the coastal town of Chioggia (IT) and successfully brought back to the lab with their original substrate in closed boxes filled with sea water. Half a dozen mussels were also collected for inoculating the husbandry system. Animals were then acclimatized in our quarantines and transferred to 76x26x1 mm microscopic slides (Marienfeld, DE). After the colonies had settled properly on the slide, they were transferred to the recirculating system where they developed vertically inside the specimen tanks using a custom attachment setup using cable ties (Netztech Handels AG) and a silicone badge gripper (Specialist ID, US). Colonies were fed using a blend of commercial algae concentrates from Instant algae (LPB Shellfish Diet, Shellfish Diet 1800, RG complete, RotiGrow Plus; US), complemented with a planktonic substitute (Zootonic, Tropic Marin, DE) and commercial bakery yeast (Rinkevich and Shapira, 1998). Feeding was controlled by 12V normally closed solenoid valves (CJV23, Conjoin, CN) controlled by a customized microcontroller (Arduino Uno). Colonies were monitored weekly by macrophotography using a Canon 5D Mark II with objective Sigma 50 mm f/2.8 EX DG macro

and a flash Canon Speedlite 430EX II. Salinity was kept constant at 35 g/L of seasalt, temperature was set at 21 °C all year round.

Live L-type rotifers (Planktovie) were bought in brackish 15 g/L seawater. We thus acclimatized them to our system's 35 g/L by slowly increasing the salinity of their medium by 5 g/L every week. Rotifers were cultures in 1 L Erlenmeyer flasks without agitation, fed with RG Complete every day and their water is changed every 2 days. Once the population became sufficiently dense, typically every other day, they were spitted in half – one half serving for continuing their expansion, the other half being fed to the specimen tanks.

4. Results

4.1. Description of the system

We designed a custom recirculating system for the in-land culture of colonial tunicates (Fig. 2A), which allows to reduce periodic water changes to minimal amounts. In such closed systems, the wastes produced by the animals (e.g. NH_4^+, NO_2^+, NO_3^+) accumulate over time and need to be continuously purified. Our modular aquaculture system is structured in three levels around a stainless stand to maximize the use of gravity for the circulation of the artificial sea water (ASW) in the aquarium. On the top, food culturing tanks distribute their content into the specimen tanks located in the middle, which overflow into the water filtration units placed at the bottom of the apparatus (Fig. 2B). The filtration unit combines mechanical and biological filtrations to reach a minimal weekly water change of 5 % of the total 450 L of ASW present in our system. In particular, filtration includes a particle filter, a so-called upflow filter (or moving bed filter), a nitrate filter, an algae scrubber, a protein skimmer, live cured stones and a UV-C unit (Fig. 2C).

To breed our animals in optimal conditions, we specifically aimed at using a maximum of food-grade plastic material, to avoid rust, wear and chemical contamination as much as possible. Four specimen tanks are custom made from 15/20mm PE/PP material – each can be filled up with approx. 40 liters. Tanks have one compartment and each has a heater which is placed at the bottom of the tank and controlled by an temperature sensor placed on the side of the tank. Aeration and mixing of the water is ensured by a circulation pump placed on the back side of each tank. Tanks are completely closed to make a light-isolated environment, with a convenient front opening (Fig. 2D) for easy access and cleaning. Water falls into the tank through a silicone tube, which flow rate can be regulated to modulate the amount of flow-

through, and leaves the tank from an outlet located below the mesh. Each tank is equipped with two lights sunken in the lid of the tank in such a way that the heat sink at the back of the LED tile is outside the tank for optimal heat transfer. The intensity and photoperiod of each LED unit can be controlled independently through a central control unit. Animals are placed in the tanks on a custom system of hangers made using plastic tubes, plastic hooks and badge grippers (Fig. 2E)

The central filtration unit (sump) has been placed on the ground. Water from the tanks is drained through a fleece filter into an aerated moving-bed biofilter, through a ceramic-beads biofilter and into the main sedimentation tank. The sedimentation tank is connected to an external protein skimmer with its submerged pump, a nitrate filter, an algae scrubber and a refugium populated with live curated stones and macro-algae. The refugium and the algae scrubber are illuminated by two LED units constantly illuminated. The sedimentation tank is connected to the main circulation pump, which provides system water to the cooling unit in a closed loop, and to the specimen tanks through a UV sterilizer. In addition, a submerged pump is activated by a timer for periodic water changes by pumping system water into the sewage through the overflow drain. This pump can also be used for complete drainage of the system. In order to reduce evaporation, the whole filtration tank is fitted with a clear lid.

Above the specimen tanks, we have installed four 10 L cylindrical feeding tanks. Each tank is made of non-transparent PVC pipes 200 mm in diameter and 300 mm long. An external tube water level made of clear PVC hose and a visit window allow to monitor the content of each tank. Content of the tanks is mixed by injecting air through a microporous tube placed at the bottom of the tank, and lighting is done by two LED rails placed inside a clear pipe through the center of the non-air tight lid of the tank. Food is drained outside of each tank by gravity

through an electronically controlled micro-valve placed close to the bottom of the tank. Air is supplied via 4 mm silicone hoses directly from the main air pump.

Besides the main system, we also maintain two quarantine containers for new comers and new subclones (Fig. 2F). This part has been designed and built up on site and is by purposefully not recirculating into the main system. However, both tanks can be filled with system water, and system water can be collected for experiments using handy garden hose nozzles. Each container can be filled with up to 30 L of saltwater, aerated and mixed by a pump placed in the back of the tank. Each tank is illuminated with photoperiod-controlled lights. Animals are placed in standing plastic staining racks (Fig. 2G) inside the quarantine tanks.

