Low pH conditions impair module capacity to regenerate in a calcified colonial invertebrate, the bryozoan *Cryptosula pallasiana*

Chiara Lombardi a, *, 1, Paul D. Taylor b, 1, Silvia Cocito a, Camilla Bertolini c, 2, Piero Calosi c, d

a ENEA Marine Environment Research Centre, PO Box 224, 19100 La Spezia, Italy

b Department of Earth Sciences, Natural History Museum, Cromwell Road, London SW7 5BD, UK

c Marine Biology and Ecology Research Centre, Plymouth University, Drake Circus, Plymouth, PL4 8AA, UK

d Département de Biologie, Chimie et Géographie, Université du Québec à Rimouski, 300 Allée des Ursulines, Rimouski, QC G5L 3A1, Canada

Corresponding author : chiara.lombardi@enea.it (C. Lombardi)

Abstract

Many aquatic animals grow into colonies of repeated, genetically identical, modules (zooids). Zooid interconnections enable colonies to behave as integrated functional units, while plastic responses to environmental changes may affect individual zooids. Plasticity includes the variable partitioning of resources to sexual reproduction, colony growth and maintenance. Maintenance often involves regeneration, which is also a routine part of the life history in some organisms, such as bryozoans. Here we investigate changes in regenerative capacity in the encrusting bryozoan *Cryptosula pallasiana* when cultured at different seawater pCO2 levels. The proportion of active zooids showing polypide regeneration was highest at current oceanic pH (8.1), but decreased progressively as pH declined below that value, reaching a six-fold reduction at pH 7.0. The zone of budding of new zooids at the colony periphery declined in size below pH 7.7. Under elevated pCO2 conditions, already experienced sporadically in coastal areas, skeletal corrosion was accompanied by the proportional reallocation of resources from polypide regeneration in old zooids to the budding of new zooids at the edge of the colony. Thus, future ocean acidification can affect colonial organisms by changing how they allocate resources, with potentially profound impacts on life-history patterns and ecological interactions.

1. Introduction

The production of multiple copies of a basic body form characterizes clonal modular organisms, whose repeated units may separate or remain connected during their lifespans

(Harper, 1977). Modular organisms are widespread, include both plants and colonial animals, and share many similar reproductive, defensive, competitive and life history traits. Marine invertebrates that grow as modular colonies, such as corals, hydroids, bryozoans and ascidians, jointly dominate the profuse sessile communities encrusting solid surfaces in the sea, and form a major component of global marine biodiversity.

The modules of colonial animals e zooids e are not always identical but may instead be polymorphic, meeting the various structural and functional needs of the colony, such as defence, feeding and sexual reproduction (Hughes, 1989). Communication and cooperation between individual zooids, which may involve neural connectivity, enables colonies to behave as integrated functional units and allows the translocation of substances/metabolites to facilitate feeding, growth, reproduction, response to threats, and recovery from localized damage (Mackie, 1986; Stuefer et al., 2004).

An important element of maintenance in modular organisms is regeneration. In fact, degeneration-regeneration cycles are characteristic of many modular organisms and enable: (1) replacement of ageing zooids; (2) excretion of waste products; and (3) shedding of fouling organisms (Hughes, 1989; Gordon, 1977). It is common place for the zooids of marine colonies to undergo degeneration-regeneration cycles, e.g. thecate hydroids, bryozoans and colonial ascidians (Crowell, 1953; Gordon, 1977; Berrill, 1935). In the exclusively colonial phylum Bryozoa, each feeding zooid possesses a polypide e the feeding structures and associated organs e that exhibits cycles of degeneration and regeneration in the majority of species. Degeneration of a bryozoan polypide results in the formation of a 'brown body', which is either expelled from the colony or retained in the coelomic cavity (Gordon, 1977; Hughes, 2005). Brown body formation can be

triggered by adverse environmental factors, embryogenesis, or simply the accumulation of residual materials in the digestive and secretory cells of the stomach, causing the entire stomach to degenerate (Gordon, 1977). Tissues of the zooid that remain after polypide degeneration are used together with materials translocated from adjacent zooids to form a replacement polypide, a process known as polypide regeneration. Cycles of polypide degeneration-regeneration are rejuvenatory and extend the lifespans of individual zooids in bryozoan colonies (Hughes, 2005; Dyrynda, 1981).

Plasticity in module regeneration has the potential to improve the ability of a species to cope with the low pH conditions sporadically experienced by coastal organisms (Arnaud-Haond et al., 2012; Hofmann et al., 2011), as well as ocean acidification (OA) predicted to occur at a greater frequency and more chronically over the coming centuries (IPCC, 2014). Resulting from anthropogenically elevated levels of atmospheric carbon dioxide (CO2), OA has been manifested by a drop of 0.1 units in average surface seawater pH, as well as a reduction in carbonate ion concentration during the past 150 years. According to expected fossil fuel consumption, a further pH decline of 0.3e0.5 units (400 matme1000 matm pCO2) is predicted by 2100 (IPCC, 2014), nd a cumulative drop of up to 0.7 units or more (540 matm to c. 1990 matm) by 2300 (Kawaguchi et al., 2013).

The aim of the present study is to investigate alterations in regenerative capacity in a calcifying colonial invertebrate under future ocean conditions. The cheilostome bryozoan *Cryptosula pallasiana*, cultured in a mesocosm for periods of up to four weeks at pH levels of 8.1 (current ocean), 7.7, 7.4 and 7.0, was used as a model organism to infer changes in the relative investment to maintaining existing zooids by polypide regeneration vs. the budding of new zooids. We here investigate: (1) frequency of

polypide regeneration (regenerated polypides/total number of polypides); (2) changes in the number of 'active generations' of zooids (i.e. the number of rows of mature zooids in the active generation band involved in polypide cycles); and (3) changes in ontogenetic zonation (i.e. the relative extent of the budding band, active generation band and moribund/corroded zooid band) under different pH scenarios. Our results identify a previously unrecognised biological response e diminution of regenerative capacity e that may occur as a consequence of OA, with likely impacts on marine functional diversity.

2. Material and methods

2.1. Study species

The cheilostome bryozoan *Cryptosula pallasiana* (Moll, 1803)is an encrusting species forming sheet-like colonies comprising numerous zooids, each about 0.8 mm long, which feed by extending the tentaculate organ (lophophore) of the polypide, produce gametes and brood embryos. New zooids are added by budding at the colony periphery, leaving increasingly older zooids at greater distances from the growing edge. The calcareous skeleton of the zooid body walls comprises calcite and aragonite, with calcite having an intermediate/low content of MgCO3 predominating (Poluzzi and Sartori, 1974; Smith et al., 2006). Polypides survive for 2e10 weeks in aquarium conditions, then completely regress in 6e17 d (Gordon, 1973). A new polypide may begin to form during the process of regression (Gordon, 1973). Widely distributed in the North Atlantic, Mediterranean and Black Sea, C. pallasiana inhabits littoral and shallow sublittoral environments (<50 m deep) and is a globally successful invasive fouling species in docks and harbours.

