

Reactive Oxygen Species and the Regulation of Hyperproliferation in a Colonial Hydroid

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ABSTRACT

Colonies of *Podocoryna carnea* circulate gastrovascular fluid among polyps via tubelike stolons. At polyp-stolon junctions, mitochondrion-rich cells in part regulate this gastrovascular flow. During competition, colonies hyperproliferate nematocytes and stolons; nematocysts are discharged until one colony is killed. Hyperproliferation then ceases, and normal growth resumes. Here, competing colonies were treated with azide, which inhibits respiration and upregulates reactive oxygen species (ROS). After the cessation of competition, azide-treated colonies continued to hyperproliferate. In azide-treated competing colonies, however, mitochondrion-rich cells were found to produce similar amounts of ROS as those in untreated competing colonies. Subsequent experiments showed that both azide treatment and competition diminished the lumen widths at polyp-stolon junctions, where mitochondrion-rich cells are found. In competing colonies, these diminished widths may also diminish the metabolic demand on these cells, causing mitochondria to enter the resting state and emit more ROS. Indeed, results with two fluorescent probes show that mitochondrion-rich cells in competing colonies produce more ROS than those in noncompeting colonies. In sum, these results suggest that competition perturbs the usual activity of mitochondrion-rich cells, altering their redox state and increasing ROS formation. Via uncharacterized pathways, these ROS may contribute to hyperproliferation.

Introduction

Competition between encrusting marine invertebrates has been extensively studied but often from very different intellectual perspectives. A long tradition in marine ecology examines the

role of community diversity and structure in this context (Connell 1961a, 1961b; Paine 1966; Dayton 1971; Lubchenco and Menge 1978; Menge et al. 1986; Bruno and Witman 1996; Connell et al. 2004). At the same time, an equally long tradition examines these competitive encounters from an immunological perspective, particularly with regard to fusion and rejection of tissues (Ivker 1972; Buss et al. 1984; McFadden 1986; Buss and Grosberg 1990; Ishii and Saito 1995; Padilla et al. 1996; Gild et al. 2003; Ferrell 2004; De Tomaso et al. 2005; Nicotra et al. 2009; Rosa et al. 2010). In the context of the latter perspective, there has been considerable interest in inducible defenses (Buss 1990; Harvell 1990), particularly as exemplified by cnidarians. Cnidarians are an early-diverging group of animals that includes corals, anemones, jellyfish, and hydroids. Inducible defenses in cnidarians include mesenterial filaments or sweeper tentacles in scleractinian corals (Lang 1971, 1973), acrorhagi or catch tentacles in anemones (Purcell 1977; Bigger 1980), and hyperplastic stolons in hydroids (Ivker 1972). Each of these specialized structures is armed with nematocytes, the stinging cells of cnidarians. Production of these structures and nematocyst firing occurs in response to histoincompatible tissue (Buss et al. 1984; Buss 1990).

Competition with inducible stolons is particularly well studied in colonial hydractiniid hydroids. A colony consists of a collection of polyps that are interconnected by tubelike stolons. Considerable recent progress has been made in understanding the functional biology of these colonies when they are growing normally in the absence of competition. Gastrovascular fluid containing food and other metabolites is pumped by polyp contractions throughout the lumen of the stolons (Schierwater et al. 1992; Blackstone 1996; Dudgeon et al. 1999). Polyp-stolon junctions form complex structures that are bound to the substratum by matrix proteins including laminin and type IV collagen (L. Buss, personal communication, February 11, 2011). Mitochondrion-rich epitheliomuscular cells are located at these junctions. Such mitochondrion-rich cells have been identified only at these junctions and not elsewhere in a colony (Blackstone et al. 2004). Note the distinction between mitochondrion-rich epitheliomuscular cells and typical epitheliomuscular cells: because mitochondrial biogenesis depends on metabolic regulation (Wu et al. 1999; Arany et al. 2008), any cell that is subject to sufficient metabolic demand will become mitochondrion rich. Hence, these mitochondrion-rich cells must have a particularly energy-demanding function, likely involving the regulation of gastrovascular flow by opening the lumen at polyp-stolon junctions. As a by-product of their function, these mitochondrion-rich cells emit high levels of reactive oxygen species (ROS) and other metabolic signals. Here, ROS are defined as partially reduced forms of oxygen and include super-

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oxide, hydrogen peroxide, and the hydroxyl radical (Thannickal and Fanburg 2000). When polyps are contracting and the mitochondrion-rich cells are active, the electron carriers of mitochondria become oxidized and the production of ROS is decreased. However, when the polyps are not contracting and the mitochondria are in the resting state, the electron carriers become reduced and more ROS are produced (Blackstone et al. 2004; Doolen et al. 2007).