The conditions in the system are monitored and logged automatically (Fig. 2H) using an APEX system from Neptune Systems, connected to a number of sensors and actuators. Temperature, salinity, pH and ORP (oxidation reduction potential) are measured every 2 min in the main sedimentation tank. Temperature is controlled by the cooling unit and a heater, salinity can be increased by pumping 270 g/L brine water located in a 75 L tank placed behind the feeding tanks using a peristaltic pump, also controlled by Apex. Brine tank is mixed four times per day using a heavy duty concrete mixer controlled by a timer. pH and ORP send an alert email if they deviate from their defined ideal range (7.0 - 8.5 pH, 300 - 400 ORP). Water level is monitored using two optical sensors driving an automatic top-off kit that delivers deionized water directly in the main sedimentation tank by opening a solenoid valve connected to the University's water system. Temperature is also measured individually in each specimen tank, and salinity is measured in one of the tanks for redundancy. Leaks and water loss are monitored using a floor sensor and an optical sensor placed a the bottom of the sedimentation

tank, both leading to the shutdown of the whole system in case of emergency. Conditions in the quarantine are monitored manually.

4.2. Initialization and maintenance of the system

Before its first usage, the aquaculture system was cured over two weeks. First, the system was filled with tap water, run for 48 h and completely emptied. This wash was repeated two more times to remove all traces of chemicals from the system. During this time, the wild mussels were cured and cleaned in our quarantine using ASW changed once per week and fed using 1 mL/day of Shellfish Diet 1800. The cleaned aquarium was then filled up with ASW and curing was started. Ammonium chloride was added (3 mg/L) together with the nitrifying bacteria, the concentrated marine biota, the mussels and the live rocks, and the system was left to run for 24 hours. 5 grams of commercial fish food mix was added in the sedimentation tank followed by 7 g every week. Water quality (pH, NH_4^+, NO_2^+, NO_3^+) was measured every other day until ammonium concentration started to decrease (1.5 weeks post inoculation). 1.5 mg/L of ammonium was then added and the monitoring continued. When the nitrite concentration started to decrease (4 weeks post inoculation), the nitrate filter was started following the manufacturer's instructions. 0.5 mg/L of ammonium was added and the system monitored until the water quality was within the range defined by commercial kits (6 weeks post inoculation in total). System was then used following our routine maintenance as described below.

Maintenance of our aquaculture system was reduced to a minimum by automatizing as many steps as possible, the bulk of the remaining manual labor being dedicated to tending to the various needs of the animals. Weekly duties include only the cleaning of the protein

skimmer and every other week the replacement of the fleece placed at the bottom of the specimen tanks for trapping solid waste as well as the cleaning of the algae scrubber. Once a month, the excess of algae growing in the refugium is removed, the live stones scrubbed clean and the Apex system checked. Every 2-3 months depending on our use of system water, the brine tank needs refilling, and once a year a general maintenance is done where all the tubing pumping brine water are thoroughly cleaned.

In our hands, the most important factors for a reliable performance of our recirculation system is the quality of the water. Not inasmuch as having perfectly low concentrations for all the noxious compounds in the system water, but in preventing these compounds from accumulating to dangerously high concentrations. Consequently, we manually measured on a weekly basis the four most important ions (NH_4^+, NO_2^-, NO_3^-, PO_4^{3-}, Fig. 2H) as well as pH and salinity to confirm the readings from our controller. In addition, we tested monthly the following other parameters: KH, O_2, I, Ca_2^+, Mg_2^+, Si and K^+.

4.3. Breeding colonial tunicates in-land

Wild isolates were collected twice a year (Spring/Autumn) in the Venetian lagoon (Fig. 1B). Animals were transferred on their original substrate into 1 L water-tight wide-mount cylindrical containers. Animals were then transported to the laboratory, which typically took between 8 h and 10 h. Important parameters for successful transportation of the collected samples are first a large animal to volume ratio (> 20 x), regular water changes during transportation (every 2-3 h), as little air in the container as possible and minimizing the turbulence during transport. However, the best predictor of a colony that will well tolerate being transferred was its size, namely medium-sized colonies composed of 50-100 zooids were

305 typically fitter once in our laboratory. Another important aspect to consider when collecting is
306 the type of substrate the animal is growing on, and its shape. Colonies growing flat on mussels
307 were the most appropriate for later transfer to our glass slides, while those intermingled with
308 brittle algae were very difficult to isolate once in the lab.

309 Upon arrival, wild specimens were transferred to 2 L tanks filled with ASW adapted to the
310 salinity and pH of the natural sea water they were transported in. Animals were cleaned, their
311 substrate reduced to a minimum and the ones without any sign of filter-feeding activity
312 discarded. Animals were placed in our quarantine tanks, fed twice daily with 1 mL of feeding
313 mix and left 24 h to adapt. On the next day, colonies were subcloned to numbered microscopy
314 glass slides using a single-edge razor blade following a customized protocol (Bugada and
315 Blanchoud, 2021). Slides were placed in plastic staining racks at a density of 7 slides per rack
316 standing at the bottom of the quarantine tank. Colonies were left for 48 h to attach to the slides,
317 carefully cleaned (Blanchoud and Wawrzyniak, 2021), the decaying pieces of tissue discarded
318 and the remaining attached colonies placed in the second quarantine tank. Water in the
319 quarantines was changed when the NH_4^+ levels rose above 1.5 mg/L, or at least once a week.
320 We have observed that in our 30 L setup, with up to two dozen colonies, this will take around 7
321 days. The colonies that detached were subcloned again into the first quarantine and the
322 process repeated until all colonies either attached or decayed. Colonies were then acclimatized
323 to the salinity of our recirculating system by lowering the salinity of our quarantine by 1 g/L per
324 week. Once the quarantines' salinity was identical to that of our system, and the colonies had
325 spent at least 2 weeks in quarantine under weekly monitoring against diseases and parasites,
326 they were transferred to the main system where they proliferated actively (Fig. 3A). The lineage
327 of all strains, their clonal offspring, lifespan, position in the system and treatments were

recorded in an online database. Approximately 50 % of the collected specimens could be transferred successfully to our recirculating system.

Over the 3 years of this experiment, we transferred around 100 strains of colonial ascidians, and of *Botrylloides diegensis* in particular, from their natural environment to our recirculating system. We repeatedly observed that some strains declined in the first few months after their acclimatization and ultimately went extinguished without any apparent reason, while neighboring slides with different animals propagated totally fine. We could not find any pattern in the premature disappearance of these isolates. However, some strains have been much more productive than others (Fig. 3B) and vastly compensated these losses. Currently, 6 strains have ended up constituting over 95 % of all the colonies present in our aquaculture. Although we could have promoted a higher genetic diversity in our lab, this was not an objective in this study. We rather favored fast propagating animals to identify candidate reference strains for in-lab studies where biological replicates and large sample numbers are important.

