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 of species of interest

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 by budding, typically leading to the formation of colonies where animals, called zooids, are interconnected through 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.

Herein we present our success in breeding *Botrylloides diegensis*, a species of colonial ascidians, for more than 3 years in recirculating artificial seawater over 600 km away from their natural habitat. We show that colonies adapt well to in-lab culturing and that a specific strain can be isolated, propagated and efficiently used for research 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.

Keywords: Aquaculture, colonial tunicates, recirculating system, Botrylloides, Botryllus, breeding

1. 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 known 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 hours 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 natural 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 used 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 is currently, 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 successfully using this setup 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 to optimize 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.

2. Materials and Methods

2.1 Aquarium structure

The complete list of material and equipment used to assemble our system is provided in Table 1 (Supplementary Figures S1-S4). 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 materials: fountain pump (universal 300, Eheim, DE), air blower (Medo LA-60B, Nitto Kohki, DE), cooling unit (Titan 4000, Aqua Medic, DE), heaters (titanium D-

D 600W, Schego, DE), motor driven protein skimmer (DC runner 1.1, AquaMedic Gmbh, DE), circulation pump (Titan-Tec 10000, Messner Gmbh, DE), UV steriliser (Vecton2, TMC Aquarium, US), lights (AquaBeam 1500 Ocean blues, TMC Aquarium, US). 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 (Gripline, Semadeni AG, CH), aerated by one pump each (Wavemaker Jecod SW-2, Jebao, CN) and illuminated by a led rack each (Marine hybrid powerLED+, Eheim, DE) was added to the main system by our departmental workshop. Our quarantine tanks are placed on a heavy-duty plastic stand (Camshelving Premium, Cambro, US). Staining plastic racks (1233, Semadei AG, CH) were used for 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 (Apex 2016, Neptune Systems, US) controller, a custom micro-controller (Pico, Raspberry Pi, UK) as well as light controllers (BioLumen, TMC Aquarium, US). In particular, salinity, pH, day/night cycles, temperature and changes of water are performed automatically and reproducibly.

2.2 Artificial sea water production and filtration

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

(K+Media, Evolution Aqua, UK), an algae scrubber (Medium Turf Scrubber, IceCap, US) and phosphate absorption resin (Aqua Crown Phosphatadsorber, Deltec, DE). Live stones cured with Coralline algae as well as macroalgae were bought 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 Thermo Fisher Scientific (US).

2.3 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 to inoculate the husbandry system. Animals were then acclimatized in our quarantines and transferred to 76x26x1 mm microscopic slides (Marienfeld, DE). After the colonies had properly settled 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, CH) and a silicone badge gripper (Specialist ID, US). Colonies were fed using a blend of commercial algae concentrates from Instant algae (Shellfish Diet 1800, 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 normal closed solenoid valves (CJV23, Conjoin, CN) controlled by a customized microcontroller (Pico, Raspberry Pi, UK, Supplementary Material 1). 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. Statistical significance between growth rates was measured using two-tailed Student's *t*-tests. Salinity was kept constant at 35 g/L of seasalt, temperature was set at 21 °C all year round.

Live L-type rotifers (Planktovie, FR) were purchased 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 cultured in 1 L Erlenmeyer flasks without agitation, fed with RG Complete every day and their water was changed every 2 days. Once the population became sufficiently dense, typically every other day, they were splitted in half. One half serving for continuing their expansion, the other half being fed to the specimen tanks.

192 ## 3. Results

193 ### 3.1. Description of the system

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

206 To breed our animals in optimal conditions, we specifically aimed at using a maximum of
207 food-grade plastic material, to avoid rust, wear and chemical contamination as much as
208 possible. Four 40 L specimen tanks were custom made from 15/20mm PE/PP material. Tanks
209 have one compartment and each has a heater which is placed at the bottom of the tank and is
210 controlled by a temperature sensor placed on the side of the tank. Aeration and mixing of the
211 water are ensured by a circulation pump placed on the back side of each tank. Tanks are
212 completely closed to make a light-isolated environment, with a convenient front opening (Fig.
213 2D) for easy access and cleaning. Water falls into the tank through a silicone tube, which flow
214 rate can be regulated to modulate the amount of flow-through, and leaves the tank from an

outlet located in the back of the tank. A fleece is placed at the bottom of the tanks to collect solid wastes. 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 (Fig. 2D). 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 (Supplementary Figure S1). 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 (Fig. 2C). 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 cured stones and macro-algae. The refugium and the algae scrubber are constantly illuminated by two LED units. The sedimentation tank is connected to the main circulation pump, which provides system water to the cooling unit, the algae scrubber and the refugium in a closed loop, as well as to the specimen tanks through a UV sterilizer. In addition, periodic water changes are controlled by a timer-activated submerged fountain pump. This pump discards system water into the sewage through the sump's overflow drain to induce its replacement with fresh seawater. 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 plastic lid.