109

110 2.2. Biological material and experimental design

111 Colonies of C. pallasiana were collected from marinas in Brixham, Plymouth and
112 Falmouth, southwest UK, during late summer 2012. In the laboratory, larvae released
113 from the wild colonies were settled onto acetate sheets, grown to 20e50 zooids while fed
114 daily with the microalgae *Isochrysis galbana* and *Rhinomonas reticulata*, and then
115 excised and glued, two colonies per slide, to 76 38 mm microscope slides using
116 cyanoacrylate adhesive (48 slides in total). The slides were then placed back in the
117 original culture vats. Twelve days later, two initial batches of 16 slides each were
118 transferred to the experimental apparatus described below and kept at constant
119 temperature (15 C) and at one of four pH levels: pH 8.1 as control (ambient) conditions,
120 and 7.7, 7.4 and 7.0, to mimic the predictions of various models of future oceanic pH
121 (Feely et al., 2004; IPCC, 2014). One of the initial batches of 16 slides was kept for one
122 week (¼ '1-week Batch'), and the second batch was kept for four weeks (¼ '4-week
123 Batch'). The third batch of 16 slides was introduced into the experimental apparatus on
124 Day 8, replacing the 1-week Batch, and maintained for the next two weeks (¼ '2-week
125 Batch'). The exposure times of one, two and four weeks used for the experiment were
126 based on our experience of growth rates in the bryozoan culture system (Pistevos et al.,
127 2011). Orthogonal designs were employed to test the null hypotheses that polypide
128 regenerative capability (regenerated polypides/total number of polypides), the number of
129 active zooid generations (i.e. the number of rows of mature zooids in the active
130 generation band involved in polypide cycles) and ontogenetic zonation (i.e. the relative
131 extent of the budding band, active generation band, and moribund/corroded zooid band)

in *C. pallasiana* did not vary when exposed to lowered pH and after different lengths of time.

2.3. Determining polypide regeneration, active zooid generations and ontogenetic zonation

Polypide degeneration is the complete regression of the polypide within a zooid, leaving a residual brown body (which may subsequently be defecated by the new polypide), whereas regeneration is the reverse transition to restore a complete polypide (Fig. 1).

Just prior to introducing the colonies of *C. pallasiana* to the experiment (start: time 0 for the different batches), they were placed on sheets of graph paper and photographed using image capture and processing software (Infinity Analyze, Lumenera, Ottawa, Canada) connected to a digital camera (Infinity 1, Lumenera, Ottawa, Canada) attached to a microscope (MZ12, Leica, Heerbrugg, CH) at 0.8 x magnification. Further images of each colony were taken after 7 d for colonies of the 1-week Batch, after 14 d for colonies of the 2-week Batch, and after 21 and 30 d for colonies of the 4-week Batch. Following photographic recording at 21d, colonies from the 4-week Batch were put back into the experimental apparatus for the final week without being exposed to air or significant temperature or pH/pCO2 fluctuations.

Polypide regeneration was here investigated only in the 4-week batch by comparing the states of the zooids at the start of the experiment and after three and four weeks. In order to quantify the frequency of polypide regenerations, zooids were separately numbered on digital images of the colonies; regeneration was scored for any zooid, previously lacking

154 a polypide, in which a new polypide appeared either between week 1 and week 3 or
155 between week 3 and week 4. This figure was then compared with the total number of
156 zooids per colony having polypides after four weeks to quantify the proportion of
157 regenerating zooids, i.e. regenerated polypides/total number of polypides.

158 Active zooid generations were estimated as the number of longitudinal rows (i.e. parallel
159 to growth direction) of mature zooids involved in polypide cycles within each colony
160 (Fig. 1). This figure was calculated in each colony for the week 1, 2 and 3 batches at the
161 end of their periods of exposure to four pH levels (8.1, 7.7, 7.4, 7.0).

162 Colony ontogenetic zonation was estimated through time (time 0, week 1, 2, 3 and 4) for
163 each pH level (8.1, 7.7, 7.4, 7.0) by counting the number of zooid generations in each of
164 the three main bands:(1) the budding band prior to polypide completion and feeding
165 (BB); (2) the active zooid generation band with functional feeding polypides (AgB); (3)
166 and the band of 'old', moribund zooid bands in which feeding had ceased (OB). The
167 proportion of zooids in the BB was calculated with respect to the AgB (BB/AgB), and the
168 proportion of zooids composing the OB with respect to those in the AgB (OB/AgB). Data
169 from all three batches were used for time 0, data from the 1-week batch for the end of the
170 first week, data from the 2-week batch for the end of the second week, and data from the
171 4-week batch for the third and fourth weeks.

172

173 2.4. Experimental set-up

174 The system used in this experiment was adapted from Melatunan et al., (2011) and
175 consisted of four header tanks, one per pH treatment, each containing 64 L of sea water,

which was gravity fed, at a rate of 20 mL min□1, from the header tanks to each of 32 exposure tubs (volume 0.23 L). The system was designed to drain all water into a common sump, which filled each header tank as needed. Therefore, the water used in the three treatments was the same and any variation in water quality associated with separate systems was minimized (see Melatunan et al., 2011). One microscope slide with two bryozoan colonies was placed in each tub. Within each batch (1-week, 2-week and 4-week), four tubs were haphazardly allocated to each pH level. The tubs were randomly allocated to one of four trays at the beginning of the experiment so that each tray contained a mixture of pH x exposure-time combinations. Water flowing out of the tubs was held in the holding trays, helping to maintain a stable temperature throughout the experiment, and drained through a biological filter into a sump where seawater was aerated to reach control pH level, then pumped up into a separate tank; the four header tanks were then individually fed from this via a submersible pump (Rioþ, Aqua pump, 1700, TAAM).