In our system, colonies are cultures on numbered microscopy glass slides hanging vertically in the water, which reproduces somewhat their natural environment where we mainly collected them from the lateral sides of floating pontoons (Fig. 1B). This breeding method has multiple advantages, first for microscopy and general handling of the animals in the lab, second for the ease of use of the badge gripper and the total absence of metal parts (Fig. 2E), and finally for the identification and localization of specific colonies within the system. Each slide hangs onto a hook placed onto a PVC tube, itself fixed in the top part of the specimen tanks. Given the space constrains of our system to allow manipulation of each animal individually, each specimen tank can contain up to 32 slides (Fig. 2D).

Colonies are checked upon at least once every two weeks, and often daily for slides participating in specific studies. In this occasion, colonies are taken out of the system, cleaned and subcloned if necessary. Subcloning was undertaken for three main reasons. First, for maintenance to compensate for the propagation of the animals away from the center of the slide in particular, because this impedes its ease of manipulation and observation. Second to produce clonal biological replicates for experiments. Third, to fuse smaller clones from the same strain to obtain bigger samples for studies where large amounts of tissue were required.

Excitingly, several *B. diegensis* strains have been observed to become gravid (Fig. 3C-D). Some of these oocytes even got fertilized as we could identify oozoids developing later on in our system (Fig. 3E). Although this result was neither expected nor aimed for, it confirms that our colonies adapted well to our recirculating system and it shows that induction of sexual reproduction is possible under controlled conditions.

4.4. Differential breeding of colonial tunicates

Colonies in our recirculating system were bred under continuous feeding following preliminary indications (Blanchoud, unpublished) that such delivery was promoting growth. In addition, this type of feeding has two advantages, first a reduced contamination of the colonies and their slides because of the overall lower concentration of food present in the water, and second a lower daily maintenance as food needs to be prepared only once for multiple days. Continuous feeding was implemented using electronically controlled valves. We optimized the frequency and duration of the opening of each valve to reach uniform amounts between all four specimen tanks. On average, valves opened 1.5 s every 3 min, thus delivering around 2 L of

373 diluted food per day. Food was prepared every 4 days to guarantee food quality after culture in
374 our feeding tanks and concentration was adapted to minimize clotting.

375 Animals were fed with a custom food mix composed of equal parts of two commercial
376 blends of concentrated micro-algae (Shellfish Diet 1800, RotiGrow Plus), supplemented with
377 live rotifers (cultured in-lab), enriched with 1 % (v/v) bakery yeast and 2 % (v/v) of planktonic
378 supplement. To monitor the well-being of our animals, 4 slides representative of the same 4
379 strains were imaged per tank once a week. We manually counted the number of zooids in each
380 picture to estimate the growth rate of our animals and the impact of the different breeding
381 conditions.

382 The favorable environmental culturing conditions presented here were identified thanks to
383 multiple differential breeding experiments. Such approach could be used to test the impact of
384 environmental conditions on specific aspects of the specimens' development, and to adapt our
385 culturing conditions to different species for their breeding. Herein we show one example of
386 such experiments. Animals were fed with four different food mixes: A) 1.6 mL/day ShellFish
387 Diet, 1.6 mL/day RotiGreen Plus, B) 1.1 mL/day ShellFish Diet, 1.1 mL/day RotiGreen Plus, C)
388 3.2 mL/day RotiGreen Plus, D) 3.2 mL/day Shellfish Diet. All food mixes were complemented
389 with bakery yeast and planktonic substitute at the same proportion. Animals were bred under
390 these conditions for four consecutive weeks while being monitored weekly. To compensate for
391 any possible bias in specific specimen tanks, we swapped the feeding diets after 4 weeks, and
392 monitored the same colonies for another 4 weeks (Fig. 3F).

393 Animals fed with food mix A and D outperformed those fed with B and C (Fig. 3G).
394 Moreover, animals fed with food mix A and D had positive growth rates (> 1), meaning that each
395 generation produces more animals than the previous one thus leading to exponential growth.

Although the differences were not significant in the first set of experiments, we measured the same trends in both sets of feeding experiments. This confirms that the impact of tank specific biases, if any, did not influence significantly our measurements. The low breeding measured with mix B indicates less food than our baseline was not sufficient to sustain a positive growth rate. This confirms that we are not overfeeding our animals. The low breeding measured with mix C indicated that feeding on a blend of two type of algae mix was more beneficial. However, the good performance of feeding with D, also a single algae mix, suggests that this food source (Shellfish Diet 1800) is the source of most growth in our species. Overall, this experiment exemplifies how differential breeding can be used in our in-land aquaculture system to study how feeding influences the growth of our colonial ascidians.

5. Discussion & Conclusions

We here present a hybrid flexible recirculating husbandry setup for the long-term in-lab culture of colonial ascidians. We developed this prototype to sustain our research on the exceptional capacity of *Botrylloides* to undergo whole-body regeneration (Blanchoud et al., 2018a). Yet, in this endeavor we also set out to establish a system that would be suitable for promoting as well as popularizing these colonial ascidians as model organisms more globally. Our achievements in breeding *Botrylloides* constantly over more than 3 years shows that this is indeed possible, and we do hope that it will initiate a renewed interest in studying these fascinating animals in novel places, including in non-coastal ones.