Above the specimen tanks, we have installed four 10 L cylindrical feeding tanks (Supplementary Figure S2). Each tank is made of non-transparent PVC pipes 200 mm in diameter and 300 mm long. An external 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

238 a microporous tube placed at the bottom of the tank, and lighting is done by two LED rails
239 placed inside a clear pipe through the center of the non-air tight lid of the tank. Food is drained
240 outside of each tank by gravity through an electronically controlled micro-valve connected to
241 the outlet of the tank (Supplementary Figure S3). Air is supplied via 4 mm silicone hoses
242 directly from the main air pump through a custom-built manifold (Supplementary Figure S4).

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

250 The conditions in the system are monitored and logged automatically (Fig. 2H) using an
251 APEX system from Neptune Systems (Supplementary Figure S3), connected to a number of
252 sensors and actuators. Temperature, salinity, pH and ORP (oxidation reduction potential) are
253 measured every 200 s in the main sedimentation tank. Temperature is controlled by the cooling
254 unit and a heater, salinity can be increased by pumping 270 g/L brine water located in a 75 L
255 tank placed behind the feeding tanks using two peristaltic pumps, also controlled by Apex.
256 Brine tank is mixed four times per day using a heavy duty concrete mixer controlled by a timer
257 (Supplementary Figure S4). An alert email is sent if some values deviate from their defined ideal
258 range (7.9-8.5 pH, 27-35 salt, 14-24 °C, 300-400 ORP). Water level is monitored using two
259 optical sensors driving an automatic top-off kit that delivers deionized water directly in the main
260 sedimentation tank by opening a solenoid valve connected to the University's water system

(Supplementary Figure S1). 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 at the bottom of the sedimentation tank, both leading to the shutdown of the whole system in case of emergency. Additional water quality parameters (pH, NH_4^+, NO_2^-, NO_3^-, PO_4^{3-}) and the conditions in the quarantine are monitored manually.

3.2. 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^+.

3.3. 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-mouth cylindrical containers. Animals were then transported to the laboratory, which typically took

306 between 8 h and 10 h. Important parameters for successful transportation of the collected
307 samples are first a large animal to volume ratio (> 20X), regular water changes during
308 transportation (every 2-3 h), as little air in the container as possible and minimizing the
309 turbulences during transport. However, the best predictor of a colony that will well tolerate
310 being transferred was its size, namely medium-sized colonies composed of 50-100 zooids
311 were typically fitter once in our laboratory. Another important aspect to consider when
312 collecting is the type of substrate the animal is growing on, and its shape. Colonies growing flat
313 on shellfish were the most appropriate for later transfer to our glass slides, while those
314 intermingled with brittle algae were very difficult to isolate once in the lab.

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

329 to the salinity of our recirculating system by lowering the salinity of our quarantine by 1 g/L per
330 week. Once the quarantines' salinity was identical to that of our system, and the colonies had
331 spent at least 2 weeks in quarantine under weekly monitoring against diseases and parasites,
332 they were transferred to the main system where they proliferated actively (Fig. 3A, Movie 1).
333 The lineage of all strains, their clonal offspring, lifespan, position in the system and treatments
334 were recorded in an online database. Approximately 50 % of the collected specimens could be
335 transferred successfully to our recirculating system.

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

348 In our system, colonies are cultured on numbered microscopy glass slides hanging
349 vertically in the water, which reproduces somewhat their natural environment where we mainly
350 collected them from the lateral sides of floating pontoons (Fig. 1B). This breeding method has
351 multiple advantages, first for microscopy and general handling of the animals in the lab, second

352 for the ease of use of the badge gripper and the total absence of metal parts (Fig. 2E), and
353 finally for the identification and localization of specific colonies within the system. Each slide
354 hangs onto a hook placed onto a PVC tube, itself fixed in the top part of the specimen tanks.
355 Given the space constrains of our system to allow manipulation of each animal individually,
356 each specimen tank can contain up to 32 slides (Fig. 2D).

