Vol. 460: 277–287, 2012
doi: 10.3354/meps09776

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published July 24

REVIEW

Zooxanthellae that open calcium channels: implications for reef corals

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ABSTRACT: Toxins that open cell membrane calcium channels have been found in the dinoflagellate genus *Symbiodinium*, and likely occur in most zooxanthellae. I used published observations to examine some potentially far-reaching consequences to reef corals. Algal toxins may stimulate coral calcification by opening Ca^{2+} channels on the calcifying ectoderm. The coral discharges the resulting protons ($Ca^{2+} + HCO_3^- \rightarrow CaCO_3 + H^+$) into its coelenteron cavity, where they improve algal bicarbonate and nutrient assimilation. Coupling calcification with autotrophic physiologies contributes to the success of highly calcareous zooxanthellar symbioses, and to their associations with nutrient-poor tropical waters. Nutrient shortages freeze zooxanthellae in the G1 phase of the cell cycle. Dinoflagellates are often most toxic at such times, perhaps because toxins modulate their nuclear mix of cations, to control DNA conformation and activity. Increased Ca^{2+} influx into host cells disrupts cell adhesion and induces apoptosis. Zooxanthellae assimilate host nutrients, complete G1, divide, and disperse to new hosts. Nutrient shortages associate with high sea surface temperatures (SST), producing correlations between SST, calcification, and algal exit. Zooxanthellae proliferate when nutrients are abundant, but when nutrients later disappear, usually as SST warms, toxins and the departure of over-abundant zooxanthellae potentially overwhelm the coral and cause coral bleaching.

KEY WORDS: Toxin · Coral · Polyketide · *Symbiodinium* · Calcification · Photosynthesis · Bleaching

INTRODUCTION

Several 'bioactive compounds' or 'toxins' that open membrane Ca^{2+} channels have been discovered in the dinoflagellate genus *Symbiodinium*. A given strain of algae may produce several structurally related toxins. Zooxanthellatoxins A and B (A: $C_{140}H_{232}O_{57}NS$) and symbiodinolide ($C_{137}H_{232}O_{57}NS$) were found in zooxanthellae strain Y-6, likely clade A2, from the acoel flatworm *Amphiscolops* (Nakamura et al. 1993, Kita et al. 2007). Clade A1 algae from a Hawaiian tidepool make zooxanthellamides C1-5 ($C_{128}H_{220}O_{53}N_2S_2$; Onodera et al. 2005). Zooxanthellamide-D ($C_{54}H_{83}O_{19}N$) occurs in clade B algae from the jellyfish *Cassiopeia* (Fukatsu et al. 2007). Although the first of these toxins were discovered nearly 20 yr ago, little is known about how zooxanthellae use them. This article provides a roadmap for experiments.

Most of these alcohol-soluble polyketides consist of long continuous looped carbon chains, about 9 nm long, decorated with alcohols and ketones, plus bis-epoxides in the zooxanthellatoxins and symbiodinolide. Zooxanthellatoxins A and B contract mammalian muscle tissues at 0.7 µM (Nakamura et al. 1993), and symbiodinolide opens mammalian N-type Ca^{2+} channels at 7 nM and immediately ruptures host cells at 2.5 µM (Kita et al. 2007). *Symbiodinium* toxins

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are, however, much weaker than the Ca^{2+} influx agonist maitotoxin, which may be the most potent dinoflagellate toxin.

Coral symbionts have not yet been examined for toxins, but all analyzed strains of *Symbiodinium* apparently make Ca^{2+} channel openers, so coral symbionts likely do also. *Symbiodinium* furthermore retains the specialized machinery for making elaborate polyketides despite being a small dinoflagellate with the smallest known dinoflagellate genome (LaJeunesse et al. 2005).

Algal toxins appear abundant enough to affect the host coral. Cultured zooxanthellae strain Y-6 contains 75 and 40 µM of zooxanthellatoxins A and B, plus 76 µM symbiodinolide (Nakamura et al. 1993, Kita et al. 2007). These values may not be maxima, due to incomplete yield on toxin extractions, and variable toxin levels in the algae. *Symbiodinium* can occupy most of the volume of infected cells and reaches densities of millions of cells cm^{-2} in the coral endoderm.

Ca^{2+} channel openers can be potent physiological modulators. Eukaryotic cells maintain sub-micromolar levels of cytosolic free Ca^{2+}. Small increases in intracellular Ca^{2+} affect many cellular functions, including motility, secretion, exocytosis, transcription, cell adhesion, apoptosis, dehydrogenation reactions, and immunological responses including the generation of reactive oxygen species (ROS) and nitric oxide (NO; Clapham 2007). Intracellular parasites and symbionts often hijack the regulation of host cell Ca^{2+}, and *Symbiodinium* may do likewise (Fang et al. 1998, Sawyer & Muscatine 2001, DeSalvo et al. 2008, Kita et al. 2010, Yuyama et al. 2011). Ca^{2+} channel openers provide a mechanism. Algal colonization presumably succeeds best in hosts with compatible physiologies, especially if toxins suppress harmful host physiologies or stimulate beneficial ones.