As pioneers in the in-land breeding of ascidians without previous experience with saltwater aquaculture, we obviously initially came across some difficulties with the maintenance of the quality of our recirculating water. Accumulation of noxious ions is the main challenge for such a system. Nitrates in particular tend to accumulate quickly and for the long term because the anaerobic bacteria that can process this ion develops much slower than the others involved in the rest of the nitrogen cycle (Arrigo, 2005). Although our nitrate filter solved this particular issue relatively well, the most common solution to this accumulation remains to increase water changes (Zebcare, personal communications). Consequently, the main strategies available to maintain the quality of our ASW were to slowly vary the biological load of our system (i.e. animals and feeding), to use periodic treatments with removable media (e.g. phosphate binding resin), and to perform large water changes (50 % of the total water amount) when needed. To avoid sudden changes in water quality, we thus focused on reducing the variations in biological load on our system. Indeed, the three major regression events we experienced (Fig. 3B arrowheads) followed a sudden increase in the size of our colonies

population that we naively combined with an increase in feeding to try supporting these additional animals. We experienced a similar issue three times consecutively because we were initially not monitoring all the parameters of water, and thus wrongly believed that we had solved the issue at the root the decline. Instead, the accumulation was transferred to different noxious ions. The third and more drastic regression (**Fig. 3B black arrowhead**) was unfortunately concurrent with a sanitary confinement that prevented us from tending properly to our aquaculture. Consequently, the latent issue lasted longer than the two previous ones. Nevertheless, colonies recovered particularly quickly and repopulated our culture setup very efficiently. Moreover, we resorted only twice over the 3 years to large water changes, confirming that the quality of our water is stable.

An important point in controlling the quality of our ASW was to understand that although a particular food source might be beneficial to the growth of our species, it might be imbalanced with respect to our target water parameter values. For instance, we observed that our food mix has a higher pH than our ideal pH 8.2. Although the important dilution that takes place during feeding renders this increase quite minimal, it similarly accumulated over time to yield to a noticeable increase in the whole system (Fig. 2H). Properly balancing the pH of the food mix before feeding it to the specimen tanks solved the problem at its root. Consequently, we advise to measure all the parameters of the food mix being delivered to the animals before starting a new feeding experiment. Feeding using live algae (Joly et al., 2007; Brown et al., 2009; Blanchoud et al., 2017) or using home-made blends of vegetable pastes (Kowarsky et al., 2021) might be more appropriate in the context of an in-lab monoculture as presented here.

Colonies of *Botrylloides diegensis* have been cultured in our laboratory for more than 3 years. We have established an in-lab all-year-round stable and fully monitored system which

can be adapted to the needs of other tunicates to further popularize this subphylum. In parallel, we have introduced for the last year another colonial ascidian in our system, *Botryllus schlosseri*. While this species has so far well adapted to our recirculating ASW aquaculture, it grows much slower than *B. diegensis*. Yet, colonies look healthy and they do propagate. One potential explanation for this behavior is that *Botryllus schlosseri* colonies thrive at temperatures lower than 20 °C (Boyd et al., 1986), above which they tend to become gravid and slow down their asexual reproduction. Our system allows to set different water temperatures in different specimen tanks and optimizing *Botryllus* culture will be attempted in this direction.

While gravid colonies confirm the good status of our colonies, the observed sexual reproduction occurred without apparent relation to any specific environmental condition. In addition, the open configuration of our specimen tanks together with our important flow-through is not adapted to handling spawning of colonial tunicates. Consequently, we will explore adapting our in-land aquaculture with published methods used in other species of colonial ascidians (Fletcher and Forrest, 2011; Joly et al., 2007), both for the induction of spawning as well as for the retrieval and settling of the motile larvae.

A surprising result from our differential breeding experiment is that only one set of data shows statistically significant effects, while the same trends can be observed in the other one. One potential explanation is that some remnant effect of the previous set of feeding experiment was influencing the monitored colonies. Indeed, because *Botrylloides* have three generations co-existing constantly within the colony (Berrill, 1947), the number of adults observed in the following generation was determined based on the breeding conditions that were in place when this new generation budded, which means around two weeks in our 21 °C system. Consequently, differential breeding experiments might need to be monitored over

periods including 3 additional initial weeks for buffering between experiments. Alternatively, incorporating only the data from the last week of the experiment could remove this issue, but more colonies would need to be monitored to yield statistically significant results.

Overall, we show that *Botrylloides diegensis* can be proficiently bred in-land and our preliminary results with Botryllus schlosseri suggest that our our system for controlled breeding can provide the necessary platform for the worldwide spreading of colonial ascidians.

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We also thank the people from our institute's workshops for helping us, teaching us and showing us how to modify, improve and develop an aquaculture system. In particular, Ruben Pooley, Alain Werro, Jean-Daniel Niederhauser and François Zosso.

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7. Author contributions

Conceptualization, Funding acquisition & Project administration: S.B.; Investigation, Methodology, Validation: L.M.S, M.W., S.B.; Visualization: S.B.; Writing - original draft: M.W.; Writing - review & editing: M.W., S.B.

8. References

Aldred, N., Clare, A.S., 2014. Mini-review: Impact and dynamics of surface fouling by solitary and compound ascidians. Biofouling 30, 259–270. https://doi.org/10.1080/08927014.2013.866653

Arrigo, K.R., 2005. Marine microorganisms and global nutrient cycles. Nature 437, 349–355. https://doi.org/10.1038/nature04159

Berrill, N.J., 1947. The developmental cycle of *Botrylloides*. Q J Microsc Sci 88, 393–407.

Blanchoud, S., Rinkevich, B., Wilson, M.J., 2018a. Whole-Body Regeneration in the Colonial Tunicate *Botrylloides leachii*. Results Probl Cell Differ 65, 337–355. https://doi.org/10.1007/978-3-319-92486-1_16

Blanchoud, S., Rutherford, K., Zondag, L., Gemmell, N.J., Wilson, M.J., 2018b. *De novo* draft assembly of the *Botrylloides leachii* genome provides further insight into tunicate evolution. Sci Rep 8, 5518. https://doi.org/10.1038/s41598-018-23749-w

Blanchoud, S., Wawrzyniak, M., 2021. Cleaning colonial ascidians. Protocols.io. https://doi.org/10.17504/protocols.io.brxem7je

Blanchoud, S., Zondag, L., Lamare, M.D., Wilson, M.J., 2017. Hematological Analysis of the Ascidian *Botrylloides leachii* (Savigny, 1816) During Whole-Body Regeneration. The Biological Bulletin 232, 143–157. https://doi.org/10.1086/692841

Boyd, H.C., Brown, S.K., Harp, J.A., Weissman, I.L., 1986. GROWTH AND SEXUAL MATURATION OF LABORATORY-CULTURED MONTEREY *BOTRYLLUS SCHLOSSERI*. The Biological Bulletin 170, 91–109. https://doi.org/10.2307/1541383