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

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

369

370 3.4. Differential breeding of colonial tunicates

371 Colonies in our recirculating system were bred under continuous feeding following
372 preliminary indications (Blanchoud, unpublished) that such delivery was promoting growth. In
373 addition, this type of feeding has two advantages, first a reduced contamination of the colonies
374 and their slides because of the overall lower concentration of food present in the water, and

375 second a lower daily maintenance as food needs to be prepared only once for multiple days.
376 Continuous feeding was implemented using electronically controlled valves (Supplementary
377 Material 1). We optimized the frequency and duration of the opening of each valve to reach
378 uniform amounts between all four specimen tanks. On average, valves opened 1.5 s every 3
379 min, thus delivering around 2 L of diluted food per day. Food was prepared every 4 days to
380 guarantee food quality after culture in our feeding tanks and concentration was adapted to
381 minimize clotting.

382 Animals were fed with a custom food mix composed of equal parts of two commercial
383 blends of concentrated micro-algae (Shellfish Diet 1800, RotiGrow Plus), supplemented with
384 live rotifers (cultured in-lab), enriched with 1 % (v/v) bakery yeast and 2 % (v/v) of planktonic
385 supplement. To monitor the well-being of our animals, 4 slides representative of the same 4
386 strains were imaged per tank once a week. We manually counted the number of zooids in each
387 picture to estimate the growth rate of our animals and the impact of the different breeding
388 conditions (Fig. 3F).

389 The favorable environmental culturing conditions presented here were identified thanks to
390 multiple differential breeding experiments. Such approach could be used to test the impact of
391 environmental conditions on specific aspects of the specimens' development, and to adapt our
392 culturing conditions to different species for their breeding. Herein we show one example of
393 such experiments. Animals were fed with four different food mixes: A) 1.6 mL/day ShellFish
394 Diet 1800, 1.6 mL/day RotiGrow Plus, B) 1.1 mL/day ShellFish Diet 1800, 1.1 mL/day RotiGrow
395 Plus, C) 3.2 mL/day RotiGrow Plus, D) 3.2 mL/day Shellfish Diet 1800. All food mixes were
396 complemented with bakery yeast and planktonic substitute at the same proportion. Animals
397 were bred under these conditions for four consecutive weeks while being monitored weekly. To

compensate for any possible bias in specific specimen tanks, we swapped the feeding diets after 4 weeks, and monitored the same colonies for another 4 weeks.

Animals fed with food mix A and D outperformed those fed with B and C (Fig. 3G). Moreover, animals fed with food mix A and D had positive growth rates (> 1), meaning that each 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 significantly influence 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 as previously published (Rinkevich and Shapira, 1998). 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.

4. 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 constantly breeding *Botrylloides* 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 with limited experience in saltwater aquaculture, we 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 on the long term because the anaerobic bacteria that can process this ion develop much more slowly 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. 3A-B shaded areas) followed a sudden increase in the number of colonies that we naively combined

with a corresponding 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 noxious ions in the system's water, and thus wrongly believed that we had solved the issue at the root of the decline. Instead, the accumulation was transferred to different noxious ions. The third and more drastic regression (Fig. 3A-B blue area) 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 and will be experimented in the future.

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 over 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 that include 3 additional 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 system for controlled breeding can provide the necessary platform for the worldwide spreading of colonial ascidians.

5. Acknowledgments

We would like to thank Dr. Megan Wilson for sharing her experience with breeding *Botrylloides*, Pr. Miles Lamar for providing space for our trials when setting up a flow-through system at the University of Otago, Pr. Loriano Ballarin for his extensive experience on breeding *Botryllus* in closed systems, Dr. Fabio Gasparini for helping us with the collection of our wild isolates, all the members of these laboratories for useful discussions on the breeding of colonial ascidians as well as Laura Bugada, Mohamed Abshir Mohamed, Margaux de Raemy, Lionel Page, Jeanne Bruelhart and Silvia Moreno Forero for their help in taking care of the husbandry setup.

We 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, Bertrand Dubois and François Zosso.

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6. Author contributions

Simon Blanchoud: Conceptualization, Funding acquisition, Project administration Visualizaton. Marta Wawrzyniak: Writing-original draft. Marta Wawrzyniak, Lluis Matas Serrato, Simon Blanchoud: Investigation, Methodology, Validation, Writing-review & editing.

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8. Declaration of Interests

The authors have declared no conflict of interest.

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10. Figure legends (all figures should be printed in color)

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 (Wilson lab, University of Otago, NZ). (**D**) Example of a closed system (Karahan lab, METU, Turkey).

Figure 2. The colonial ascidian husbandry setup. (**A**) Schematic blueprint of our system. (**B**) Picture of the developed prototype. The main system is composed of a shelf of tanks for 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 in our quarantine tanks. (**H**) Parameters of the water quality measured in our system between March 2018 and March 2021. From left to right: ORP – oxidation reduction potential (mV); salinity (g/l); water temperature in the sump and tanks 1 – 4 (°C); pH in the sump and measured with a kit; levels of noxious substances: NH_4^+, NO_2^-, NO_3^- and PO_4^{3-} (mg/l).