Many of *Symbiodinium*'s prominent hosts are highly calcareous, including foraminifera, sponges, giant clams, and corals. Symbiotic corals generally calcify faster than non-symbiotic corals, especially during the daytime (e.g. Gattuso et al. 1999). Opening Ca^{2+} channels on the coral's calcifying ectoderm would stimulate calcification more directly than feeding the coral and raising pH and O_2 levels. Calcification generates protons ($Ca^{2+} + HCO_3^- \rightarrow CaCO_3 + H^+$) which the coral discharges into its semi-enclosed coelenteron cavity. That potentially improves algal HCO_3^- and nutrient uptake (Fig. 1). The following

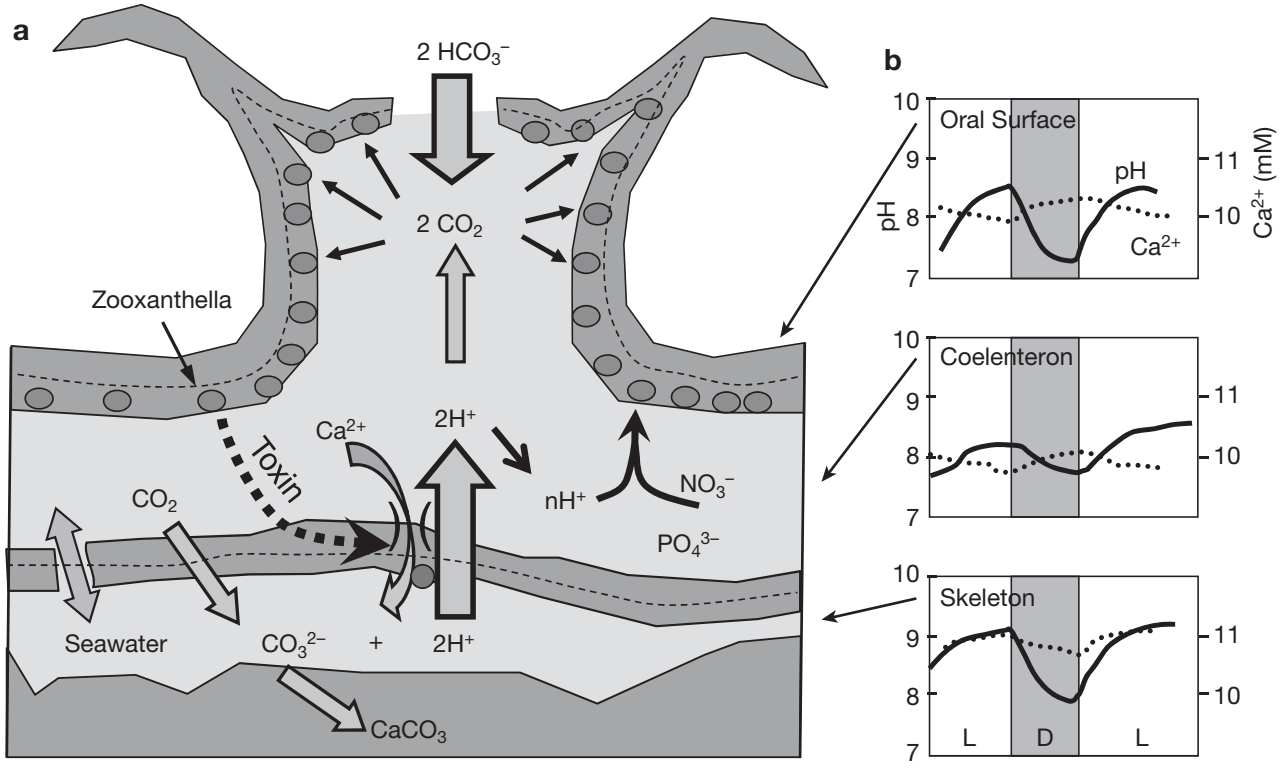

Fig. 1. Coral physiological model. (a) Coral calcification generates protons, which the coral discharges into its coelenteron cavity. Zooxanthellae inhabit adjacent cells of the oral endoderm, and potentially benefit from improved CO_2 and nutrient uptake resulting from this proton discharge. (b) pH and Ca^{2+} measured above the coral, in the coelenteron, and at the calcification site, in light (L) and dark (D), based on Al-Horani et al. (2003a,b). Ries (2011) reported pH values exceeding 10 at the calcification site

sections explore these ideas. Later sections explore how algal toxins may contribute to algal dispersal, coral bleaching, and algal cell cycles.

CALCIFICATION AND PHOTOSYNTHESIS

Corals bring seawater into the calcification site (Bentov et al. 2009, Tambutté et al. 2012), and apparently raise its [Ca^{2+}] and pH through $Ca^{2+}/2H^+$ exchange pumping by Ca^{2+} ATPase (Niggli et al. 1982, Dixon & Haynes 1989). [Ca^{2+}] increases <10%, while pH rises 1 to 2 pH units (Al-Horani et al. 2003a,b, Ries 2011; Fig. 1b bottom panel). CO_2 diffuses into this alkaline fluid and reacts to produce $CO_3^=$ (McConnaughey 1989, 2003). This raises the [Ca^{2+}] [CO_3^{2-}] ion product and speeds calcification (Cohen & McConnaughey 2003).

Corals have abundant Ca^{2+} channels (Zoccola et al. 1999), which probably concentrate on the coral's calcifying ectoderm, along with Ca^{2+} ATPase (Zoccola et al. 2004). This localization of Ca^{2+} channels may allow zooxanthellae to selectively stimulate coral calcification.

The coral discharges the protons from calcification into its coelenteron cavity. There they react with HCO_3^- to produce CO_2, which is captured by zooxanthellae in the surrounding endoderm (Fig. 1a). CO_2 levels below ambient (~10 µM) strongly inhibit photosynthesis in freshly isolated zooxanthellae (Leggat et al. 2002; Fig. 2a). High coral photosynthetic rates and mild coelenteron pH values (Al-Horani et al. 2003a,b; Fig. 1b, middle panel) suggest that the protons from calcification largely counteract photosynthetic alkalinization and CO_2 depletion.