CO2 gas was released into the header tanks using a multistage CO2 regulator (EN ISO 7291; GAS-ARC Group, Diss, Norfolk, UK) connected to a flip-flop control solenoid valve (ORIFICE 3/16 Closed System, Farmington, CT, USA) controlled by calibrated pH controllers (pH-201 Digital; Dream Reef, UK). In order to maintain stable pH treatments throughout the experiment, a submersible aquarium circulation pump was placed in each of the four header tanks. Bryozoans were fed with 250 mL of *Isochrysis galbana* and *Rhinomonas reticulata* in each header tank twice a week. Seawater pH and temperature were measured daily with a hand-held meter (YSI 85, YSI Inc. Yellow Springs, USA, Metter Toledo Technical buffers: 4.01, 7, 9.21) in all parts of the system (tubs, header

tanks, trays and sump) but since the values at all the locations were found to be the same on a given day, it was afterwards measured only in the four header tanks in order to minimize disturbance in the tubs. Seawater samples for the determination of total alkalinity AT were collected twice a week in borosilicate bottles, poisoned with 30 ml of HgCl2 (0.02%), and kept in the dark until measured by Gran titration using an alkalinity titrator (AS-ALK2, Apollo SciTech Inc. Newark, DE, USA). On the same day as AT sampling, in the same containers, salinity was measured with a refractometer (H2Ocean D-D The Aquarium Solution Ltd. Ilford, Essex, UK).

Carbonate system parameters that were not directly measured were calculated using CO2SYS (Pierrot et al., 2006), employing constants from Mehrbach et al. (1973) refitted to the NBS pH scale by Dickson and Millero (1987) and the KSO4 dissociation constant from Dickson (1990). Carbonate system parameters are summarised in Table 1.

2.5. Physical-chemical parameters

Differences in the chemical data of the systems among treatments were estimated using ANOVA. A post-hoc Tukey HSD test was performed whenever a significant difference was found. These statistical analyses were performed using R®.

The analyses of physico-chemical parameters revealed that both pH and p CO2 were significantly different across treatments (pH: F3.80 ¼ 1226, $p < 0.01$, pCO2:F3.80 ¼ 651.7, $p < 0.01$), and post-hoc tests shows all treatments to be significantly different from each other ($p < 0.01$) (Table 1).

DIC was significantly different across treatments (F3.80 ¼ 17.85, $p < 0.0001$), and post-hoc tests showed significant differences in 8.1 vs. 7.7 ($p < 0.05$), 8.1 vs. 7.4 ($p < 0.01$), 8.1 vs. 7.0 ($p < 0.01$) and 7.7 vs.7.0 ($p < 0.01$), but not in 7.7 vs. 7.4 and 7.4 vs. 7.0.

HCO3 was significantly different across treatments (F3.80 ¼ 17.85, $p < 0.0001$), and post-hoc tests revealed significant differences in 8.1 vs. 7.7 ($p < 0.01$), 8.1 vs. 7.4 ($p < 0.01$) and 8.1 vs. 7.0 ($p < 0.01$) but not in 7.7 vs. 7.4, 7.7 vs. 7.0 and 7.4 vs. 7.0. CO3 was significantly different across treatments (F3.80 ¼ 651.7, $p < 0.01$) and between all treatments (Tukey test for all combinations $p < 0.01$).

Regarding saturation states, both U calcite and U aragonite were significantly different across treatments (F3.80 ¼ 651.7, $p < 0.01$), while the post-hoc tests show all treatments to be significantly different from each ($p < 0.01$).

2.6. Data analyses

For each of the four pH treatments, four slides were analysed at the end of each exposure period (the data from the two colonies on each slide being pooled). Univariate analysis of variance (ANOVA) was used to test statistical differences in mean polypide regeneration rates among pH levels after four weeks. Student-Newman-Keuls (SNK) tests were performed a posteriori whenever a significant difference was found. Levene's test was performed as an a priori evaluation of homogeneity of variance.

Differences in the size of the budding band relative to the number of active generations (BB/AgB) in the size of the old band over active generations (OB/AgB) among pH levels and exposure times (week 1, week 2 and week 4: random factor) were analysed by using

a General Lineal Model (GLM). In addition, two-way ANOVA for repeated measures tests were performed to investigate the effect of exposure to different pH levels and exposure times in independent samples from week 1, 2 and 4 batches (Start-week 1, Start-week 2, Start-week 3, Start-week 4). Student-Newman-Keuls (SNK) tests were performed a posteriori whenever a significant difference was found. Prior to analysis, Levene's test was employed to assess the homogeneity of variance. When the test was significant ($p < 0.05$), transformations (square root, logarithmic, arcsin) were applied in order to achieve homogeneity of variance (Levene $p > 0.05$). In the case of failure of the transformation, after the residuals check, the more stringent criterion of $a < 0.01$ was applied (Underwood, 1997). All statistical analyses were performed using Statistica® v.7.

3. Results

3.1. Polypide regeneration

After four weeks, colonies of *Cryptosula pallasiana* grown under decreasing pH conditions showed a progressive reduction in polypide regeneration ratio, i.e. regenerated polypides/total number of polypides. The mean ratio ±s. e. was 0.15 ± 0.04 at pH 8.1; 0.03 ± 0.02 at pH 7.7; 0.03 ± 0.02 at pH 7.4; 0.02 ± 0.02 at pH 7.0 (ANOVA: F3 ¼ 4.93, p ¼ 0.02, Fig. 2 and Table 2A).

3.2. Active zooid generations

Cryptosula pallasiana colonies were able to bud new zooids with complete polypides (Fig. 1) in all four pH treatments throughout the four week duration of the experiment (Fig. 3). However, the number of active generations was greatest in the control and decreased progressively with decreasing pH (Fig. 1)(F_3 ¼ 44.81, $p < 0.01$)(Table 2B) (SNK test: 8.1 > 7.7 > 7.4 > 7.0). In control conditions, (pH ¼ 8.1) the number of active zooid generations increased through time (min: 4, max: 7) (Fig. 3). At pH 7.7, the number of active generations was less than in the control (min. 2, max. 5), but did not vary significantly across time (Fig. 3). At both pH 7.4 and 7.0, a decrease in the number of active generations over time was observed (min. 0.5, max. 4) (Fig. 3).

The number of active zooid generations within the same colony through time did not differ significantly (two-way ANOVA for repeated measures) from the start to week 1 in all treatments. However, significant differences were observed subsequently among treatments and between times (starte2 weeks: treatment F_3 ¼ 11.49, $p < 0.01$; time F_1 ¼ 36.25, $p < 0.01$; starte3weeks: treatment F_3 ¼ 8.65, $p < 0.01$; time F_1 ¼ 20.22, $p < 0.01$; starte4 weeks: treatment F_3 ¼ 17.03 $p < 0.01$; time F_1 ¼12.63 $p < 0.01$), and treatment * time interaction after 3 weeks (F_3 ¼ 10.73 $p < 0.01$) and 4weeks(F_3 ¼ 22.55 $p < 0.01$).