Figure 3. Breeding of colonial ascidians. (**A**) Lineage of a successful strain (S131) showing its propagation by subcloning (from February 2019 till March 2021). Major recession events are pointed at by shaded areas (grey and blue), the one concurrent with a sanitary confinement is highlighted in blue. (**B**) Evolution of the number of clones bred in our system shown from March 2019 till February 2021, color-coded by strain ID. Major recession events are depicted as in **A**.

657 (C) Picture of a relatively large colony composed of over 300 zooids, which became gravid in
658 2019. (D) Picture of a gravid bud with its oocyte visible (arrowhead). (E) Picture of a young
659 oozoid recently metamorphosed. Visible organs including its oral siphon (s), ampullae (a) and
660 bud (b). (F) Sample pictures taken on the specified days during our differential feeding
661 experiment. Depicted pictures correspond to the same colony fed with the combination Mix A /
662 Mix B. (G) Relative mean number of zooids counted per colony (n=3) averaged over the
663 indicated number of weeks. P * < 0.05; ** < 0.01; *** < 0.001.
664
665 **Table 1. Components use in our husbandry system for colonial ascidians.** Most components
666 are visible in **Supplementary Figures S1-S4**. Note that the given prices have been gathered from
667 a variety of different providers and currencies and will likely vary. Transportation, taxes and
668 assembly costs were not taken into account. a) these components were custom-build and their
669 costs are thus estimates only. b) these components are consumables and the given price
670 corresponds to what is required for assembling one complete setup.
671
672 **Movie 1. In-land breeding of *B. diegensis*.** Time-lapse imaging of a colony developing over a
673 period of 25 days.
674
675 **Supplementary Figure S1. Detailed structure of the sump unit.** (A) Moving-bed filter, (B)
676 refugium and (C) main sedimentation tank composing the sump of our husbandry system. (D)
677 Fleece filter separated from its underlying moving-bed unit shown in A. (E) Components of the
678 refugium filter and of the ceramic beads filtration unit visible in B. (F) Area between the sump
679 and the chiller where the main circulation pump, the nitrate filter and the leak detection probe

are located. The main components visible in the panels are identified using their ID as listed in Table 1.

Supplementary Figure S2. Detailed structure of the feeding unit. (A) Feeding tank connected to the delivery valve for dispensing food in the specimen tank located underneath. Each feeding tank is composed of (B) a 10 L cylindrical container, (C) a micro-porous air diffuser and (D) a lid with a clear compartment for hosting the lighting bars. Components ID are specified in Table 1.

Supplementary Figure S3. Detailed structure of the controlling units. (A) Main control panel with the Apex controller and its various modules. (B) Behind the husbandry system where the solenoid valve controls the input of deionized water for topping-up the sump. (C) Components of the custom micro-controller unit for continuous feeding of our colonies. (D) Lights and feeding control units are installed behind the feeding tanks. Components ID are specified in Table 1.

Supplementary Figure S4. Detailed structure of the air and salts units. (A) The main air pump is isolated from the husbandry system and connects to a custom manifold. The pH unit is located on the left side of the system. (B) The manual control valve for the pH buffer is accessible from behind the main system. (C) The brine tank is located on the right side of the system and is pumped automatically by the two peristaltic pumps visible in A. Components ID are specified in Table 1.

Supplementary File 1. Design and control of our custom micro-controller for continuous feeding. This archive contains all the documents required to reproduce our micro-controller. The file used to design and produce the custom PCB board (controller_design.fzz) using Fritzing, a dedicated Open Source software. A schematic representation of that PCB board (controller_design_scheme.pdf) and two vectorized visualizations of the PCB connections, one for each side of the board (controller_design_pcb_top.pdf, controller_design_pcb_bottom.pdf). A list of the electronic components required to assemble the PCB board (controller_components.txt) and the MicroPython code used to control the valves (controller_code.py).