Fig. 2b estimates how calcification and photosynthesis affect seawater CO_2 and pH. Fig. 2c estimates carboxylation efficiency (%Vmax), based on kinetics in Fig. 2a. The shaded diagonal arrows depict a 1.3 ratio of calcification to net photosynthesis (C:P). This was the average for several corals examined by Gattuso et al. (1999), and for corals not supplemented with nutrients by Tanaka et al. (2007; see Fig. 4c). Photosynthetic removal of 30% of the dissolved inorganic carbon (DIC) without calcification raises pH from 8.0 to 8.8, reduces CO_2 from 10 to 1 µM, and reduces carboxylation efficiency to 8%Vmax. Yet the same amount of photosynthesis at C:P = 1.3 reduces CO_2 only to 5 µM, and carboxylation efficiency to 30% Vmax. In this extreme example, calcification triples photosynthetic efficiency.

Coral calcification is generally considered a weak photosynthetic stimulus (Tanaka et al. 2007). Most experiments minimize CO_2 stress, however, and that minimizes the stimulus. Gattuso et al. (2000) also showed that reduced-Ca^{2+} seawater prevents net calcification without inhibiting photosynthesis. These experiments probably did not stop the proton transport into the coelenteron. Finally, branching and foliose corals calcify fastest in their apical polyps, and

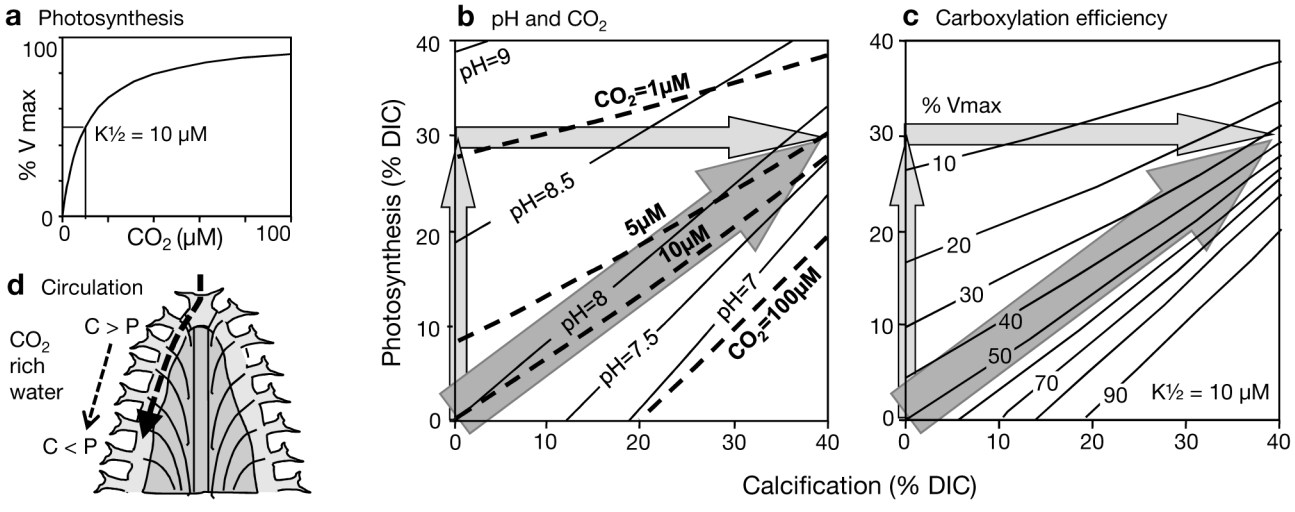

Fig. 2. Photosynthesis, calcification, and CO_2. (a) Photosynthetic kinetics of freshly isolated zooxanthellae (Leggat et al. 2002). Photosynthesis is half saturated at ~10 µM CO_2, which is close to ambient CO_2 levels. (b) pH (solid lines) and CO_2 (heavy dashed lines) in warm seawater, subject to calcification and photosynthesis (*x*- and *y*-axes, as % of total dissolved inorganic carbon, DIC). Shaded diagonal arrow depicts 1.3 ratio of calcification to photosynthesis. Seawater initially contains 10 µM CO_2 at pH 8. (c) Carboxylation efficiency (% Vmax), calculated from CO_2 levels from (a), and photosynthetic kinetics of Leggat et al. (2002). (d) Coelenteron water circulation hypothesized for branching coral, which brings CO_2-rich water from rapidly calcifying branch tips to photosynthetic later polyps, C = Calcification, P = Photosynthesis

photosynthesize mainly in their lateral polyps (Fang et al. 1989). For calcification to stimulate photosynthesis, the coral would need to transport CO_2-rich water from the apical to the lateral polyps. In support of this idea, Gladfelter (1983) detected inward ciliary currents at the branch tips of *Acropora* (Fig. 2d).

Summarizing, coral calcification likely stimulates photosynthesis when CO_2 is depleted, as might occur under stagnant conditions, with massive and encrusting morphologies, and when non-calcareous algae lower ambient CO_2 levels. Calcification may also improve photosynthesis in dim light, judging from the prevalence of calcification among deep-living algae (Aponte & Ballantine 2001).

