Breakdown of coral colonial form under reduced pH conditions is initiated in polyps and mediated through apoptosis

Hagit Kvitt^{a,b,c}, Esti Kramarsky-Winter^d, Keren Maor-Landaw^e, Keren Zandbank^b, Ariel Kushmaro^d, Hanna Rosenfeld^c, Maoz Fine^{b,e}, and Dan Tchernov^{a,b,1}

^aMarine Biology Department, Leon H. Charney School of Marine Sciences, University of Haifa, Mount Carmel, Haifa 31905, Israel; ^bInteruniversity Institute for Marine Science, Eilat 88103, Israel; ^cIsrael Oceanographic and Limnological Research, National Center for Mariculture, Eilat 88112, Israel; ^dAvram and Stella Goldstein-Goren Department of Biotechnology Engineering, Ben-Gurion University, Beer-Sheva 8410501, Israel; and ^eThe Mina & Everard Goodman Faculty for Life Sciences, Bar-Ilan University, Ramat Gan 52900, Israel

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Certain stony corals can alternate between a calcifying colonial form and noncalcifying solitary polyps, supporting the hypothesis that corals have survived through geologic timescale periods of unfavorable calcification conditions. However, the mechanisms enabling this biological plasticity are yet to be identified. Here we show that incubation of two coral species (*Pocillopora damicornis* and *Oculina patagonica*) under reduced pH conditions (pH 7.2) simulating past ocean acidification induce tissue-specific apoptosis that leads to the dissociation of polyps from coenosarcs. This in turn leads to the breakdown of the coenosarc and, as a consequence, to loss of coloniality. Our data show that apoptosis is initiated in the polyps and that once dissociation between polyp and coenosarc terminates, apoptosis subsides. After reexposure of the resulting solitary polyps to normal pH (pH 8.2), both coral species regenerated coenosarc tissues and resumed calcification. These results indicate that regulation of coloniality is under the control of the polyp, the basic modular unit of the colony. A mechanistic explanation for several key evolutionarily important phenomena that occurred throughout coral evolution is proposed, including mechanisms that permitted species to survive the third tier of mass extinctions.

apoptosis | ocean acidification | corals

The origin of scleractinian corals preceded the onset of the Permian Triassic mass extinction, as indicated by phylogenetic analysis (1, 2). However, the first fossil record of scleractinian corals is evident only 10 Ma after the Permian Triassic mass extinction (i.e., “reef gap”). In addition, after the Triassic Jurassic extinction, a second “reef gap” is reported, extending for a period of at least 4 Ma (3). In light of this inconsistent fossil record, it was hypothesized that stony corals have the plasticity to alternate between soft bodies that do not leave fossil records and calcifying forms (4–6). This hypothesis was experimentally supported (7) by exposing the stony corals *Oculina patagonica* and *Madracis pharensis* to reduced pH conditions, such as are expected during periods of elevated CO₂ levels (8, 9). Accordingly, significant differential morphologies in both polyps (feeding, reproducing units) and coenosarcs (the tissue bridge between the polyps of the coral colony) were observed. Whereas the polyps elongated, the coenosarc tissues disintegrated, concomitant with rapid dissolution of the skeleton. On reacclimation to normal, present-day seawater conditions, the solitary polyps commenced calcifying and the coenosarc tissue reformed (7). These tissue-specific responses could denote that alternating between colonial and solitary forms is governed by controlled cellular processes, particularly programmed cell death (PCD) mechanisms that are essential in the maintenance of tissue homeostasis. One such PCD mechanism, apoptosis, is characterized by the orderly activation of caspases, a family of cysteine proteases (10). Caspases cleave a variety of cellular substrates and give rise to several

distinct characteristic morphologic features of apoptosis (10). Apoptosis has been remarkably well-conserved within metazoan phyla, both in terms of morphologic cellular features and in the repertoire of genes involved. The presence of appropriate cellular and molecular machinery in Anthozoa has been established (11); however, the involvement of this mechanism after stress events is still unclear (12–15). In corals, regulated, tissue-specific apoptosis after thermal stress has been experimentally established (14, 15), and its induction/reduction in the coral tissues matched the profiles of caspase-3 activity and caspase-3 gene expression (14). Under reduced pH conditions, changes in apoptosis-related gene expression, including up-regulation of caspase 3, were reported for the stony coral *Acropora millepora* (16). However, to date, this pathway has not been experimentally established in corals or any calcifying marine organisms under reduced pH conditions.