Figure 1

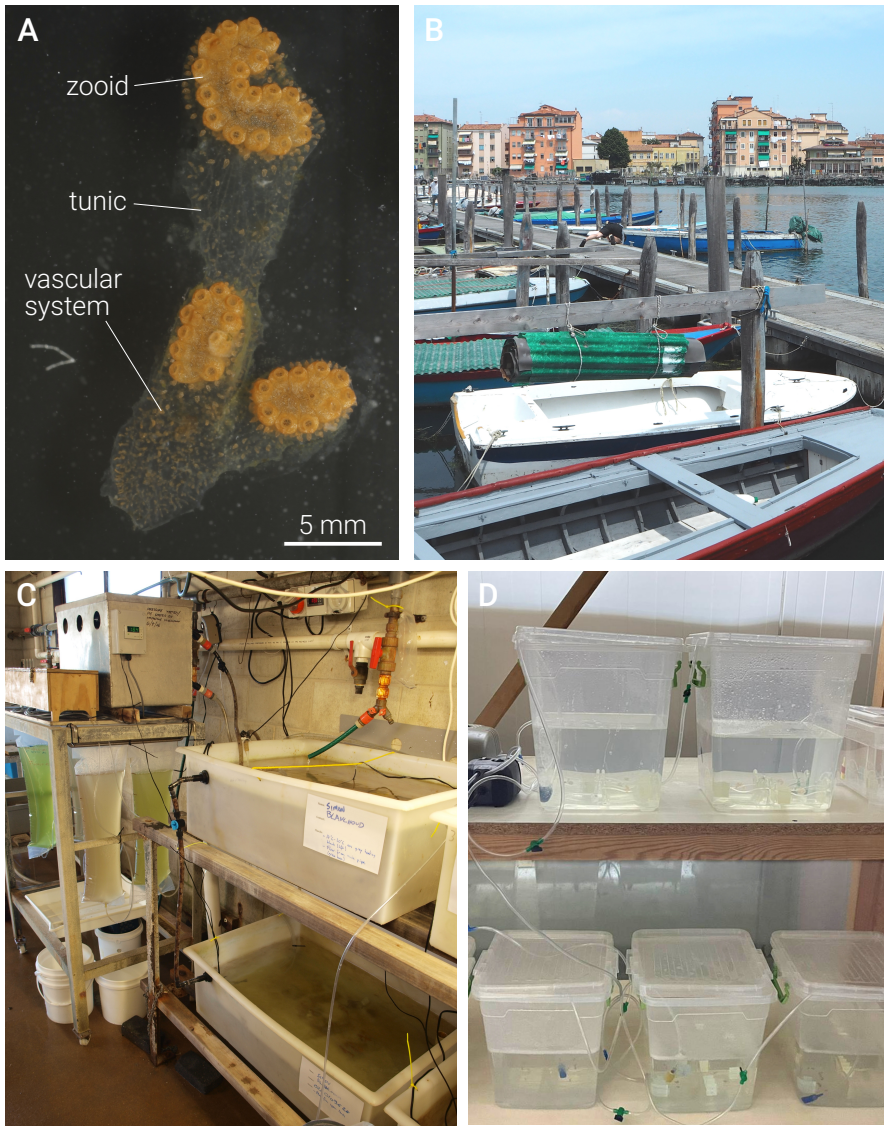
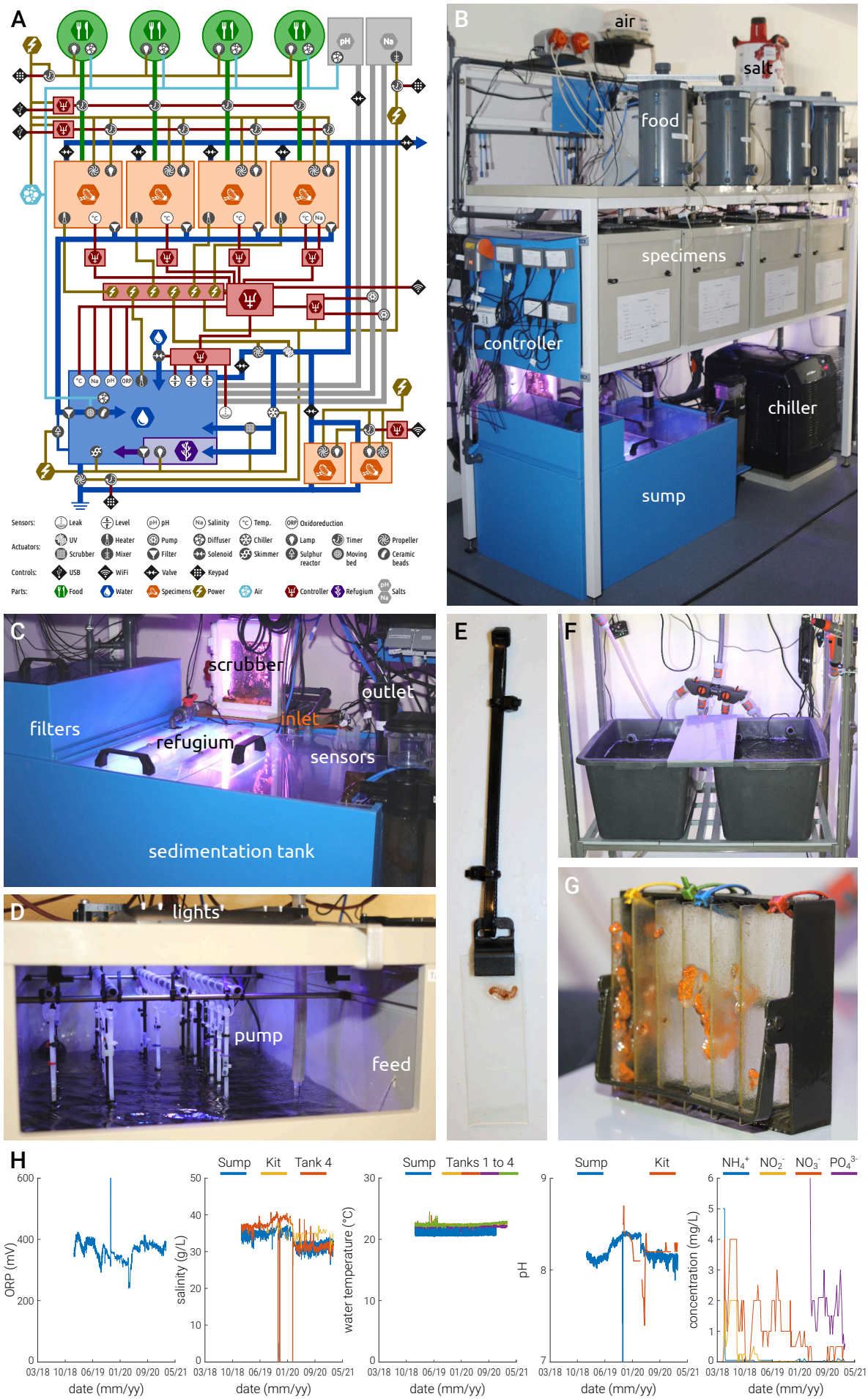