NUTRIENT UPTAKE

Corals inhabit some of the earth's most oligotrophic environments (Fig. 3a). Thermal stratification impedes nutrient inputs to surface waters, so nutrients correlate negatively with temperature (Fig. 3b,c). Turbulence and thermocline shoaling bring nutrients to the surface and promote the growth of zooxanthellae. Conversely, sunny, warm, calm conditions stratify the water column and intensify surface nutrient depletion.

Nutrient scarcity often accelerates calcification. Corals calcify faster at high sea surface temperatures (McNeil et al. 2004, Silverman et al. 2006, Cooper et al. 2012), when nutrients are generally depleted. Corals also calcify faster without nutrient supplements (Fig. 4) (Kinsey & Davies 1979, Marubini & Davies 1996, Marubini & Thake 1999, Ferrier-Pagès et al. 2000, 2001, Renegar & Riegl 2005, Holcomb et al. 2010). Even in contrary examples (Tanaka et al. 2007), nutrient supplements increase photosynthesis and biomass more than calcification. Relative to photosynthesis and biomass, nutrient shortages induce faster calcification (Fig. 4c).

Connections between calcification and nutrient uptake are also apparent in various plants. Calcareous reef algae show enhanced phosphate uptake (Demes et al. 2010). Coccolithophorids often dominate the summertime plankton, when nutrients are depleted, and calcify faster when nutrients are scarce (Paasche & Brubak 1994). Calcareous plants dominate in oligotrophic alkaline, Ca^{2+}-rich lakes (McConnaughey

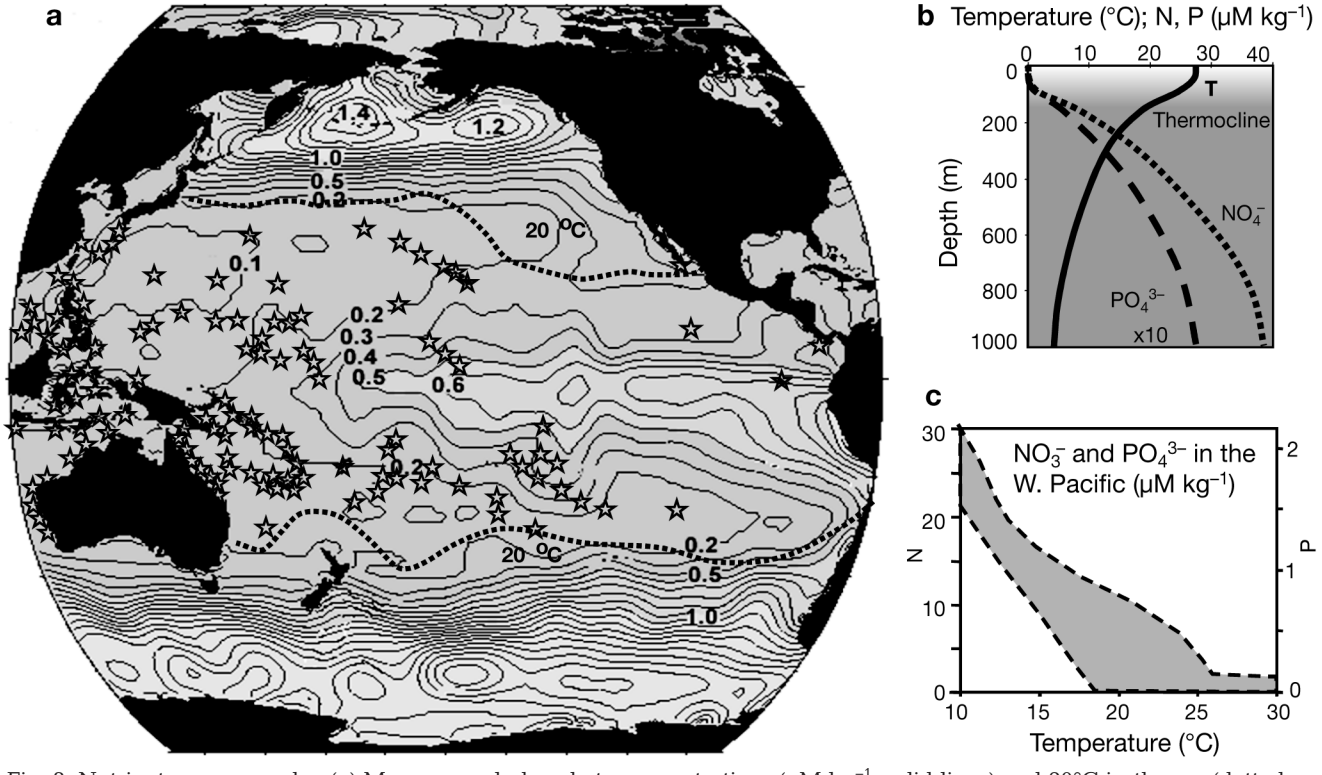

Fig. 3. Nutrient oceanography. (a) Mean annual phosphate concentrations (µM kg^{-1}, solid lines) and 20°C isotherms (dotted lines) at 10 m depth in the Pacific (based on Talley 2007). Reefs (☆) are most developed where nutrients are scarce. (b) Typical vertical profiles of nitrate, phosphate, and temperature in the western Pacific. (c) Envelope of typical nitrate and phosphate concentrations plotted against temperature for the western Pacific