Results and Discussion

To study the differential tissue-specific changes occurring in stony corals after subjection to reduced pH conditions (pH 7.2), we incubated fragments of *Pocillopora damicornis* (from the Gulf of Eilat) and *O. patagonica* (from the Mediterranean Sea) in pH 7.2, and both morphologic and molecular changes were

Significance

Stony corals have survived through geologic timescale periods of unfavorable calcification conditions, including low-pH regimes, possibly by alternating between a calcifying colonial form and noncalcifying soft body solitary polyps. Although experimentally supported, the mechanisms enabling this biological plasticity are unidentified. We show that incubating two coral species under reduced pH conditions induced tissue-specific apoptosis that leads to loss of coloniality because of the dissociation of polyps (the basic modular units of the colony) from their connective tissue (coenosarc). Apoptosis was initiated in the polyps and subsided once the dissociation between polyps and coenosarc terminated. When conditions returned to normal, these solitary polyps initiated calcification and regenerated coenosarcs. These results indicate that regulation of coloniality is under the control of the polyp.

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¹To whom correspondence should be addressed. Email: dtchernov@univ.haifa.ac.il.

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monitored. Two to 4 wk after incubation in pH 7.2, gross morphologic changes were observed in both *P. damicornis* and *O. patagonica* (Fig. 1). In *P. damicornis*, after 3 wk, the coenosarc completely disintegrated (Fig. 1B) and the polyps detached from the skeleton (Fig. 1C and Fig. S1). The detached polyps showed no gross structural or morphologic anomalies, and their tentacles were regularly extended (Fig. 1C and Fig. S1). In *O. patagonica*, the onset of major changes in morphology occurred after 4 wk, when most of the coenosarc tissue disintegrated (Fig. 1F). The solitary polyps remained attached to the skeletal matrix, and their tentacles were fully extended for the duration of the experiment (Fig. 1G). In both coral species, although the coenosarc completely disintegrated, almost 100% of the polyps remained morphologically intact.

We used caspase activity measurements and transmission electron microscopy (TEM) to follow pH-induced, tissue-specific, cellular responses in the various tissue compartments of both coral species. We monitored these responses throughout the 7 wk of experimental incubation at pH 7.2 in tandem with the gross morphologic changes observed in colony shape. In both coral species, caspase activity increased significantly during weeks 2–4, parallel to the coenosarc's disintegration (Fig. 2). In polyps, this response appears to be constrained to the site of detachment from the coenosarc, as in both coral species, apoptotic cells in the polyp's body were observed mainly in the calicoblastic epidermis adjacent to the coenosarc, but not in the polyp's gastroderm (Fig. 3B and F). In *O. patagonica*, apoptotic cells were evident in tissues of gastrodermal and epidermal origin exclusively in the coenosarc (Fig. 3C). In *P. damicornis*, both apoptosis and necrosis were observed in coenosarcs (Fig. 3G). These findings suggest that both an abrupt and prolonged reduction of pH results in the induction and progression of site/tissue-specific apoptosis, resulting, in turn, in the dissociation of the polyps from coenosarcs and the demise of the latter. Interestingly, apoptotic cells were not observed in the calicoblastic epidermis of solitary polyps after the disintegration of the coenosarc (*O. patagonica*) or in polyps detached from the skeleton (*P. damicornis*) (Fig. 3D and H). Indeed, in *O. patagonica*, caspase activity declined to basal levels in the solitary polyps, concomitant with complete disintegration of the coenosarcs (Fig. 2B). Although caspase activity in the solitary polyps of *P. damicornis* could not be measured, the combined results (TEM and caspase activity in *O. patagonica* and TEM in *P. damicornis*) indicate that once the dissociation between polyps and coenosarc

terminates, apoptosis subsides, further suggesting that this highly controlled mechanism ensures the survival of the polyp fraction of the colony. Moreover, in *O. patagonica*, caspase activity at pH 7.2 initially increases significantly in the polyps and is consistently higher in polyps than in the coenosarc (Fig. 2B), further indicating that the apoptotic cascade leading to the disruption of the colonial form is initiated and controlled by the polyps.