Brown, F.D., Keeling, E.L., Le, A.D., Swalla, B.J., 2009. Whole body regeneration in a colonial ascidian, *Botrylloides violaceus*. J. Exp. Zool. 312B, 885–900. https://doi.org/10.1002/jez.b.21303

Bugada, L., Blanchoud, S., 2021. Subcloning colonial ascidians. Protocols.io. https://doi.org/10.17504/protocols.io.brw9m7h6

Chen, L., Wang, X.-N., Fu, C.-M., Wang, G.-Y., 2019. Phylogenetic Analysis and Screening of Antimicrobial and Antiproliferative Activities of Culturable Bacteria Associated with the Ascidian Styela clava from the Yellow Sea, China. BioMed research international 2019, 7851251. https://doi.org/10.1155/2019/7851251

Coma, R., Ribes, M., Gili, J.-M., Zabala, M., 2000. Seasonality in coastal benthic ecosystems. Trends in Ecology & Evolution 15, 448–453. https://doi.org/10.1016/S0169-5347(00)01970-4

Comeau, L.A., Filgueira, R., Guyondet, T., Sonier, R., 2015. The impact of invasive tunicates on the demand for phytoplankton in longline mussel farms. Aquaculture 441, 95–105. https://doi.org/10.1016/j.aquaculture.2015.02.018

Delsuc, F., Brinkmann, H., Chourrout, D., Philippe, H., 2006. Tunicates and not cephalochordates are the closest living relatives of vertebrates. Nature 439, 965–8. https://doi.org/10.1038/nature04336

Epelbaum, A., 2009. Botryllid tunicates: Culture techniques and experimental procedures. AI 4, 111–120. https://doi.org/10.3391/ai.2009.4.1.12

Fletcher, L., Forrest, B., 2011. Induced spawning and culture techniques for the invasive ascidian *Didemnum vexillum* (Kott, 2002). AI 6, 457–464. https://doi.org/10.3391/ai.2011.6.4.11

Hiebert, L.S., Vieira, E.A., Dias, G.M., Tiozzo, S., Brown, F.D., 2019. Colonial ascidians strongly preyed upon, yet dominate the substrate in a subtropical fouling community. Proc. R. Soc. B. 286, 20190396. https://doi.org/10.1098/rspb.2019.0396

Hirose, E., Saito, Y., Watanabe, H., 1995. Regeneration of the tunic cuticle in the compound ascidian, Botrylloides simodensis. Developmental & Comparative Immunology 19, 143–151. https://doi.org/10.1016/0145-305X(94)00062-K

Holland, L.Z., 2016. Tunicates. Current Biology 26, R146–R152. https://doi.org/10.1016/j.cub.2015.12.024

Joly, J.-S., Kano, S., Matsuoka, T., Auger, H., Hirayama, K., Satoh, N., Awazu, S., Legendre, L., Sasakura, Y., 2007. Culture of Ciona intestinalis in closed systems. Dev Dyn 236, 1832–1840. https://doi.org/10.1002/dvdy.21124

Kassmer, S.H., Langenbacher, A.D., De Tomaso, A.W., 2020. Integrin-alpha-6+ Candidate stem cells are responsible for whole body regeneration in the invertebrate chordate *Botrylloides diegensis*. Nat Commun 11, 4435. https://doi.org/10.1038/s41467-020-18288-w

Kowarsky, M., Anselmi, C., Hotta, K., Burighel, P., Zaniolo, G., Caicci, F., Rosental, B., Neff, N.F., Ishizuka, K.J., Palmeri, K.J., Okamoto, J., Gordon, T., Weissman, I.L., Quake, S.R., Manni, L., Voskoboynik, A., 2021. Sexual and asexual development: two distinct programs producing the same tunicate. Cell Reports 34, 108681. https://doi.org/10.1016/j.celrep.2020.108681

Lambert, G., 2005. Ecology and natural history of the protochordates. Canadian Journal of Zoology 83, 34–50. https://doi.org/10.1139/Z04-156

Nagar, L., Shenkar, N., 2016. Temperature and salinity sensitivity of the invasive ascidian Microcosmus exasperatus Heller, 1878. AI 11, 33–43. https://doi.org/10.3391/ai.2016.11.1.04

Navon, G., Kaplan, A., Avisar, D., Shenkar, N., 2020. Assessing pharmaceutical contamination along the Mediterranean and Red Sea coasts of Israel: Ascidians (Chordata, Ascidiacea) as bioindicators. Marine pollution bulletin 160, 111510. https://doi.org/10.1016/j.marpolbul.2020.111510

Palanisamy, S.K., Rajendran, N.M., Marino, A., 2017. Natural Products Diversity of Marine Ascidians (Tunicates; Ascidiacea) and Successful Drugs in Clinical Development. Nat. Prod. Bioprospect. 7, 1–111. https://doi.org/10.1007/s13659-016-0115-5

Rinkevich, B., Shapira, M., 1998. An improved diet for inland broodstock and the establishment of an inbred line from *Botryllus schlosseri*, a colonial sea squirt (Ascidiacea). Aquatic Living Resources 11, 163–171. https://doi.org/10.1016/S0990-7440(98)80113-7

Rinkevich, Y., Rosner, A., Rabinowitz, C., Lapidot, Z., Moiseeva, E., Rinkevich, B., 2010. *Piwi* positive cells that line the vasculature epithelium, underlie whole body regeneration in a basal chordate. Developmental Biology 345, 94–104. https://doi.org/10.1016/j.ydbio.2010.05.500

Roberts, B., Davidson, B., MacMaster, G., Lockhart, V., Ma, E., Wallace, S.S., Swalla, B.J., 2007. A complement response may activate metamorphosis in the ascidian Boltenia villosa. Dev Genes Evol 217, 449–458. https://doi.org/10.1007/s00427-007-0157-0

Scelzo, M., Alié, A., Pagnotta, S., Lejeune, C., Henry, P., Gilletta, L., Hiebert, L.S., Mastrototaro, F., Tiozzo, S., 2019. Novel budding mode in *Polyandrocarpa zorritensis*: a model for comparative studies on asexual development and whole body regeneration. EvoDevo 10, 7. https://doi.org/10.1186/s13227-019-0121-x