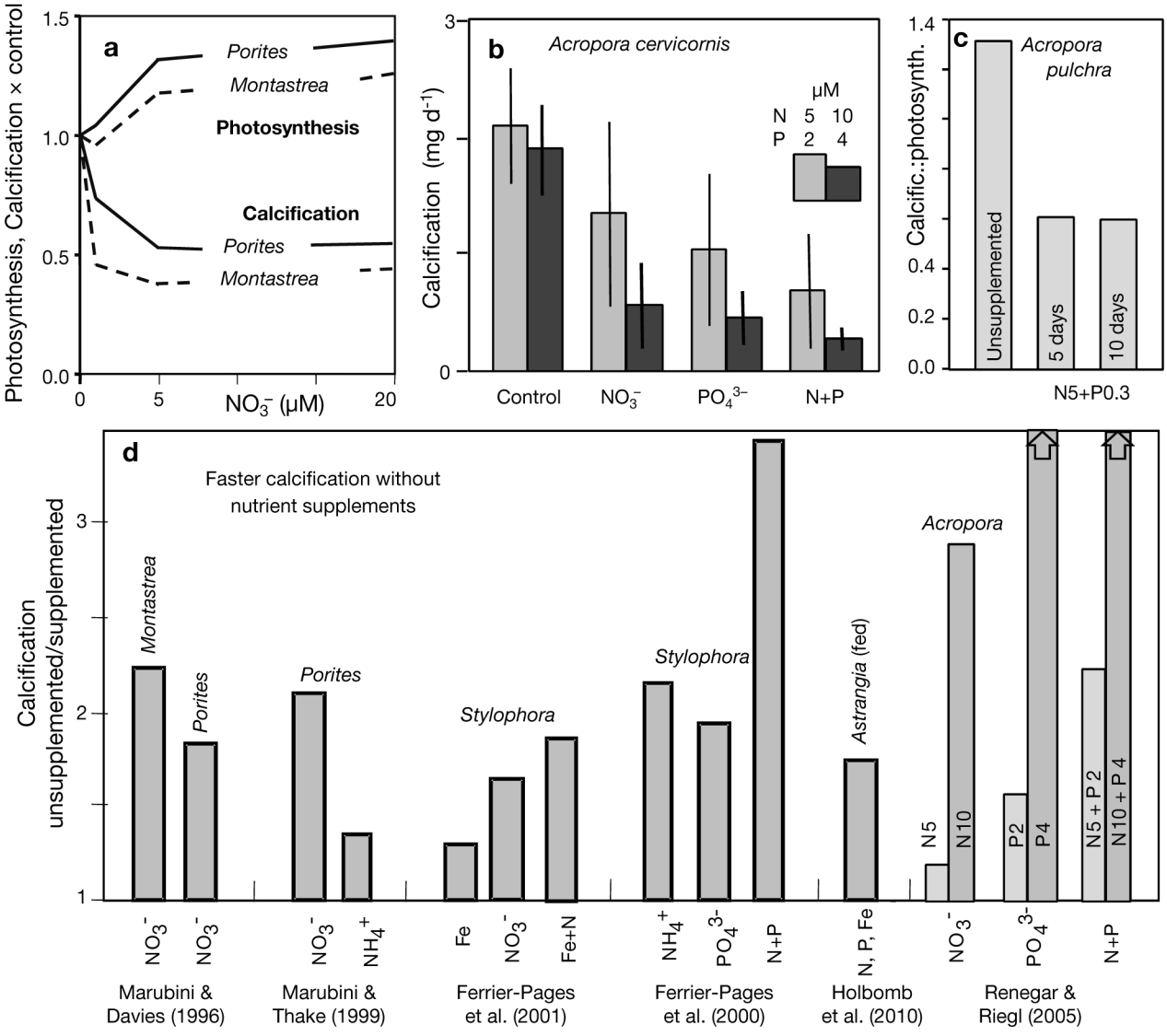

Fig. 4. Faster calcification during nutrient scarcity. (a) Nitrate supplementation stimulated photosynthesis but inhibited calcification in an experiment by Marubini & Davies (1996). (b) N or P supplementation suppresses calcification in *Acropora* (Renegar & Riegl 2005). (c) N+P supplementation (5 μM nitrate and 0.3 μM phosphate) reduces the ratio of calcification to photosynthesis in *Acropora* (Tanaka et al. 2007). (d) Faster calcification without nutrient supplements (after several authors)

et al. 1994). Land plants and their symbiotic fungi often calcify in nutrient-poor alkaline soils (Jaillard et al. 1991, Khan 1995). Calcium carbonates and oxalates sometimes completely fill the vacuoles of root cortical cells, close to the sub-apical region of proton export and absorptive root hairs (Fig. 5a).

The physiology linking proton export to nutrient uptake has been most studied in land plants. Proton secretion leaches nutrients from the soil, and strengthens the cell membrane electrical gradient. That drives the electrophoretic uptake of cations, including Fe^{2+} and NH_4^+. NO_3^- and PO_4^{3-} may be taken up in (nH^+, anion) combinations (Sakano 1990, Ullrich & Novacky 1990, Mistrik & Ullrich 1996, Blatt et al. 1997, Wollenweber 1997, Zvyagilskaya et al. 2001). Lowering external pH by 1 unit theoretically improves the thermodynamics of NO_3^- and PO_4^{3-} uptake at least 10-fold (Figs. 5b,c). Fig. 1 applies this idea to corals.

Algal toxins may speed coral calcification during nutrient shortages. Nutrient shortages freeze zooxanthellae in the G1 phase of the cell cycle (Muscatine et al. 1989, Muller-Parker et al. 1996, Smith & Muscatine 1999, Wang et al. 2008; Fig. 6a), where dinoflagellates are often most toxic (Pan et al. 1999, Taroncher-Oldenburg et al. 1999, Guisande et al.