According to the latest report on ocean acidification, oceanic pH is not expected to decrease during this century to values as low as pH 7.2, as predicted by climate change projections (9, 17, 18). However, pH 7.2–7.6 is relevant for coral evolution, as it has been that low before the Permian Triassic mass extinction event and through the Cretaceous (9), as well as in the early Eocene (8). Interestingly, throughout most of geologic history, corals were under a relatively low pH regime (compared with contemporary pH), with the exception of most of the Cenozoic era (9). It must be noted that our experiments did not include countering the low pH with higher Ca concentrations, simulating past conditions; thus, we cannot claim that the response we have reported is a mechanistic solution to any past event. Attenuation in calcification may have resulted from unfavorable water chemistry [low Ω_{arg} (5, 18–22)] or as part of a set of physiologic changes associated with extremely low pH (7). In the contemporary ocean, the ongoing acidification has been causing a significant decrease in the concentration of carbonate ions, compromising the ability of multiple taxa of marine calcifiers to precipitate calcium carbonate (23). Similar indications originate from multiple laboratory experiments under high- CO_2 conditions, with a wide variety of calcifying marine species, including corals, that exhibit reduced calcification and growth rates (17, 24). Under these conditions, corals must invest more energy to maintain homeostasis (25) and to continue depositing skeleton (26). Therefore, the occurrence of increased apoptosis resulting in tissue loss of the coenosarc in pH-stressed corals could be a mechanism whereby colonial corals rid themselves of energetically costly processes (e.g., calcification) and tissues.

Intercellular acidification has been reported to be an important controller of PCD in mammals, affecting caspase activation, the expression of anti- and proapoptotic genes of the Bcl-2 family, intracellular Ca^{2+} homeostasis, and the activation of low-pH-dependent endonucleases (27, 28). It was recently reported that *Stylophora pistillata* is able to regulate intracellular pH in its calcifying tissue (calicoblastic epithelium), even under

Fig. 1. Loss of coral colonial form at pH 7.2, degradation of the coenosarc, and its reversibility. Coral fragments of *P. damicornis* and *O. patagonica* were placed together in control (pH 8.2) and treatment (pH 7.2) aquaria. (A and E) Control colonies at pH 8.2. (B and F) Coenosarc degradation after 2–4 wk incubation in pH 7.2. (C and G) Intact solitary polyps after 4–7 wk incubation in pH 7.2. (D and H) Solitary polyps showing regeneration of coenosarc tissue and return to calcification after being transferred back to control conditions (pH 8.2). cal, calcification; co, coenosarc; p, polyp.

Fig. 2. Caspase activity is significantly increased within 2–4 wk at pH 7.2. Coral fragments of *P. damicornis* and *O. patagonica* were placed together in control (pH 8.2) and treatment (pH 7.2) aquaria and sampled over the indicated time points ($n = 12$ per coral species per time point). (A) Caspase activity in *P. damicornis* at pH 7.2 (dark gray) compared with controls at pH 8.2 (light gray). (B) Caspase activity in *O. patagonica* polyps (dark gray) and coenosarc tissues (checked dark gray) at pH 7.2 compared with controls at pH 8.2 (polyps, light gray; coenosarc tissues, checked light gray). Results are expressed as means $\pm$ SE of independent extractions from distinct fragments. Caspase activity was measured as DEVD cleavage (in relative fluorescence units, RFU) and expressed as RFU/ μ g protein $^{-1}$ h $^{-1}$. Values were tested by one-way ANOVA and a t test. Asterisks indicate significant differences between pH 7.2 and pH 8.2 of the same time point and tissue. * $P < 0.05$ and ** $P < 0.01$.

reduced pH conditions (26). The extent of this capacity remains to be ascertained for other tissues of *S. pistillata* and other coral species. The latter report could explain the discrepancy of the tissue viability between the polyp and the coenosarc as a reflection of the buffering capacity of the two areas; that is, it might be that only the tissue that can regulate intracellular pH will survive this stress.

The induction of calicoblastic epidermal-specific apoptosis (Fig. 3F) resulting in detachment of viable polyps from *P. damicornis* observed under unfavorable pH conditions (Fig. 1C and Fig. S1) suggests this PCD response to environmental stress is carefully controlled. This could provide a mechanistic explanation for the reported “programmed release” of single polyps including or

devoid of skeleton after environmental stress (29–32). Furthermore, in the calicoblastic epithelium of *P. damicornis* polyps after 2–3 wk at pH 7.2, the desmocytes attaching the polyp to the skeleton (Fig. 4A) gradually changed morphology. The desmosomes disappeared (Fig. 4B) concomitant with the appearance of cilia and microvilli (Fig. 4C) protruding from the calicoblastic epithelium into the area of detachment. The development of cilia and microvilli in polyps detaching from the skeleton facilitates their motility, as well as the ensuing reattachment to the substrate, further supporting the hypothesis of a controlled nature of this stress response. In *O. patagonica*, which does not release polyps under an unfavorable pH regime (Fig. 1G), all the desmocytes observed in the base of the polyps remained morphologically intact throughout the experiment (Fig. 4D).