Figure 2


A
Sensors: Leak Level pH Salinity Temp. Oxidoreduction
Actuators: UV Heater Pump Diffuser Chiller Lamp Timer Propeller Scrubber Mixer Filter Solenoid Skimmer Sulphur reactor Moving bed Ceramic beads
Controls: USB WiFi Valve Keypad
Parts: Food Water Specimens Power Air Controller Refugium Salts
B
air
salt
food
specimens
controller
chiller
sump
C
scrubber
outlet
inlet
filters
refugium
sensors
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ORP (mV)
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Sump Kit Tank 4
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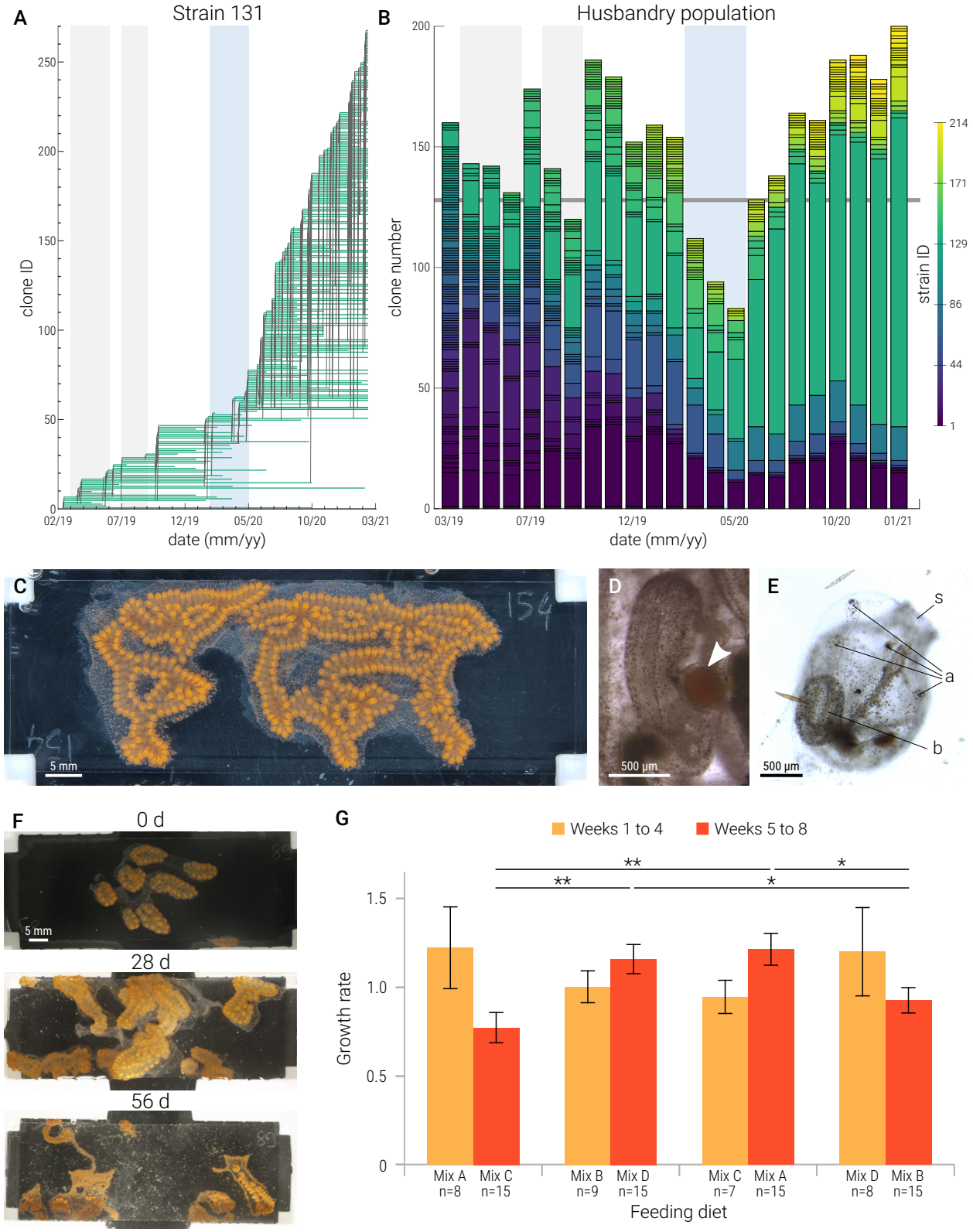
Figure 3