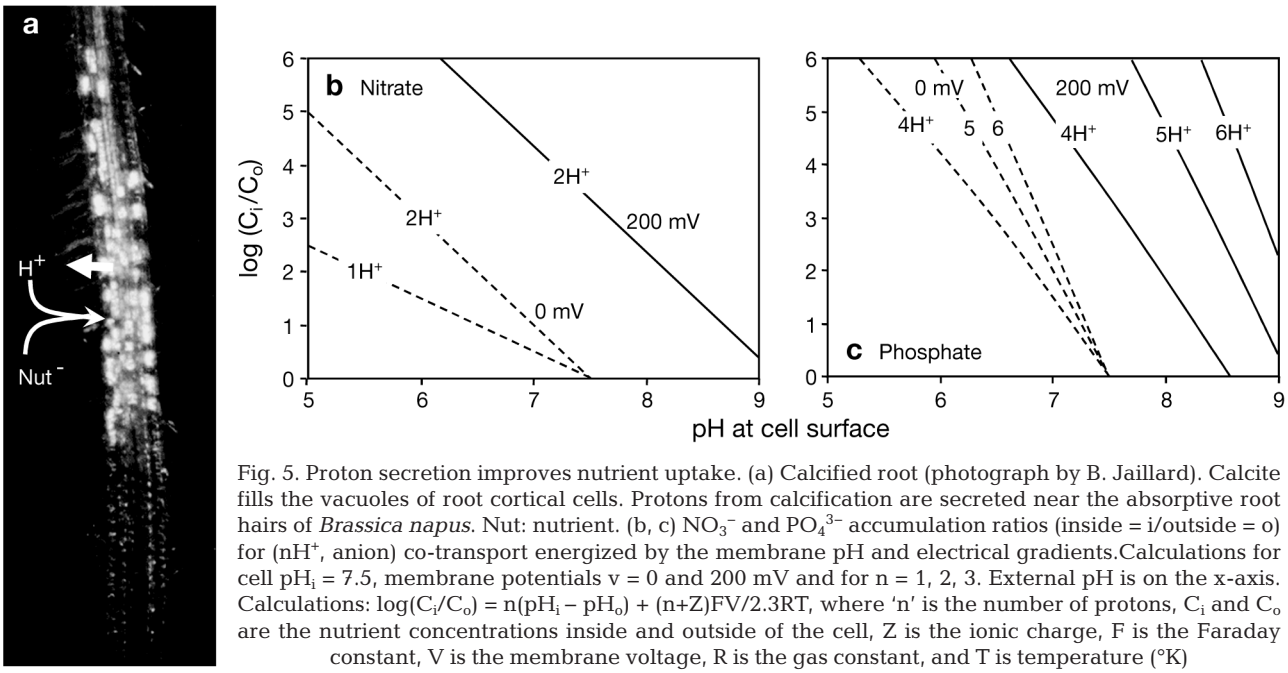

Fig. 5. Proton secretion improves nutrient uptake. (a) Calcified root (photograph by B. Jaillard). Calcite fills the vacuoles of root cortical cells. Protons from calcification are secreted near the absorptive root hairs of *Brassica napus*. Nut: nutrient. (b, c) NO_3^- and PO_4^{3-} accumulation ratios (inside = i/outside = o) for (nH^+, anion) co-transport energized by the membrane pH and electrical gradients.Calculations for cell pH_i = 7.5, membrane potentials v = 0 and 200 mV and for n = 1, 2, 3. External pH is on the x-axis. Calculations: $\log(C_i/C_o) = n(pH_i - pH_o) + (n+Z)FV/2.3RT$, where 'n' is the number of protons, C_i and C_o are the nutrient concentrations inside and outside of the cell, Z is the ionic charge, F is the Faraday constant, V is the membrane voltage, R is the gas constant, and T is temperature (°K)

2002, Paz et al. 2004, Varkitzi et al. 2010). When cultures of *Gambierdiscus* (Chinain et al. 2010) and *Ostreposis* (Guerrini et al. 2010) transition from exponential growth to a nutrient-limited stationary phase, the polyketides maitotoxin, ovatoxin, and a putative palytoxin approximately double.

ALGAL EXIT

Departing zooxanthellae likely obtain nutrients from the host coral. Zooxanthellae become more mitotic and progress faster through the cell cycle in heat-stressed corals, and after release from the coral (Baghdasarian & Muscatine 2000, Strychar et al. 2005; Fig. 6c–e). Warm seawater is generally nutrient depleted, so the nutrients needed to complete G1 phase and enter mitosis probably come from the host coral. Zooxanthellae also hyper-accumulate NH_4^+ from corals injured by *Vibrio* infections (Toren et al. 1998, Banin et al. 2001), and may similarly absorb nutrients from necrotic or apoptotic coral cells (Fig. 6f).

Several factors favor departure of nutrient-starved algae: they cannot easily reproduce in the coral (Fig. 6a), they can take coral nutrients as they leave, and they will likely die if they remain inside a bleaching coral (Dunn et al. 2004). The coral may also evict the algae, if they become too toxic, and mainly produce lipids (Muller-Parker et al. 1996).