In both *P. damicornis* and *O. patagonica*, the detached/solitary polyps reformed coenosarcs and started recalcifying when placed again in water at pH 8.2 (Fig. 1H). In *O. patagonica*, the solitary polyps reformed a colony (Fig. 1D), as reported previously (7). This provides important evidence for the reversibility of this process and suggests this plasticity might enable coral species to shift from a colonial form to a solitary one, and vice versa. This mechanism of alternating phenotypes ensures an increased fitness. Moreover, these phenotypic changes may enable some coral species to physically move from an unfavorably stressed local environment to a more favorable one. On the basis of our results (Fig. 5), we suggest a “two-stage response” model explaining loss/gain of coloniality in coral species that respond to reduced pH conditions via disintegration of the coenosarc and survival of the polyps. These include (i) the onset of PCD mechanisms, resulting in the dissociation of the polyps from the coenosarcs and disintegration of the latter, and (ii) the subsiding of PCD concomitant with loss of the colonial form and acclimation of the solitary polyps to the chronic lower pH conditions. When the solitary polyps are returned to normal pH conditions, *O. patagonica* polyps revert to the colonial form, whereas in *P. damicornis* polyps, coenosarc formation and resumption of calcification could represent the onset of a return to the colonial form. A similar controlled cellular response was also reported in corals that withstood thermal stress and bleaching (14). This response included rapid induction of tissue-specific apoptosis with the onset of thermal stress, subsiding concomitant with the

Fig. 3. TEM of the cellular changes observed in *P. damicornis* and *O. patagonica* at pH 7.2. (A and E) Cells in coenosarc tissues of controls (pH 8.2) showing nuclei with dispersed, lightly stained nuclear chromatin. (B, C, F, and G) After 2–4 wk incubation in pH 7.2, apoptotic cells, characterized by nuclear chromatin forming crescent-like caps at the periphery of the nuclei, were observed in calicoblastic epidermal cells of polyps at the point of attachment to coenosarcs (B and F) and in coenosarcs tissue (C and G). (G) Necrotic cells in coenosarc of *P. damicornis*, characterized by ruptured cell membranes with little or no evidence of cytoplasm or organelles remaining. (D and H) Cells in the calicoblastic epidermis of solitary *O. patagonica* and detached *P. damicornis* polyps at pH 7.2 showing normal nuclei. apo, apoptosis; n, nucleus; nec, necrosis.

Fig. 4. TEM of the morphology of the cells that anchor the calicoblastic epithelium to the skeleton (desmocytes) in the calicoblastic epithelium of solitary polyps at pH 7.2. (A) In *P. damicornis*, desmocytes still attached to the skeleton show intact desmosomes (the cellular protrusions that attach the desmocytes to the skeleton) and tonofibrillae (a cytoplasmic bundle of fine filaments that converge at desmosomes). (B and C) In *P. damicornis* in the area of detachment, disappearance of desmosomes (B) is occurring concomitantly with the appearance of cilia and microvilli (C). (D) Desmocytes of *O. patagonica* remain morphologically intact. ci, cilia; ds, desmosomes; mi, microvilli; tf, tonofibrillae.

acclimation of the coral to the chronic thermal stress and the breakdown of symbiosis (bleaching). Moreover, when these corals were returned to ambient temperature, symbiosis was reestablished and the coral reverted to its original phenotype (14). In contrast to thermal stress, where the symbionts could be harmful to the coral host (14), under reduced pH conditions, the symbionts apparently remain intact and the symbiosis is maintained (7). Indeed, apoptosis in both thermal stress and reduced pH conditions seems to provide the corals with an option to rid themselves rapidly of suboptimal, and sometimes also harmful, symbionts (14), or of energetically costly processes (e.g.,

calcification) and tissues, respectively. Therefore, controlled apoptosis provides an essential mechanism enabling the coral to respond to changing environmental conditions, emphasizing the importance of this process in these sessile, simple metazoans.