How these normal colony functions are perturbed by competition has been little studied. Rather, research on competing colonies has focused on inducible defenses. When incompatible colonies encounter one another, they compete by producing defensive structures called hyperplastic stolons (Fig. 1). These specialized stolons are produced only from peripheral stolons in the area of contact (Ivker 1972). Hyperplastic stolons are not produced by the tissue of the stolon mat (Buss et al. 1984; Buss and Grosberg 1990). These stolons form when approaching stolon tips from one colony induce the formation of new tips in the other colony (Müller et al. 1987). These tips then grow, contact additional stolons, and induce more tips (Müller et al. 1987). Via some combination of proliferation and migration, nematocytes and interstitial cells accumulate in the epidermis of the proliferating stolons (Buss et al. 1984; Lange et al. 1989). A tangled mass of raised stolons packed with nematocytes thus emerges in the zone of contact. These hyperplastic stolons then discharge their nematocysts into the foreign tissue, which causes destruction and death of the tissue in the region of contact (Buss et al. 1984). Production of hyperplastic stolons continues until one colony is killed, at which point the excess nematocytes are digested by gastrodermal cells (Lange et al. 1989). Normal tissue growth of the surviving colony then resumes (Ivker 1972; Buss et al. 1984; McFadden 1986).

In this investigation, we focus on the resumption of normal growth subsequent to this competition-related proliferation of cells and stolons. Cnidarians appear capable of regulating this hyperproliferation, although how they do so remains largely unstudied and as yet unconnected to normal colony physiology. These questions are examined in colonies of *Podocoryna carnea* during competitive encounters with colonies of *Hydractinia symbiolongicarpus*. When these hydractiniid hydroids encounter one another, colonies of both species initially produce hyperplastic stolons in the area of contact. In most encounters, the colony of *P. carnea* will prevail over the colony of *H. symbiolongicarpus* (McFadden 1986). Under normal physiological conditions, *P. carnea* is capable of regulating cellular and stolon proliferation and will stop producing hyperplastic stolons once it has defeated *H. symbiolongicarpus* (McFadden 1986). Given the importance of the gastrovascular system in regulating responses to perturbation (Buss 2001), a reasonable hypothesis is that it is this system that allows hydractiniid hydroids to regulate hyperproliferation, both of cells and of stolons. In particular, we hypothesize that the actions of the mitochondrion-rich cells and their release of ROS are involved in this regulation. A reasonable starting point for our investigation is thus to treat competing colonies of hydractiniid hydroids with azide. Azide functions as a mimic of molecular oxygen, binding

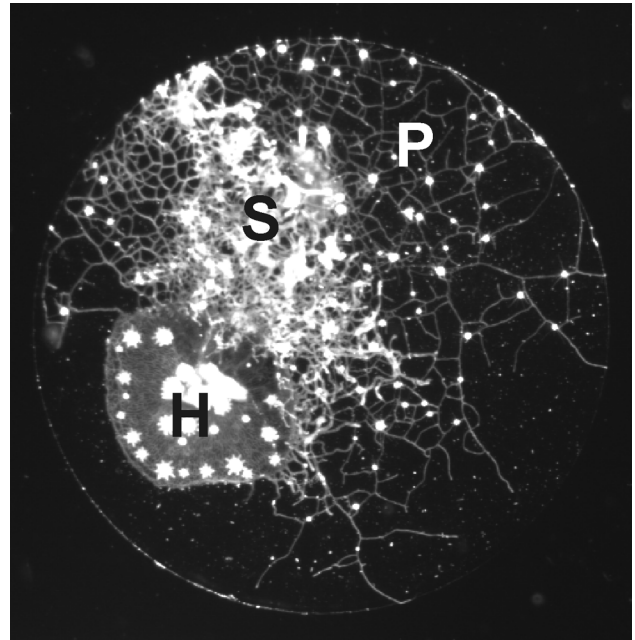


Figure 1. *Podocoryna carnea* and *Hydractinia symbiolongicarpus* competing on 15-mm round coverglass. Encounters between a colony of *H. symbiolongicarpus* (H) and a colony of *P. carnea* (P) result in the production of hyperplastic stolons (S).

to cytochrome c oxidase, and thus inhibits respiration and upregulates ROS (Blackstone 1999). Given their structural simplicity, azide (N_3^-) and its active inhibitory form (HN_3) are expected to have few pharmacological effects that are unrelated to the role of oxygen mimic. As described below, azide treatment results in continued hyperproliferation of the *P. carnea* colony even after the demise of the competing colony of *H. symbiolongicarpus*. Nevertheless, mitochondrion-rich cells of competing colonies of *P. carnea* treated with azide do not produce more ROS than those of untreated competing colonies. Further experiments reveal that competition itself perturbs the normal physiology of the gastrovascular system, increasing the ROS emitted by mitochondrion-rich cells. Via as yet uncharacterized pathways, these ROS may allow the regulation of hyperproliferation in colonies of *P. carnea*.