A
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Husbandry population
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date (mm/yy)
03/19
07/19
12/19
05/20
10/20
01/21
strain ID
214
171
129
86
44
1
C
5 mm
D
500 µm
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a
b
500 µm
F
0 d
5 mm
28 d
56 d
G
Weeks 1 to 4
Weeks 5 to 8
Growth rate
Mix A n=8
Mix C n=15
Mix B n=9
Mix D n=15
Mix C n=7
Mix A n=15
Mix D n=8
Mix B n=15
Feeding diet

ID	Component	Manufacturer	Num.	Total (€)	
1	Specimen compartments				
1.01	Stainless frame	Fleuren&Nooijen	1	1000	a
1.02	Specimen tanks 40 L	Fleuren&Nooijen	4	1200	a
1.03	Flow regulation valve (VM DN 8)	FIP	4	112	
1.04	Heaters (titanium D-D)	Schego	4	196	
1.05	Wavemaker pump (Jecod SW-2)	Jebao	4	240	
1.06	Light AquaBeam 1500 Ocean blue	TMC Aquarium	8	800	
1.07	Light BioLumen Power Pod 2	TMC Aquarium	4	392	
1.08	Stainless support bar with stoppers	Fleuren&Nooijen	8	80	a
1.09	Plastic tubing for colony racks	Fleuren&Nooijen	16	8	a
1.10	Plastic J-hooks for hanging colonies	Sinopos	128	5	
1.11	Silicone badge gripper	Specialist ID	128	141	
1.12	Various cable ties	Netztech Handels	200	6	
1.13	Filter wool	Finest aquatics	2 m/w	6	b
2	Quarantine compartments				
2.01	Heavy-duty plastic stand	Cambro	1	700	a
2.02	Quarantine container 45 L (Gripline)	Semadeni AG	2	18	
2.03	Plastic staining rack (1233)	Semadeni AG	10	54	
2.04	Wavemaker pump (Jecod SW-2)	Jebao	2	120	
2.05	360 mm marine hybrid LED (powerLED+)	Eheim	2	218	
2.06	Wireless LED controller (control+)	Eheim	1	122	
2.07	LED power supply	Eheim	1	33	
3	Feeding compartments				
3.01	Feeding tanks 10 L	Fleuren&Nooijen	4	800	a
3.02	Light Aquabar T-series Colorplus 438mm	TMC Aquarium	8	624	
3.03	Aquabar 2 channel controller	TMC Aquarium	1	58	
3.04	4.5 mm barbed micro-valve (AC42155)	Antelco	4	4	
3.05	4 mm inner diameter silicone tubing	Cortaillod	20	32	
3.06	Micro-porous tubing	Gardena	4	10	