Algal Ca^{2+} channel openers likely facilitate algal exit. Zooxanthellae sometimes depart within intact sloughed endodermal cells (Gates et al. 1992, Smith & Muscatine 1999). Cell-cell adhesion depends on Velcro-like cadherin molecules, whose intracellular anchors detach when cell Ca^{2+} rises (Ito et al. 1999, Marambaud et al. 2002). Ca^{2+} also regulates exocytosis and apoptosis (Demaurex & Distelhorst 2003, McMahon & Gallop 2005, Harr & Distelhorst 2010). Gene expression in heat-stressed corals suggests disrupted Ca^{2+} control, leading to cytoskeletal failure and loss of cell adhesion (DeSalvo et al. 2008). Ca^{2+} influxes also trigger egress in apicomplexan parasites, a sister group to the dinoflagellates (Farrell et al. 2012).

CORAL BLEACHING

Coral bleaching resembles algal dispersal in terms of its association with warm, nutrient-deficient waters, and symptoms like host cell apoptosis and sloughing (Weis 2008, Lesser 2011). Toxin-induced ion leakage across electron transport membranes and host immunological responses may increase the production of ROS and NO. Corals may suppress algal populations (Falkowski et al. 1993) to limit such pathology. Symbiotic zooxanthellae can nevertheless double in ~1 wk when nutrients are plentiful (Smith & Muscatine 1999, Tanaka et al. 2007; Fig. 6a), and

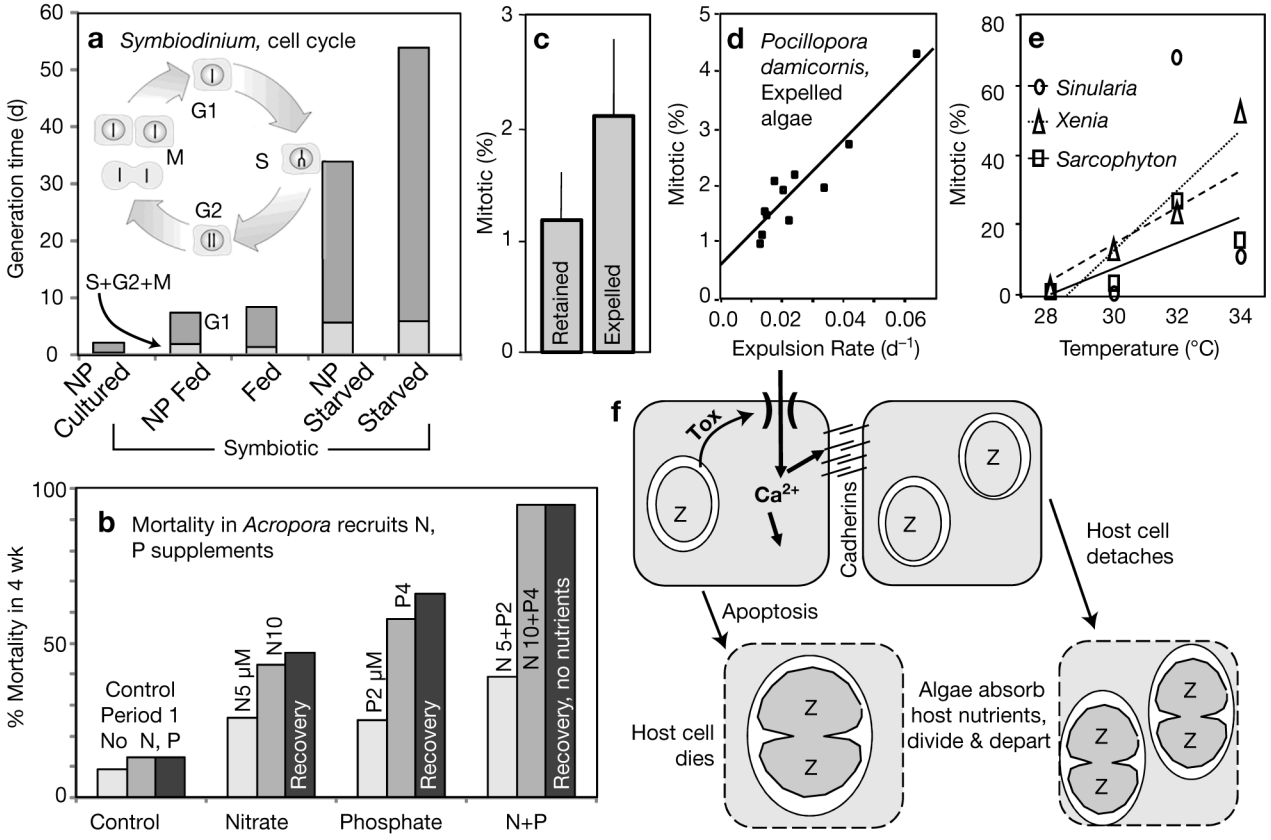

Fig. 6. Zooxanthellae life cycle. (a) Nutrient shortages prolong the G1 phase of the cell cycle (Smith & Muscatine 1999). (b) Nutrient supplements increase mortality in *Acropora* recruits (Renegar & Riegl 2005). (c, d) Expelled zooxanthellae are more mitotic (Baghdasarian & Muscatine 2000). (e) Zooxanthellae are more mitotic during bleaching at high temperatures (Strychar et al. 2005). (f) Two calcium-dependent mechanisms of algal release: apoptosis or sloughing of infected cells

coral mortality sometimes increases markedly (Renegar & Riegl 2005; Fig. 6b).

Slow but steady nutrient supplies probably reduce algal population explosions and exoduses. The diverse coral communities of the Red Sea illustrate this scenario. Deep waters there contain relatively few nutrients, but vertical mixing is fast for a tropical sea, and continues even in summer (Jean-Baptiste et al. 2004). Except in phosphorus-polluted areas (Walker & Ormond 1982), the corals seldom bleach (Karako-Lampert et al. 2004).