Material and Methods

Study Species and Culture Conditions

All experiments were carried out using a single clone of each of *Podocoryna* (= *Podocoryne*) *carnea* and *Hydractinia symbiolongicarpus*. *Podocoryna carnea* have runnerlike stolons that display a network of polyps from which many long peripheral stolons extend. In contrast, *H. symbiolongicarpus* appear sheetlike because of the formation of a stolon mat early in development from which projects varying amounts of peripheral stolons.

Colonies were grown on 15-mm round coverglasses and were cultured under standard conditions (e.g., Blackstone 1996). Ge-

netically identical colonies were produced by surgically explanting onto a coverglass one to three polyps and the connecting tissue from a source colony. A single coverglass thus represents the experimental unit; throughout, "replicate" refers to this unit. Depending on the experiment, a replicate may include a colony of *P. carnea* and a colony of *H. symbiolongicarpus* growing and competing with each other on a single coverglass or a single noncompeting colony of *P. carnea* growing on a single coverglass. Throughout, samples size (n) indicates the number of replicates used in a particular experiment. Colony growth was restricted to one side of the coverglass by removing stolons from the reverse side with a razor blade. Experiments were carried out at $\sim 20.5^\circ\text{C}$ in aquariums except when colonies were kept in glass finger bowls in incubators. Colonies were fed three times per week. Stolon proliferation experiments were carried out over a period of months, while all other experiments were performed on colonies that were just beginning to cover the available surface. These latter experiments were done shortly following feeding.

Effects of Azide on Stolon Proliferation in Competing Colonies

Competition between a colony of *P. carnea* and a colony of *H. symbiolongicarpus* was initiated by explanting both onto the same coverglass. As both colonies grew and encountered each other, they began producing hyperplastic stolons. Some replicate pairs of colonies progressed through competitive interactions more rapidly than others, so the week during which one colony prevailed was not the same for all replicates, and not all colonies were done competing by the end of each trial.

Treated replicates were perturbed using sodium azide. In its active inhibitory form, hydrogen azide (HN_3) "mimics" molecular oxygen in the sense that it blocks the electron transport chain by binding to and inhibiting cytochrome c oxidase. Inhibition of cytochrome c oxidase occurs because azide cannot be reduced and thus remains in the active site. As such, azide mimics oxygen only crudely in its molecular dimensions; it does not serve as a substitute for oxygen in chemical reactions. The inhibition of cytochrome c oxidase in turn causes a decrease in the rate of ATP formation and oxygen uptake, a relatively reduced redox state, and an increase in the formation of ROS (Erecinska and Wilson 1981; Blackstone 1999; Scheffler 1999). Azide is expected to have few pharmacological effects that are unrelated to its role as an oxygen mimic. Further, because it is the protonated form of azide that is active, relatively large quantities of azide in seawater (pH 8.2) will have relatively small effects in contrast to, for instance, cyanide. Replicates were incubated in the appropriate treatment for ~ 6 – 8 h d^{-1} . Treated replicates were incubated in a 1 mmol L^{-1} solution of azide and seawater, while controls were incubated in seawater without azide. Between treatments, replicates were maintained in normal culture conditions. On feeding days, replicates were fed before treatment.

Out of 33 total replicates (both control and azide treated), the colony of *P. carnea* prevailed by week 10 in the vast majority (25, of which 12 were controls and 13 were azide treated). In

three replicates, the competition was unresolved by week 10. In five replicates (two controls and three azide treated), the *H. symbiolongicarpus* colony prevailed by week 10. When one colony prevailed, its competitor was dead or nearly dead. Death of a competing colony occurred when all of its polyps had been reabsorbed and were covered by hyperplastic stolons of its competitor (Ivker 1972; McFadden 1986). Each week, on a non-feeding day, images were acquired before treatment. The first image was taken the week that treatment began, and the last image was taken at week 10. Whole-colony images were acquired with a Hamamatsu Orca-100 cooled CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan) attached to a macro lens and interfaced with an image analysis system. Images were acquired and subsequently analyzed using Image Pro Plus software (Media Cybernetics, Silver Spring, MD). The total combined colony area and the total hyperplastic area of both colonies were measured for each image, and the total number of polyps and reproductive polyps for each colony were counted. Total hyperplastic area was divided by total colony area (referred to as relative hyperplastic area). For the week that one colony emerged victorious, the mean of this ratio was compared between control and azide-treated replicates, using a t -test. If the variances for the two groups were equal, a standard t -test was used. If variances were unequal, a t -test for unequal variances was used. This latter test adjusts the degrees of freedom to make the test more conservative. To meet the assumptions of a t -test, data were normalized before analysis using an angular arcsine transformation, although untransformed data show the same results. Statistical analyses were performed using PC-SAS software (SAS, Cary, NC).