3.07	Polyamide cable gland	Wiska	8	6	
3.08	4.5 mm joiners barbed	Antelco	16	2	
3.09	500 g waterproof weight	Tunturi	4	27	
4	Water compartment				
4.01	Sump tank 200 L	Fleuren&Nooijen	1	600	a
4.02	Fountain pump (universal 300)	Eheim	1	55	
4.03	Digital timer IP20	Steffen AG	1	17	
4.04	Circulation pump (Titan-Tec 10000)	Messner	1	700	
4.05	UV steriliser (Vecton2 600)	TMC Aquarium	1	78	
4.06	Bi-stop ball valve	Plasson	2	74	
4.07	Cooling unit Titan 4000	Aqua Medic	1	3400	
4.08	Heaters (titanium D-D)	Schego	1	49	
4.09	Protein skimmer (DC runner 1.1)	Aqua Medic	1	205	
4.10	Filter wool	Finest aquatics	0.2 m/w	1	b
4.11	Moving bed (K+media)	Evolution aqua	10 L	19	
4.12	Cermanic beads	TetraTec	3	34	
4.13	Bacteria (Bio reefclear)	Sera	100 mL	11	
4.14	Algae scrubber	IceCap	1	240	
4.15	Denitrifyer	Korallin	1	250	
4.16	25/30/40 mm PVC tubing	Van de Lande	20	80	
4.17	Various PVC connectors and tees	Van de Lande	25	75	
4.18	19 mm tubing	Angst + Pfister AG	2	16	
4.19	4-channels water distributor (8194-20)	Gardena	1	36	
4.20	Various hose connectors and fittings	Gardena	15	75	
4.21	Water trigger nozzle (18301-20)	Gardena	2	56	
5	Refugium compartment				
5.01	Light Aquabar T-series Colorplus 438mm	TMC Aquarium	2	156	
5.02	Aquabar Dimmer Switch	TMC Aquarium	1	24	
5.03	Meshed filter box	Semadeni	1	8	
5.04	Live rock with Coralline algae	Aqua Home	8 kg	182	
5.05	Macroalgae (Chaetomorpha sp.)	Aqua Home	1	9	

6	Air compartment				
6.01	Air blower (Medo LA-60B)	Nitto Kohki	1	240	
6.02	18 mm tubing	Angst + Pfister AG	3 m	24	
6.03	18 mm to 4 mm air manifold	Fleuren&Nooijen	1	100	
6.04	4 mm inner diameter tubing	Cortaillod	20 m	33	
6.05	Air stones	Sera	3	3	
7	Salt compartments				
7.01	Mortar mixer	Einhell AG	1	80	
7.02	Digital timer IP20	Steffen AG	1	17	
7.03	Salt tank 75 L	Fleuren&Nooijen	1	180	
7.04	Seawater salt (SYN-BIOTIC)	Tropic Marin	260 g/L	57	b
7.05	pH tank 20 L	Fleuren&Nooijen	1	100	
7.06	Sodium bicarbonate	Fisher Chemicals	16 g/L	3	b
7.07	4.5 mm barbed micro-valve (AC42155)	Antelco	1	1	
8	Controller systems				
8.01	Apex 2016 Lab Grade Kit	Neptune Systems	1	680	
8.02	Magnetic holder	Neptune Systems	1	41	
8.03	Automatic top-off kit	Neptune Systems	1	200	
8.04	Solenoid valve	Neptune Systems	1	31	
8.05	Optical sensor	Neptune Systems	1	36	
8.06	Leak detection probe	Neptune Systems	1	30	
8.07	Temperature probe	Neptune Systems	4	112	
8.08	pH/ORP/Redox module	Neptune Systems	2	194	
8.09	Salinity module	Neptune Systems	2	194	
8.10	Dosing system	Neptune Systems	1	311	
8.11	Conductivity probe	Neptune Systems	1	132	
8.12	Microcontroller (Pico RP2040)	Raspberry Pi	1	4	
8.13	Custom valve controller	Aisler	1	50	
8.14	12V normal closed liquid valve (CJV23)	Xiamen Conjoin	4	8	
8.15	Rocker switch (16Q7522)	Hornbach	1	3	
8.16	Biolumen control unit	TMC Aquarium	1	413	

Total	**16741**

Table 1. Components use in our husbandry system for colonial ascidians. Most components are visible in **Supplementary Figures S1-S4**.Note that the given prices have been gathered from a variety of different providers and currencies and will likely vary. Transportation, taxes and assembly costs were not taken into account. **a)** these components were custom-build and their costs are thus estimates only. **b)** these components are consumables and the given price corresponds to what is required for one complete setup.

5 mm

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