Sheets, E.A., Cohen, C.S., Ruiz, G.M., da Rocha, R.M., 2016. Investigating the widespread introduction of a tropical marine fouling species. Ecol Evol 6, 2453–2471. https://doi.org/10.1002/ece3.2065

Shenkar, N., Swalla, B.J., 2011. Global diversity of Ascidiacea. PloS one 6, e20657. https://doi.org/10.1371/journal.pone.0020657

Taketa, D.A., Nydam, M.L., Langenbacher, A.D., Rodriguez, D., Sanders, E., De Tomaso, A.W., 2015. Molecular evolution and in vitro characterization of Botryllus histocompatibility factor. Immunogenetics 67, 605–623. https://doi.org/10.1007/s00251-015-0870-1

Tzafriri-Milo, R., Benaltabet, T., Torfstein, A., Shenkar, N., 2019. The Potential Use of Invasive Ascidians for Biomonitoring Heavy Metal Pollution. Front. Mar. Sci. 6, 611. https://doi.org/10.3389/fmars.2019.00611

Ueki, T., Koike, K., Fukuba, I., Yamaguchi, N., 2018. Structural and Mass Spectrometric Imaging Analyses of Adhered Tunic and Adhesive Projections of Solitary Ascidians. Zoological Science 35, 535–547. https://doi.org/10.2108/zs180051

Vered, G., Kaplan, A., Avisar, D., Shenkar, N., 2019. Using solitary ascidians to assess microplastic and phthalate plasticizers pollution among marine biota: A case study of the Eastern Mediterranean and Red Sea. Marine pollution bulletin 138, 618–625. https://doi.org/10.1016/j.marpolbul.2018.12.013

WoRMS Editorial Board, 2017. World Register of Marine Species. Available from http://www.marinespecies.org at VLIZ. Accessed 2020-09-29. https://doi.org/10.14284/170

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

9. Figure legends

Figure 1. Current status of colonial ascidian husbandry. (A) A typical colony of *Botrylloides diegensis* settled on a glass slide. (B). The sampling site where colonies were collected (Chioggia, IT). (C). Example of a flow-through system. (D). Example of a closed system.

Figure 2. The colonial ascidian husbandry setup. (A) Schematic blueprint of our system. (B) Picture of the prototype developed. The main system is composed of a shelf of tanks for growing food flowing into the specimens' tanks recycling through the sump (C). View inside the sump where water is filtered and rectified automatically. (D) Vertical culture of colonial ascidians - each specimens' tank is equipped with racks of hooks. (E) Colony hanger used to hold the glass slides vertically. (F) Two quarantine containers. (G) Staining racks are used during quarantine. (H) Parameters of the water quality measured in our system between March 2018 and November 2020. ORP – oxidation reduction potential (mV). salinity (g/l). water temperature in the sump and tanks 1 – 4 (Celsius). pH in the sump and measured with a kit. Levels of noxious substances: NH4, NO2/NO3 and PO4 (mg/l).

Figure 3. Breeding of colonial ascidians. (A) Lineage of a successful strain (S002) showing its propagation by subcloning. (B) Evolution of the number of clones bred in our system shown from March 2019 till November 2020, color-coded by strain ID. Major recession event are pointed at by arrowheads, the one concurrent with a sanitary confinement is further highlighted in black. (C) Picture of a relatively large colony composed of over 300 zooids, which became gravid in 2019. (D). Picture of a gravid bud with its oocyte visible (arrowhead). (E) Picture of a

young oozoid recently metamorphosed. Visible organs including its oral siphon (OS), ampullae (a) and bud (b). (F) Sample pictures taken on the specified day during our differential feeding experiment. Depicted pictures correspond to the same colony fed with the combination Mix A / Mix B. (G) Relative mean number of zooids counted per slide (n=3) averaged over the indicated number of weeks.

Table 1. Components use in our husbandry system for colonial ascidians.