3.3. Ontogenetic zonation

Colonies cultured under control conditions (pH 8.1) had a budding band (BB) of incomplete buds comprising two developing generations from the start up to week 4 of the experiment, a band of active zooid generations (AgB) undergoing polypide cycles which increased from the start to week 4, and a band of old, moribund and inactive

zooids (OB) which increased through time (Table 3). Under pH 7.7, sizes of the ontogenetic zones were different, with relatively fewer active generations compared to the control condition at week 4, although the budding band (BB) and moribund zooid band (OB), the latter with no obvious corrosion of the skeleton, showed similar trends to those in control colonies. In contrast, the old zooids in colonies exposed to lower pH (7.4 and 7.0) had corroded skeletons, but colonies retained ontogenetic bands of budding and active generations (Table 3)(Fig. 4).

The relative proportion of BB with respect to AgB, and of OB with respect to AgB, within the same colony through time did not differ significantly (two-way ANOVA for repeated measures) from the start to week 1 in all treatments. However, significant differences were observed subsequently among treatments and time for the ratio BB/AgB (starte2 weeks: treatment F3 ¼ 9.11 $p < 0.01$; time F1 ¼ 7.71 $p < 0.01$; starte3 weeks: treatment F3 ¼ 13.37 $p < 0.01$; time F1 ¼ 7.65 $p < 0.01$; starte4 weeks: treatment F3 ¼ 7.66 $p < 0.01$; time F1 ¼ 5.00 $p < 0.01$), and treatment*time interaction after 3 weeks (F3 ¼ 27.78 $p < 0.01$) and 4 weeks (F3 ¼ 5.42 $p < 0.01$). For the ratio OB/AgB, significant differences were observed only among treatments (starte2 weeks: treatment F3 ¼ 5.94 $p < 0.01$; starte3 weeks: treatment F3 ¼ 7.38 $p < 0.01$; starte4weeks: treatment F3 ¼ 7.38 $p < 0.01$), and treatment*time interaction after 2weeks(F3 ¼ 11.28 $p < 0.01$), 3 weeks (F3 ¼ 5.74 $p < 0.01$) and 4 weeks (F3 ¼ 5.74 $p < 0.01$).

4. Discussion

Regeneration is widespread among animal phyla (Sanchez Alvarado and Tsonis, 2006). Numerous studies suggest that regeneration is limited by both intrinsic (e.g. size, age, morphology, genotype) and extrinsic (e.g. temperature, pH, food availability, sedimentation) factors, and that other life history processes may compete with regeneration for resources (Dupont and Thorndyke, 2006; Sanchez Alvarado, 2000; Schram et al., 2011; Wood et al., 2010). Key questions are the evolutionary and biological reasons for different modes of regeneration, the differences and similarities between regeneration and normal development, and the factors that limit regeneration.

Levels of pH expected to occur in the open ocean by the end of the century according to the IPCC prediction (e.g. pH 7.7 for the year 2100 and 7.4 for the year 2300) are already occurring naturally in some coastal areas due to seasonal variations in pH, but also in the vicinity of estuaries, in upwelling areas, and around CO2 vent systems. Here we show the detrimental effects of low seawater pH on the regenerative capability of the bryozoan *Cryptosula pallasiana* cultured for four weeks. When exposed to low pH levels, colonies of *C. pallasiana* showed a striking decline in polypide regeneration. Even a slight pH reduction caused a substantial drop in the number of zooids regenerating new polypides. The zone of moribund, inactive zooids was consequently more extensive in colonies grown at low pH than in control colonies. However, colonies grown in low pH conditions continued to bud new zooids. Therefore, our results suggest a relative shift in the energy budget of the colony away from maintenance, which includes regeneration, and towards growth through the budding of new zooids.

Corrosion of skeletons was evident in the old zooids of *C. pallasiana* colonies cultured at pH 7.4 and 7.0, as has been found previously in other bryozoan species transplanted to a

low pH environment (Rodolfo-Metalpa et al., 2010; Lombardi et al., 2011, 2015). It is conceivable that corrosion, along with other physiological challenges brought about by living at low pH and elevated pCO2, makes polypide regeneration difficult and energetically costly compared to budding new zooids at active growing edges. In the bryozoan *Membranipora membranacea*, colonies with damage to the older, central zooids retained a capacity for growth and recovery across a range of temperatures, while the effects of damage to younger, peripheral zooids were exacerbated by elevated temperature (Denely and Metaxas, 2015). In *C. pallasiana*, lowered pH significantly reduced polypide regeneration and resulted in skeletal corrosion of older zooids but did not prevent budding of new zooids at the colony periphery. In the study of *M. membranacea*, however, regeneration relates to recovery from physical damage, while in the present study of *C. pallasiana* it is part of regular colony maintenance.

Whatever the reasons for the apparent bias in favour of budding new zooids over polypide regeneration in *C. pallasiana* colonies that were cultured at low pH, life histories changed significantly. In a bryozoan colony growing across a solid surface, the budding of new zooids around the periphery and the decline of older zooids distant from the growing edge into a moribund state leads to movement of the currently active colony regions across the substrate. The rate of outward growth will influence the outcome of interactions between the colony and neighbouring sessile organisms, particularly the result of overgrowth interactions during competition for space, which is frequently a strong factor structuring sessile communities: faster-growing organisms will generally have an advantage in overgrowth competition (Taylor, 2016). The active lifespan of a zooid, reflecting the number of polypide degeneration-regeneration cycles it undergoes,

will directly influence its total reproductive output, while areas of dead or moribund zooids within a colony are more vulnerable to fouling and overgrowth by competitor species, as reported in colonies of the bryozoan *Steginoporella* (Palumbi and Jackson, 1983). Thus it can be seen that alteration of the balance between polypide renewal (rejuvenating zooids remote from the growing edge) and the budding of new zooids at the growing edge will potentially have marked effects on ecological interactions between bryozoan colonies and the rest of the sessile community.

Pistevos et al. (2011) showed that genetic variation among colonies of the bryozoan *Celleporella hyalina* was responsible for phenotypic variability in life-history parameters such as growth, reproductive investment and gender allocation, which include also regenerate polypides. This inherent genetic variability may be important in enabling future adaptations to OA via natural selection.