Our results could provide a mechanistic model explaining the biological plasticity behind coloniality gain/loss in corals (7), which is suggested to have occurred repeatedly at least six times throughout the history of *Scleractinia* (33). This may further elucidate possible mechanisms of survival after mass extinction events found in corals over the eons (1, 2, 5, 6), providing new insight into mechanisms occurring in nature's discontinuities (i.e., the third tier in "the paradox of the first tier") (34). Hence, studies of mechanisms involved in the response of corals to changing environmental conditions may lead to better understanding of their evolution, as well as to their future under global climate change.

Materials and Methods

Collection and Maintenance of Corals. Four colonies each of *P. damicornis* (Red Sea, Israel) and *O. patagonica* (Mediterranean, Israel) were fragmented, and the fragments were acclimated for a period of 2 wk in a single aquarium. The fragments were then divided evenly between six aquaria, resulting in each aquarium containing fragments from each genotype of each species. Subsequently, in three aquaria, carbonate chemistry was manipulated by bubbling with CO₂ to reach pH 7.2_{NBS} (NBS, National Bureau of Standards) (35). The other three aquaria were kept at ambient seawater pH (8.2) as controls. Temperature and light intensity were regulated as previously described (35). Once a week, one fragment from each colony from both species from each aquarium was sampled ($n = 12$ per each time point, treatment, and coral species). Coral fragments were sampled at 1–3 wk (*P. damicornis*) and 1–7 wk (*O. patagonica*). For TEM, one fragment from each coral species was sampled per aquarium per treatment per sampling time point.

Tissue Extractions. Tissue from individual fragments was extracted and processed as described previously (14). In *P. damicornis*, the small size of polyps prevented their separation from coenosarc tissue; therefore, total animal tissue was removed off the skeleton (14). Also, because of the small size of *P. damicornis* polyps and the fact that they detach from the skeleton, a sufficient amount of protein for the caspase activity assay could not be extracted. In *O. patagonica*, the coenosarc tissue was removed off the skeleton with a fine-tooth brush (14), leaving only polyp tissues that were then extracted using air pressure.

Caspase Assay. Caspase activity was assayed fluorometrically from each fragment/tissue, using the specific substrate Ac-DEVD-AFC (*N*-acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethylcoumarin) (Calbiochem), as described (15). Caspase activity was expressed as relative fluorescence units/ μ g protein⁻¹·h⁻¹.

Statistical Analysis. Statistical significance of caspase activity between all sampling points was tested using one-way ANOVA and a *t* test (14). Analysis was undertaken with JMP (ver. 7.0.1) statistical software (SAS Institute Inc.) (14). Data are presented as the mean $\pm$ SE of independent measurements

Fig. 5. A schematic representation of the results outlining the involvement of apoptosis in loss of coloniality in corals under reduced pH conditions. A "two-stage" response: (i) the onset of apoptosis in the calicoblastic epidermis resulting in the dissociation of the polyps from the coenosarc tissue and its degradation, (ii) subsiding apoptosis, concomitant with loss of colonial form and acclimation of the solitary polyps to chronic reduced pH conditions. When exposed to normal pH, the solitary polyps of both coral species reform coenosarcs and start calcifying. In *O. patagonica*, the solitary polyps revert to their original colonial form. In *P. damicornis*, coenosarc formation and resumption of calcification could represent the onset of a return to the colonial form.

from distinct coral fragments and are compared with controls of the same time point and tissue (14).

TEM. Coral fragments were fixed individually in 2.5% (vol/vol) glutaraldehyde in filtered sea water (FSW; pH 8.2) for 8 h at 4 °C, decalcified using 20% (wt/vol) EDTA in FSW (pH 8.2), and then stored in 0.5% glutaraldehyde in FSW at 4 °C. Fragments were postfixed in osmium tetroxide, dehydrated, and embedded in Araldite. Triplicate sections from representative stages (i.e., start of coenosarc disintegration, midend of coenosarc disintegration, and solitary polyps) and

controls were sectioned, using an ultramicrotome and mounted on 300 mesh copper grids. From each sample, the whole depth of the tissue was examined. The ultrathin sections were stained with lead citrate and examined using a JEOL JEM-1230 TEM at 80 kV. Images were taken using TVIPS TemCam-F214.

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