Figure 1

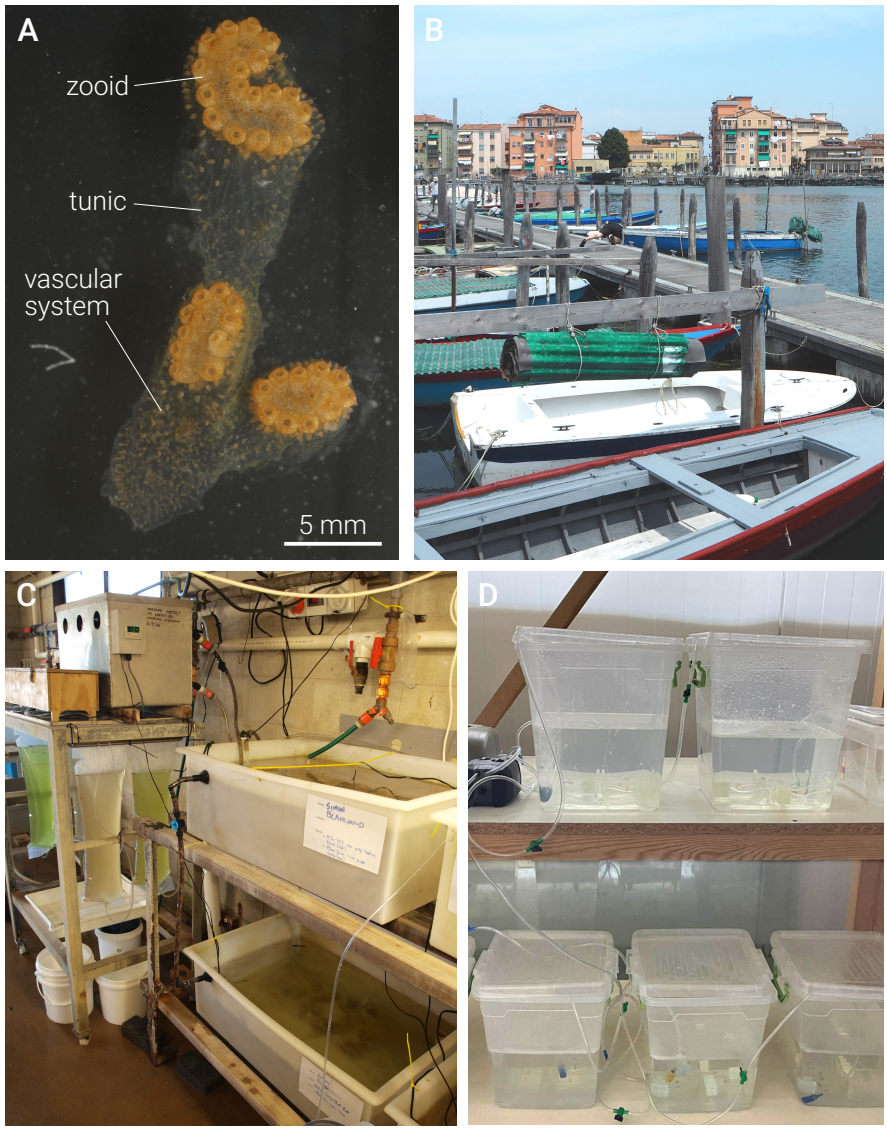


Figure 2

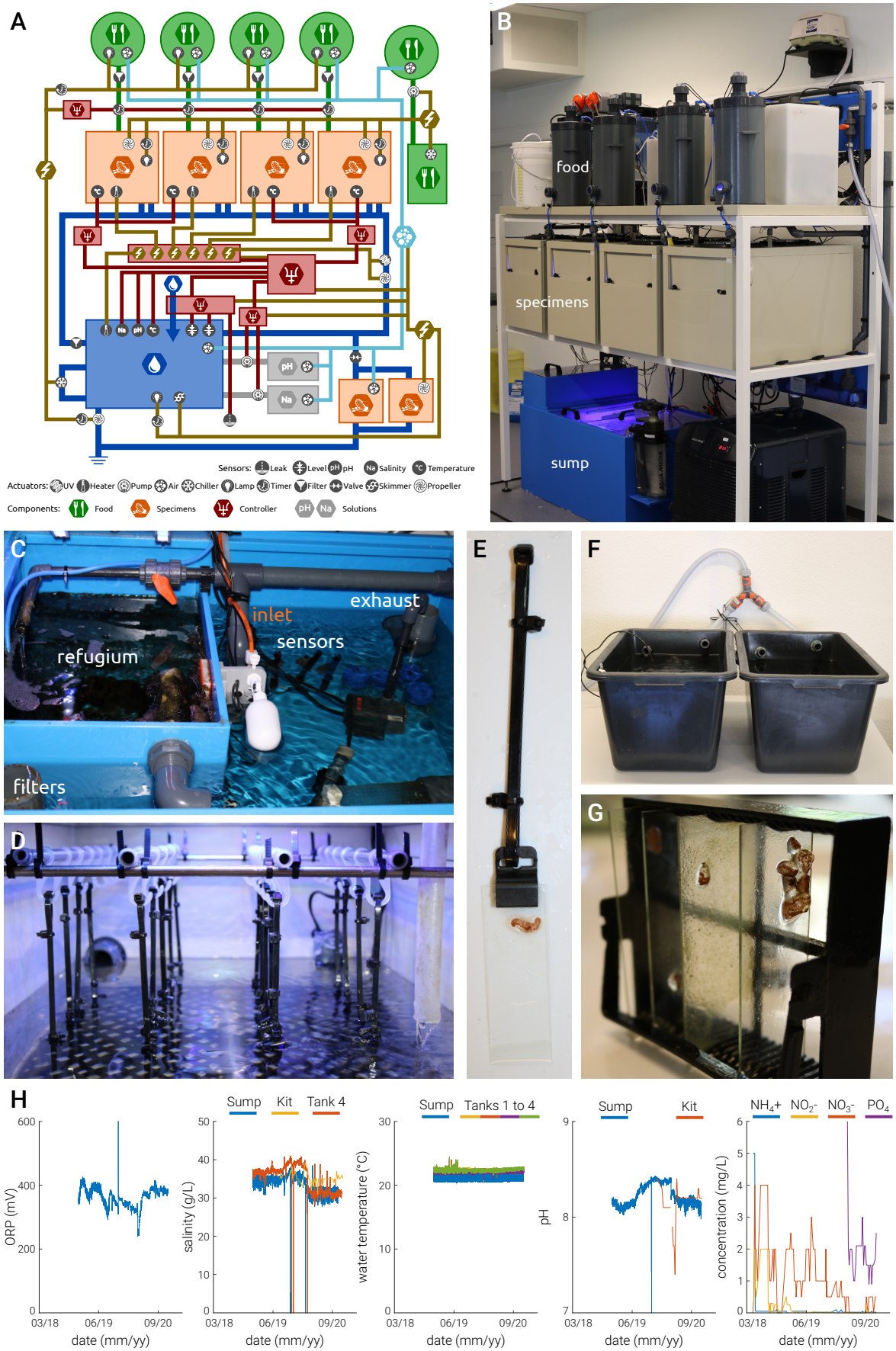
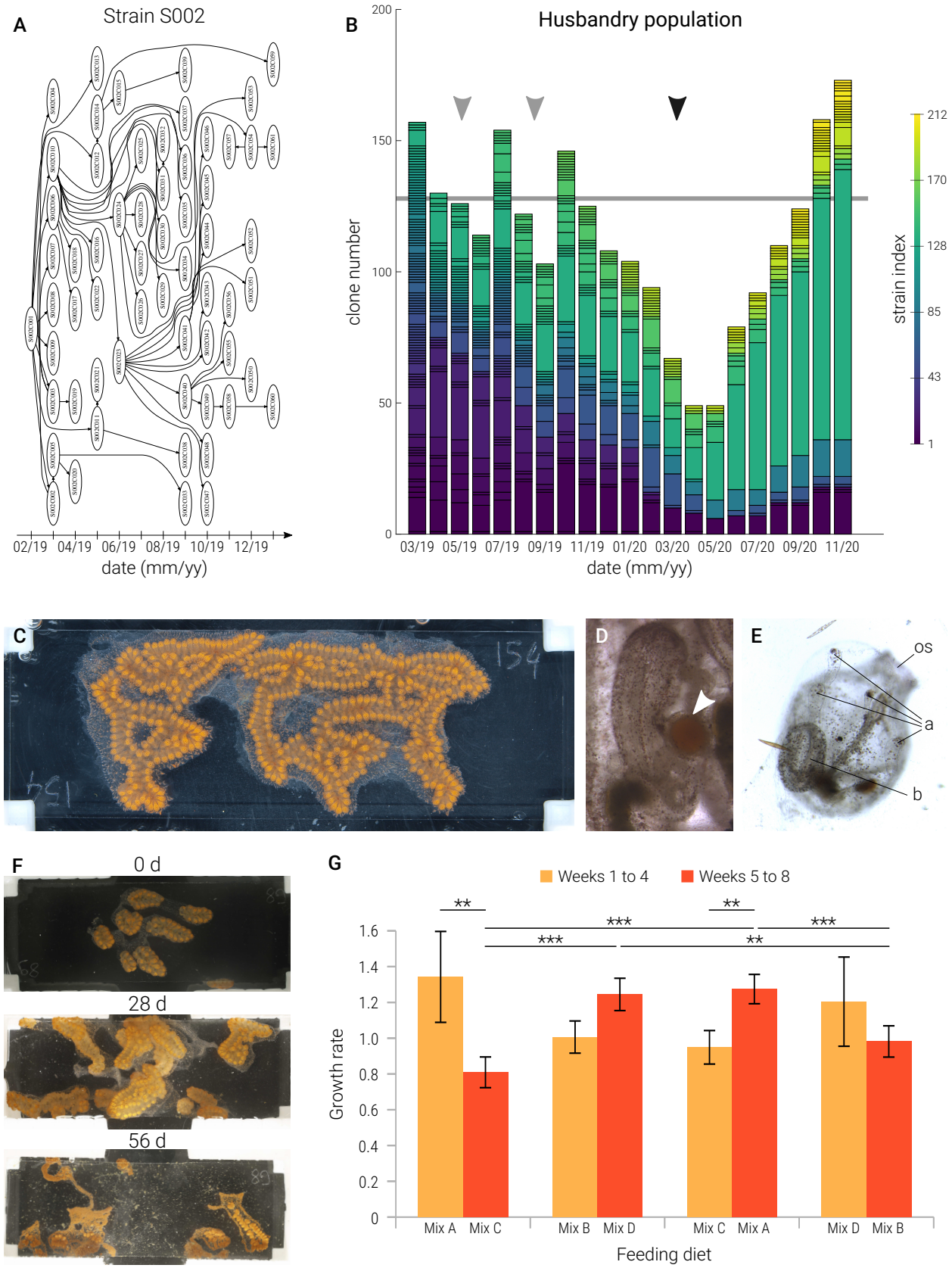