Low pH conditions already occurring seasonally and sporadically in coastal areas, due to increase in nutrient fluxes observed in the recent decades (Howarth et al., 2011), and future chronic ocean acidification have the potential to affect calcifying colonial organisms by lowering their capacity to rejuvenate zooids. As a cascade effect, this change may have a profound impact on fundamental biological processes and consequently on population and ultimately ecosystem dynamics. The effect of impaired regeneration due to OA may manifest itself at higher levels of biological assembly, for example by reducing epifaunal biomass production, reduced recruitment and altered biotic associations, thus impacting marine community ecosystem services.

Authors' contributions

CL and PC designed the study. CL carried out the experimental work with help from CB and PC and morphological studies with help from CB, performed statistical analyses with help from SC and PC, and drafted the first version of this manuscript assisted by PDT. All authors contributed to the later versions of the manuscript and approved the final version for publication.

Funding

This project was supported by the NERC UK Ocean Acidification Research Programme (NE/H017127/1) to PC and the 'ENEA prize for the best annual productivity' to CL, ENEA internal funding to CL and University of Plymouth internal funding to PC. PC is also supported by an NSERC Discovery grant and a FRQ-NT New University Researchers Start Up Program Grant.

Data accessibility

The original data has been deposited in Dryad Digital Repository and they will be available after the publication of the manuscript.

Acknowledgements

We wish to thank J.D. Bishop and C.A. Wood for the valuable help provided in specimen collection, experimental stock culturing, algal food provision during the experimental

394 phase, discussion of the experimental design, and contributions to earlier drafts of the
395 manuscript; and M. Hawkins for technical support during the experiment. C. Lombardi
396 thanks K. Hiscock for providing logistic support, the Department of Earth Sciences at the
397 Natural History Museum (London) for providing facilities and hospitality, and C.
398 Vasapollo for support with statistical analyses. All authors would like to thank the two
399 anonymous reviewers who contributed to the manuscript improvement.

400

401 **References**

402 Arnaud-Haond, S., Duarte, C.M., Diaz-Almela, E., Marb□a, N., Sintes, T., Serrano, E.A.,
403 2012. Implications of extreme life span in clonal organisms: millenary clones in
404 meadows of the threatened seagrass *Posidonia oceanica*. PLoS ONE 7 (2),
405 e30454. http://dx.doi.org/10.1371/journal.pone.0030454.

406 Berrill, N.J., 1935. Studies in tunicate development. Part IV - Asexual reproduction.
407 Philos. Trans. R. Soc. B 225, 327-379.

408 Crowell, S., 1953. The regression-replacement cycle of hydranths of Obelia and
409 Campanularia. Physiol. Zool. 26, 319-327.

410 Denely, D., Metaxas, A., 2015. Recovery capacity of the invasive colonial bryozoan
411 *Membranipora membranacea* from damage: effects of temperature, location, and
412 magnitude of damage. Mar. Biol. 162, 1769-1778.

413 Dickson, A.G., Millero, F.J., 1987. A comparison of the equilibrium constants for the

414 dissociation of carbonic acid in seawater media. Deep-Sea Res. 34, 1733e1743.
415 Dickson, A.G., 1990. The oceanic carbon dioxide system: planning for quality
416 data. U. S. JGOFS News 2, 2.

417 Dyrynda, P.E.J., 1981. A preliminary study of the patterns of polypide generation-
418 regeneration in marine cheilostome Bryozoa. In: Larwood, G.P., Nielsen, C. Eds.),
419 Recent and Fossil Bryozoa. Olsen and Olsen, Fredensborg, pp. 76-80.

420 Dupont, S., Thorndyke, M.C., 2006. Growth or differentiation? Adaptive
421 regeneration in the brittlestar *Amphiura filiformis*. J. Exp. Biol. 209, 3873-3881.

422 Feely, R.A., Wood, H.I., Kendal, M.A., Berelson, W., Kleypas, J., Fabry, V.J., Millero,
423 F.J., 004. Calcification, a physiological process to be considered in the context of
424 the whole organism. Biogeosc. Discuss. 6, 2267e2284.

425 Gordon, D.P., 1973. The Fine Structure of Normal and Regressing Polypides of the
426 Fouling Bryozoan *Cryptosula Pallasiana* (Moll). PhD Thesis. Dalhousie
427 University.

428 Gordon, D.P., 1977. The aging processes in bryozoans. In: Woollacott, R.M.,
429 Zimmer, R.L. (Eds.), The Biology of Bryozoans. Academic Press, New York, pp.
430 335-374.

431 Harper, J.L., 1977. Population Biology of Plants. Academic Press, London.

432 Hofmann, G.E., Smith, J.E., Johnson, K.S., Send, U., Levin, L.A., Micheli, F., et al.,
433 2011. High-Frequency dynamics of ocean pH: A multi-ecosystem comparison.
434 PLoS ONE 6 (12), e28983. http://dx.doi.org/10.1371/journal.pone.0028983.

435 Howarth, R., Chan, F., Conley, D.J., Doney, S.C., Garnier, J., Marino, R., Billen, G.,
436 2011.Coupled biogeochemical cycles: eutrophication and hypoxia in temperate
437 es-tuaries and coastal marine ecosystems. Front. Ecol. Env. 9 (1), 18e26.

438 Hughes, R.N., 1989. A Functional Biology of Clonal Animals. Chapman and Hall,
439 London.

440 Hughes, R.N., 2005. Lessons in modularity: the evolutionary ecology of colonial
441 invertebrates. Scie. Mar. 69, 169-179.

442 IPCC, 2014. In: Pachauri, R.K., Meyer, L.A. (Eds.), Climate Change 2014: Synthesis
443 Report. Contribution of Working Groups I, II and III to the Fifth Assessment
444 Report of the Intergovernmental Panel on Climate Change. IPCC, Geneva,
445 Switzerland, p. 151.

446 Kawaguchi, S., Ishida, A., King, R., Raymond, B., Waller, N., Constable, A., Nicol, S.,
447 Wakita, M., Ishimatsu, A., 2013. Risk maps for Antarctic krill under projected
448 Southern Ocean acidification. Nat. Clim. Chan 3, 843-847.

449 Lombardi, C., Rodolfo-Metalpa, R., Cocito, S., Gambi, M.C., Taylor, P.D., 2011. Struc-
450 tural and geochemical alterations in the Mg calcite bryozoan Myriapora truncate
451 under elevated seawater pCO2 simulating ocean acidification. Mar. Ecol. Evol.
452 Persp 32, 211-222.