Nutrients are more abundant near the bottom of the euphotic zone, but low zooxanthellar densities there (McCloskey & Muscatine 1984) may prevent toxin overload. Nutrients are more plentiful and variable near rivers and upwelling zones, and corals are correspondingly less diverse, and reefs less developed. Human fertilization of the oceans (Szmant 2002, Kim et al. 2011) raises similar issues. When nutrients disappear, the over-abundant algae may suddenly become more toxic and depart. Hence nutrient pollution may contribute to recent increases in coral bleaching.

DISCUSSION

It will take considerable research to establish how algal toxins affect symbiotic corals. Toxins must be isolated and characterized, and their abundances monitored with respect to zooxanthellae densities, nutrient levels, and algal cell cycles. Toxin effects on coral calcification, algal exit, cell sloughing, and apoptosis should be tested. Toxins may also affect physiologies not discussed here, like algal invasion of the coral, evading its immune system, and poisoning coral predators. Zooxanthellae furthermore produce several toxins beyond Ca^{2+} channel openers. Timescales also matter, since algal densities change, and their toxicities likely change even faster.

Most dinoflagellate toxins modulate membrane cation transport (Rein & Borrone 1999, Murata & Yasumoto 2000, Shimizu 2003). Saxitoxins block Na^+ channels, brevetoxins and ciguatoxins open Na^+ channels, yessotoxins promote Ca^{2+} influx, and okadaic acid inhibits enzymes that control Ca^{2+} transport. Large, potent toxins seem to focus on Ca^{2+} in-

flux. Palytoxin admits various cations including Ca^{2+} into cells through the Na^+/K^+ ATPase, ovatoxins may work similarly, and maitotoxin, zooxanthellatoxins, and symbiodinolide admit Ca^{2+}. Why do dinoflagellates make such toxins?

Dinoflagellates maintain a permanent nuclear membrane. Their chromosomes appear condensed, but lack typical histones and nucleosome structure (Hackett et al. 2004, Costas & Goyanes 2005). Divalent cations, mainly Ca^{2+} and Mg^{2+}, largely balance the negative charges on the DNA (Sigee 1986, Levi-Setti et al. 2008). Ca^{2+} binds tightly and may condense and inactivate the DNA for mitosis, while monovalents reactivate it during G1. Toxins may facilitate this cation exchange, with Ca^{2+} channel openers possibly letting Ca^{2+} out of the nucleus during G1. Such a histone-free cationic switch on DNA conformation and activity has interesting implications to the origins of the eukaryotic cell cycle and cytosolic calcium signaling.

Zooxanthellae may have secondarily adapted their cell cycle controls to external uses. However, a symbiosis dependent on toxins naturally has rough spots. When nutrients become scarce, algal toxins may prod the coral to calcify faster. If ambient waters simply lack nutrients, the algae may injure their hosts, steal their nutrients, reproduce, and seek new hosts. The coral survives this stress at low algal densities, but the sudden exit of overpopulated algae endangers the coral. Abundant zooxanthellae, and abundant nutrients, are a mixed blessing.

Other algae sometimes show similar toxic behavior. The haptophyte *Prymnesium* also makes Ca^{2+} influx agonists (Igarashi et al. 1998, Murata & Yasumoto 2000). *Prymnesium* becomes most toxic when nutrients are scarce (Johansson & Granéli 1999, Granéli & Johansson 2003). It then kills and eats organisms ranging from plankton to fish (Skovgaard & Hansen 2003, Tillmann 2003). Nutrient shortages seemingly transform the protist from a plant to a predator. Related algae colonize calcareous foraminifera (Gast et al. 2000).

Corals calcify beyond their immediate skeletal needs. Ultimately, a few millimeters of coral tissue may sit atop several meters of vacant skeleton. This continuing calcification reflects a continuing need for protons. Symbiotic foraminifera, calcareous sponges, giant clams, and calcareous algae use calcification similarly. Over millions of years, they produce limestone reefs many kilometers thick. Reefs concentrate in the tropics because that is where nutrient acquisition demands special physiologies. Zooxanthellae may also be easier to control when nutrients are scarce.

Dinoflagellate toxins invite new perspectives on many issues: how zooxanthellae communicate with their hosts, why calcareous animals make good hosts, why symbiotic corals calcify fastest at low nutrient levels and at high temperatures, how reefs flourish amidst nutrient scarcity, why dinoflagellate symbioses and coral reefs concentrate in the tropics, why high temperatures encourage algal exit and coral bleaching, and why bleaching has recently increased. Toxins seem like odd agents for promoting symbioses, but it would be even more odd if such potent agents had no effect.

Acknowledgements. L. Muscatine blazed most of the trails that converge here, and B. Sweeney nurtured my interests in dinoflagellates. Both were wonderful hosts. V. Weis, B. Buddemeier, G. Muller-Parker, F. Marubini, K. Strychar, C. Ferrier-Pagès, D. Sigee, A. Cohen, D. Allemand, J. Erez, and others provided insight. B. Jaillard provided a photograph. C. Birkeland helped immensely with editing. This paper is dedicated to the Japanese chemists who so skillfully characterized *Symbiodinium*'s toxins.

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Editorial responsibility: Charles Birkeland, Honolulu, Hawaii, USA

Submitted: January 26, 2012; Accepted: April 23, 2012
Proofs received from author(s): July 5, 2012