A
Sensors: Leak Level pH Salinity Temperature
Actuators: UV Heater Pump Air Chiller Lamp Timer Filter Valve Skimmer Propeller
Components: Food Specimens Controller Solutions
B
food
specimens
sump
C
inlet
exhaust
sensors
refugium
filters
D
E
F
G
H
ORP (mV)
salinity (g/L)
water temperature (°C)
pH
concentration (mg/L)
date (mm/yy)
03/18
06/19
09/20
Sump
Kit
Tank 4
Tanks 1 to 4
NH4+
NO2-
NO3-
PO4

Figure 3


A
Strain S002
02/19 04/19 06/19 08/19 10/19 12/19
date (mm/yy)
B
Husbandry population
clone number
0 50 100 150 200
03/19 05/19 07/19 09/19 11/19 01/20 03/20 05/20 07/20 09/20 11/20
date (mm/yy)
strain index
1 43 85 128 170 212
C
D
E
os
a
b
F
0 d
28 d
56 d
G
Weeks 1 to 4
Weeks 5 to 8
Growth rate
0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6
Mix A Mix C Mix B Mix D Mix C Mix A Mix D Mix B
Feeding diet

Unit	Manufacturer	No. of pieces
Specimen compartment		
Specimen tanks 40L	Fleuren&Nooijen	4
Heaters (titanium D-D)	Schego	4
Wavemaker pump (Jecod ECO SW2)	Jecod Co	4
Light AquaBeam 1500 Ocean blue	TMC Aquarium	8
Biolumen control unit	TMC Aquarium	1
Feeding compartment		
Feeding tanks 10L	Fleuren&Nooijen	4
Light Aquabar Tseries Colorplus 438mm	TMC Aquarium	8
Aquabar 2 channel controller	TMC Aquarium	1
Arduino Uno (delivery food controller)	Arduino CC	1
SUMP and Filtering compartment		
Filter pump FP 350	Sera Gmbh	1
Fountain pump 1800	Eheim	1
Diaphragm pump (Air400)	AniOne	1
Air blower (Medo LA60B)	Nitto Kohki	1
Circulation pump (Titan-Tec 10000)	Messner	1
UV steriliser (Vecton2)	TMC Aquarium	1
Bi-stop ball valve	Plasson	1
Light Aquabar Tseries Colorplus 438mm	TMC Aquarium	2
Aquabar 2 channel controller	TMC Aquarium	1
Cooling unit Titan 4000	Aqua Medic	1
Protein skimmer	Aqua Medic	1
Cermanic beads	Sera	1
Algae scrubber	IceCap	1
Denitrifyer	Korallin	1
Others		
Mortar mixer	Einhell AG	1
Salt tank	Fleuren&Nooijen	1
pH tank	Fleuren&Nooijen	1
Quarantine compartment		
Quarantine container	Semadeni AG	2
Wavemaker pump (Jecod ECO SW2)	Jecod Co	2
Monitoring system Apex		
Apex 2016 Grade Lab Kit	Neptune Systems	1
Automatic top-off kit	Neptune Systems	1
Dosing system	Neptune Systems	1
Optical sensor	Neptune Systems	1
Leak detection probe	Neptune Systems	1
Temperature probe	Neptune Systems	4
pH/ORP/Redox probe	Neptune Systems	2
Salinity module	Neptune Systems	2
Solenoid valve	Neptune Systems	1
Magnetic probe	Neptune Systems	1
Conductivity probe	Neptune Systems	1

Table 1. Components use in our husbandry system for colonial ascidians.