453 Lombardi, C., Cocito, S., Gambi, M.C., Taylor, P.D., 2015. Morphological plasticity in a
454 calcifying modular organism: evidence from an *in situ* transplant experiment in a

455 natural CO2 vent system. Roy. Soc. Open Scie 2, 140413. http://dx.doi.org/10.
456 1098/rsos.140413

457 Mackie, G.O., 1986. From aggregates to integrates: physiological aspects of modularity
458 in colonial animals. Phil. Trans. R. Soc. Lond. B 313, 175-196.

459 Mehrbach, C., Culberson, C.H., Hawley, J.E., Pytkowicz, R.M., 1973. Measurement
460 ofthe apparent dissociation constants of carbonic acid in seawater at atmospheric
461 pressure. Limn. Ocean. 18, 897-907.

462 Melatunan, S., Calosi, P., Rundle, S.D., Moody, A.J., Widdicombe, S., 2011. Exposure to
463 elevated temperature and PCO2 reduces respiration rate and energy status in the
464 periwinkle *Littorina littorea*. Phys. Biochem. Zool. Ecol. Evol. App 84, 583-594.

465 Moll, J.P.C., 1803. Eschara zoophytozoorum zeu phytozoorum ordine pulcherrima ac
466 notatu dignissima, pp. 1-70 pl.1-4, Vindobonae.

467 Palumbi, S.R., Jackson, J.B.C., 1983. Aging in modular organisms: ecology of zooid
468 senescence in *Steginoporella* sp. (Bryozoa; Cheilostomata). Biol. Bull. 164, 267-
469 278.

470 Pierrot, D.E., Lewis, E., Wallace, D.W.R., 2006. MS Excel Program Developed for CO2
471 System Calculations, ORNL/CDIAC- 105aCarbon Dioxide Information Analysis
472 Centre. Oak Ridge National Laboratory, US Department of Energy.

473 Pistevos, J.C.A., Calosi, P., Widdicombe, S., Bishop, J.D.D., 2011. Will variation among
474 genetic individuals influence species responses to global climate change? Oikos
475 120, 675-689.

476 Poluzzi, A., Sartori, R., 1974. In: Pouyet, S. (Ed.), Report on the Carbonate Mineralogy
477 of Bryozoans. Documents des Laboratoires de Géologie, Lyon.
478 Rodolfo-Metalpa, R., Lombardi, C., Cocito, S., Gambi, M.C., Taylor, P.D., 2010. Effects
479 of acidification and high temperature on the bryozoan *Myriapora truncata* at
480 natural CO2 vents. Mar. Ecol. Evol. Persp 31, 447-456.
481 Sanchez Alvarado, A., 2000. Regeneration in the metazoans: why does it happen?
482 Bioessays 22, 578-590.
483 Sanchez Alvarado, A., Tsonis, P.A., 2006. Bridging the regeneration gap: genetic
484 insights from diverse animal models. Nat. Rev. Gen. 7, 873-884.
485 Schram, J.B., McClintock, J.B., Angus, R.A., Lawrence, J.M., 2011. Regenerative ca-
486 pacity and biochemical composition of the sea star *Luidia clathrata* (Say)
487 (Echinodermata: Asteroidea) under conditions of near-future ocean acidification. J.
488 Mar. Biol. Ecol. 407, 266-274.
489 Stuefer, J.F., Gomez, S., Van Molken, T., 2004. Clonal integration beyond resource
490 sharing: implications for defence signalling and disease transmission in clonal plant
491 networks. Evol. Ecol. 18, 647-667.
492 Smith, A.M., Key, M.J., Gordon, D.P., 2006. Skeletal mineralogy of bryozoans:
493 taxonomic and temporal patterns. Earth- Scie. Rev. 78, 7-306.
494 Taylor, P.D., 2016. Competition between encrusters on marine hard substrates and
495 its fossil record. Palaeontology 59, 481-497.

Underwood, A.J., 1997. Experiments in Ecology: their Logical Design and Interpretation Using Analysis of Variance. Cambridge University Press, Cambridge.

Wood, H.L., Spicer, J.I., Lowe, D.M., Widdicombe, S., 2010. Interaction of ocean acidification and temperature; the high cost of survival in the brittlestar *Ophiura ophiura*. Mar. Biol. 157, 2001-2013.

Figures

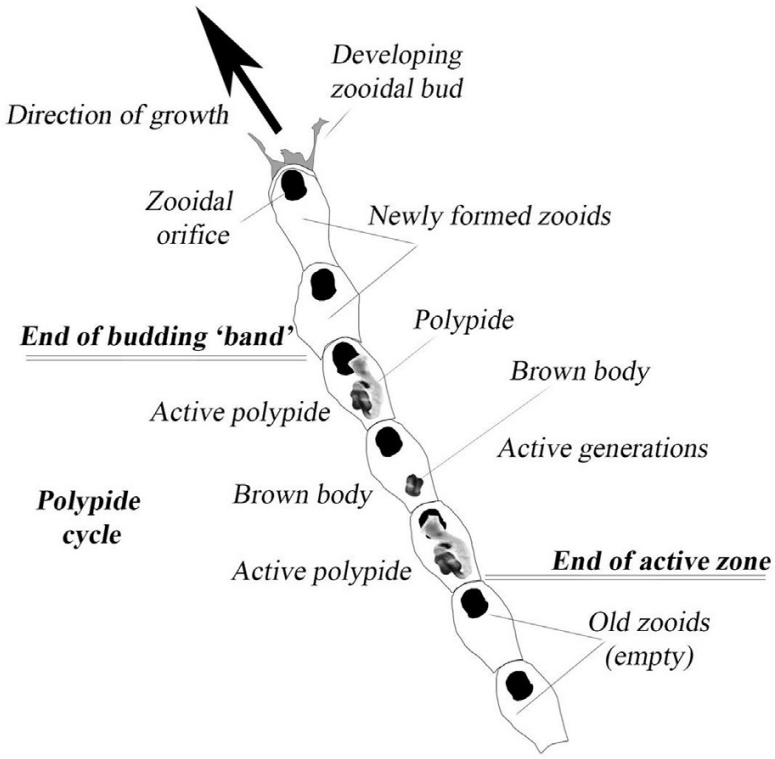

Fig. 1. Scheme of a zooidal linear series in *Cryptosula pallasiana.* Budding band e distal part of the colony e with developing bud and incomplete zooids (1st and 2nd generation). Active generations (3rd,4th and 5th) with alternations of active polypides and brown bodies (small grey masses) indicating polypide cycling. Arrow indicates the direction of growth.

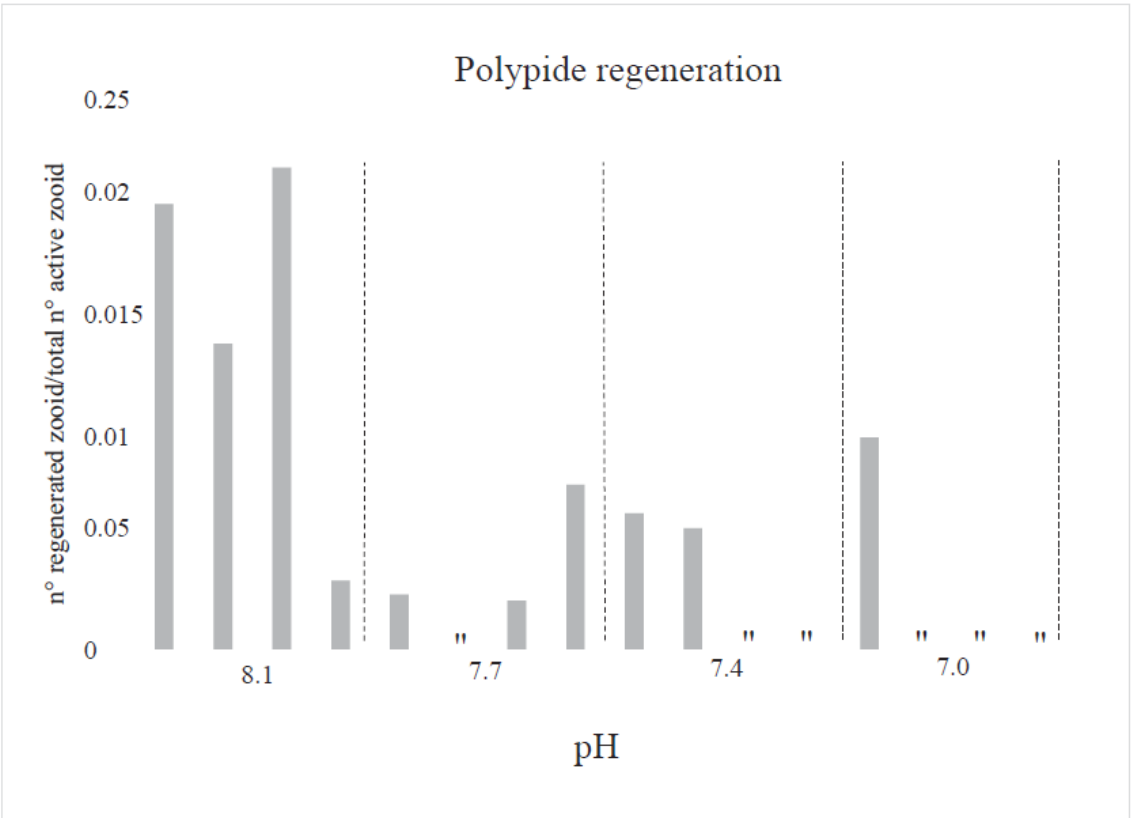

Fig. 2. The effect of four weeks exposure to lowering pH (pH 8.1, 7.7, 7.4 and 7.0) on polypide regeneration (regenerated zooids/total active zooids) within *Cryptosula pallasiana* colonies (n ¼ 4 samples per treatment). Symbol (“) indicates regeneration ¼ 0.

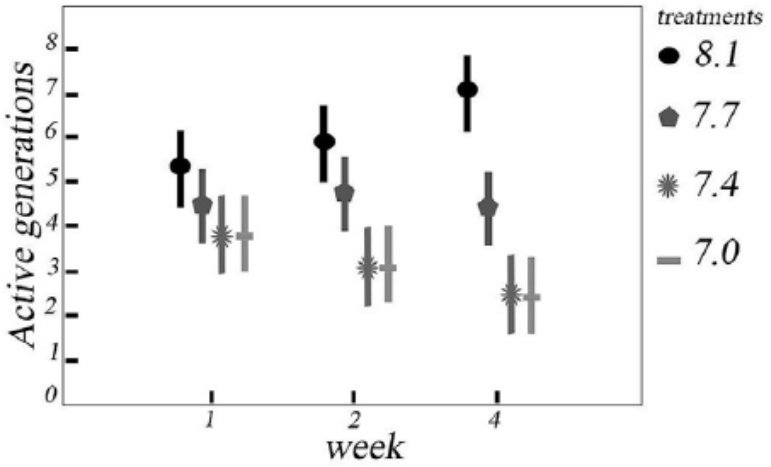

Fig. 3. Trend of active generations in *C. pallasiana* within each pH treatment (8.1, 7.7, 7.4, 7.0) through time (1, 2 and 4 weeks). Vertical bars denote 0.99 confidence intervals (n ¼ 4 samples per treatment).

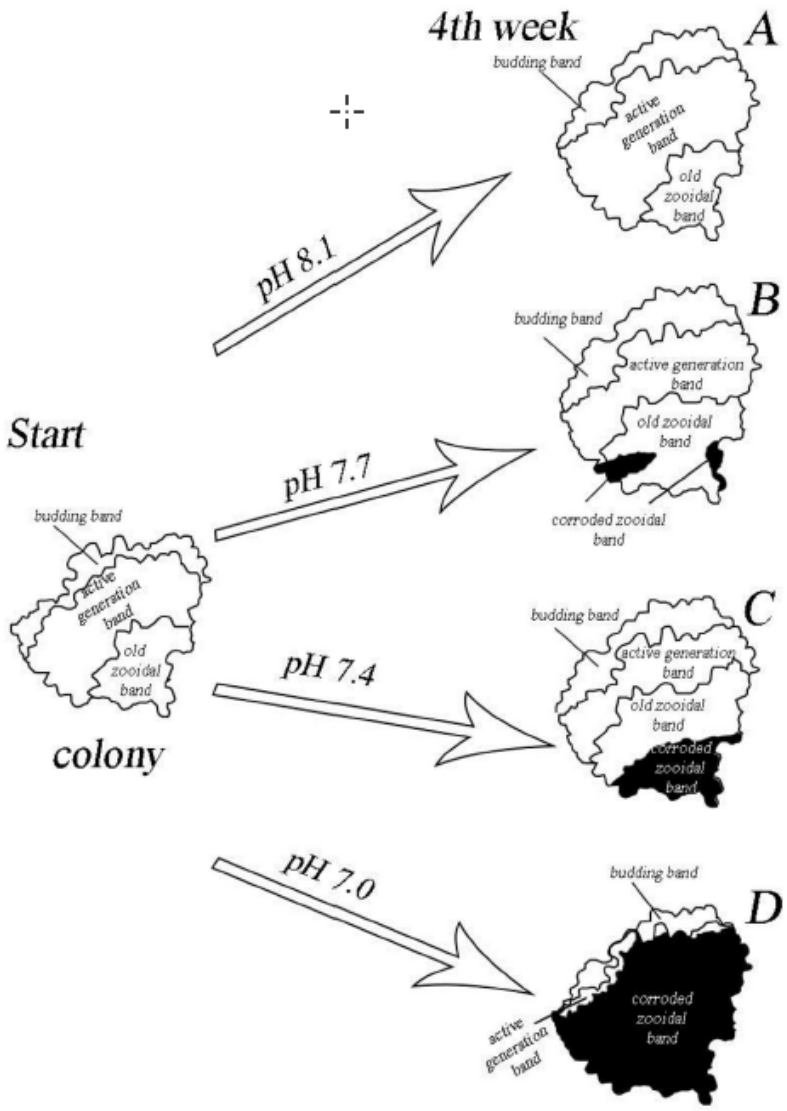

Fig. 4. Schematic representation of colony ontogenetic zonation. Budding band (BB), active generation band (AgB) and old/corroded zooid band (OB) through time (start-4 weeks) in *Cryptosula pallasiana* colonies grown under different pH conditions (A ¼ 8.1, B ¼ 7.7, C ¼ 7.4, D ¼ 7.0).

Tables

Table 1

Physical-chemical parameters of the different pH treatments (mean ± S.E.). Letters in superscripts represent statistical significant differences between treatments. Asterisks indicate parameters that have been calculated using CO2SYS.

	8.1	7.7	7.4	7.0
pH	8.15 ± 0.01^{a}	7.73 ± 0.02^{b}	7.42 ± 0.01^{c}	7.07 ± 0.01^{d}
Temp (°C)	15.12 ± 0.05^{a}	15.12 ± 0.05^{a}	15.12 ± 0.05^{a}	15.12 ± 0.05^{a}
Salinity	34.57 ± 0.25^{a}	34.57 ± 0.25^{a}	34.57 ± 0.25^{a}	34.57 ± 0.25^{a}
A_T (µmol Kg^{-1})	2.081 ± 0.031^{a}	2.078 ± 0.035^{a}	2.069 ± 0.034^{a}	2.058 ± 0.032^{a}
pCO_2 (µatm)*	381.6 ± 15.4^{a}	1118.9 ± 52.3^{b}	2314.1 ± 69.62^{c}	5223.87 ± 138.61^{d}
DIC (µmol Kg^{-1})*	1884.5 ± 31.5^{a}	2029.13 ± 34.76^{b}	2109.27 ± 36.77^{bc}	2231.59 ± 34.53^{c}
$[HCO_3^-]$ (µmol Kg^{-1})*	1731.7 ± 30.44^{a}	1927.5 ± 33.27^{b}	1993.1 ± 47.36^{b}	2023.2 ± 31.80^{b}
$[CO_3^{2-}]$ (µmol Kg^{-1})*	138.52 ± 3.45^{a}	59.91 ± 2.66^{b}	29.92 ± 0.39^{c}	13.76 ± 0.043^{d}
$\Omega_{calcite}$*	3.31 ± 0.08^{a}	1.43 ± 0.06^{b}	0.71 ± 0.01^{c}	0.033 ± 0.01^{d}
$\Omega_{aragonite}$*	2.12 ± 0.05^{a}	0.92 ± 0.04^{b}	0.46 ± 0.01^{c}	0.21 ± 0.01^{d}

Table 2

A Results of one-way ANOVA: the effect of pH (pH 8.1, 7.7, 7.4 and 7.0) on the mean ratio between regenerated zooids/total active zooids. Samples per pH ¼ 4. SNK: 8.1 > 7.7 > 7.4 > 7.1.

B. Results of GLM: the effect of duration of exposure (1, 2 and 4 weeks) and pH on active generations. Samples per pH ¼ 4. SNK test: 8.1 > 7.7 > 7.4 > 7.0. F: fixed factor, R: random factor.

*, ** ¼ significant differences.

A				
	df	MS	F	*p*
pH	3	0.06	4.93	0.02*
Error	12	0.02		
Levene		0.21	1.74	0.21

B				
	df	MS	F	p
pH (F)	3	44.81	11.13	<0.01**
Week (R)	2	0.81	0.20	0.82
pH x Week (R)	6	4.03	2.67	0.02
Error	79	1.50		
Levene		0.411	3.85	0.00

Table 3

Effect of time of exposure to different pH levels on the colony ontogenetic bands. Number of zooids per zooidal linear series (mean ± s.e.) characterizing the budding band (BB), active generation band (AgB) and old zooid band (OB) through time (start, after 1wk, 2wk, 3wk and 4wk) and across pHs. Linear series per colony: min 6- max10.

Time	pH											
	8.1			7.7			7.4			7.0		
	BB	AgB	OB	BB	AgB	OB	BB	AgB	OB	BB	AgB	OB
Start	2.00 ± 0.11	5.13 ± 0.29	2.00 ± 0.50	2.00 ± 0.00	5.00 ± 0.71	3.88 ± 0.61	2.00 ± 0.00	4.88 ± 0.35	3.00 ± 0.63	1.98 ± 0.54	4.63 ± 0.53	2.75 ± 0.25
1wk	1.92 ± 0.13	5.38 ± 0.26	2.88 ± 0.61	1.88 ± 0.13	5.50 ± 0.82	4.25 ± 0.73	1.96 ± 0.13	4.86 ± 0.38	3.71 ± 0.34	1.99 ± 0.01	4.88 ± 0.52	2.88 ± 0.23
2wk	2.00 ± 0.13	5.88 ± 0.35	4.63 ± 0.65	2.00 ± 0.00	4.75 ± 0.59	4.75 ± 0.62	2.00 ± 0.00	3.13 ± 0.29	4.75 ± 0.41	1.13 ± 0.35	3.13 ± 0.29	1.00 ± 0.50
3wk	1.89 ± 0.43	6.13 ± 0.44	4.00 ± 0.44	1.88 ± 0.13	4.38 ± 0.46	5.00 ± 0.38	1.00 ± 0.11	3.63 ± 0.26	2.63 ± 0.42	1.13 ± 0.29	2.50 ± 0.19	1.88 ± 0.35
4wk	1.93 ± 0.33	7.00 ± 0.35	4.86 ± 0.59	1.86 ± 0.13	4.43 ± 0.45	5.43 ± 0.40	0.75 ± 0.16	3.38 ± 0.19	2.25 ± 0.49	0.75 ± 0.25	2.13 ± 0.35	1.13 ± 0.29