Effects of Azide on ROS in Competing Colonies

Under physiological conditions, hydrogen peroxide represents a major component of ROS (Chance et al. 1979). The compound 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA ; Molecular Probes, Eugene, OR) is a probe that is often used to visualize and measure hydrogen peroxide (Jantzen et al. 1998; Nishikawa et al. 2000; Pei et al. 2000; Das 2010). Outside of the cell, H_2DCFDA is nonfluorescent and cannot be oxidized. Once inside the cell, esterases remove the acetate groups and form H_2DCF . Oxidation of H_2DCF results in the production of a fluorescent product, 2',7'-dichlorofluorescein (DCF), which is visualized with the Hamamatsu camera attached to a Zeiss Axiovert 135 inverted microscope (Carl Zeiss, Jena, Germany) and interfaced with an image analysis system. When excited at 450–490 nm, emission at 515–565 nm corresponds to the amount of DCF produced. Considerable debate exists as to what this fluorescence is actually measuring (Finkel 2001). Molecules that oxidize H_2DCF will typically also oxidize cysteine residues, histidine residues, and iron-sulfur clusters of proteins. Because the oxidation of these residues and clusters is the primary mechanism by which ROS affect signaling pathways (Filomeni et al. 2005), the extent to which H_2DCF is oxidized likely reflects the extent to which ROS affect signaling pathways.

Competing replicates were grown as described above. Treated

replicates ($n = 19$) were placed in a 2-mmol L⁻¹ solution of azide and seawater for ~3 h, while controls ($n = 19$) were similarly incubated in seawater. After 2 h, H₂DCFDA (solubilized in dimethyl sulfoxide [DMSO]) was added to each treatment to a concentration of 10 μmol L⁻¹, and replicates were incubated in the dark for an additional hour. Colonies were imaged in seawater in disposable chambers, with frequent water changes to maintain temperature.

The mitochondrion-rich cells of *P. carnea* were imaged at three polyp-stolon junctions per colony. Junctions imaged were outside of the hyperplastic area. In such images, the mitochondrion-rich cells are brightly fluorescent (Fig. 2). At these junctions, fluorescence from H₂DCF strongly colocalizes with fluorescence from mitochondrial probes as well as native fluorescence of NAD(P)H (Blackstone et al. 2004). Negative controls (colonies treated only with DMSO) exhibit nearly undetectable amounts of fluorescence at wavelengths used for mitochondrial and ROS probes. For each image, the luminance and area of each mitochondrion-rich cell was determined. Within a circular region of interest, Image Pro Plus software automatically identified the bright (foreground) region and the complementary dark (background) region. From this, the area and relative luminance (foreground minus background) of each mitochondrion-rich cell were calculated (Blackstone et al. 2005). Data were analyzed using a nested ANOVA (mitochondrion-rich cells nested within polyp-stolon junctions, polyp-stolon junctions nested within replicates, replicates nested within treatments). These data should be interpreted cautiously. The goal is to compare foreground luminance (i.e., the fluo-

rescence of the mitochondrion-rich cells) between replicates and treatments. Nevertheless, some standardization is necessary because luminance can be affected by artifacts (e.g., the position of the polyp above the polyp-stolon junction). Background subtraction is intended to provide this standardization. However, in some cases background subtraction may be an over-correction, because some species of ROS (e.g., peroxide) easily diffuse through membranes. Fluorescence due to peroxide from mitochondrion-rich cells can thus affect background luminance (Fig. 2). Hence, analyses were done for foreground luminance, background luminance, and relative luminance (foreground minus background). If none of these exhibited a between-treatment effect, it was concluded that such an effect was absent or too weak to be detected.

Effects of Competition on Gastrovascular Flow

While colonial hydroids are simple animals, the gastrovascular system sometimes seems anything but simple. In part, this apparent complexity stems from the challenges of visualizing all the components of the gastrovascular system—polyps, polyp-stolon junctions, stolon bifurcations, stolon anastomoses, stolon tips—simultaneously and with sufficient resolution to permit quantification. We do not pretend to resolve these difficulties here. Rather, we focus on aspects of the gastrovascular system that are relevant to the function of mitochondrion-rich cells. Two complementary experiments were carried out. In the first experiment, noncompeting colonies were used, and control colonies of *P. carnea* were compared with those treated

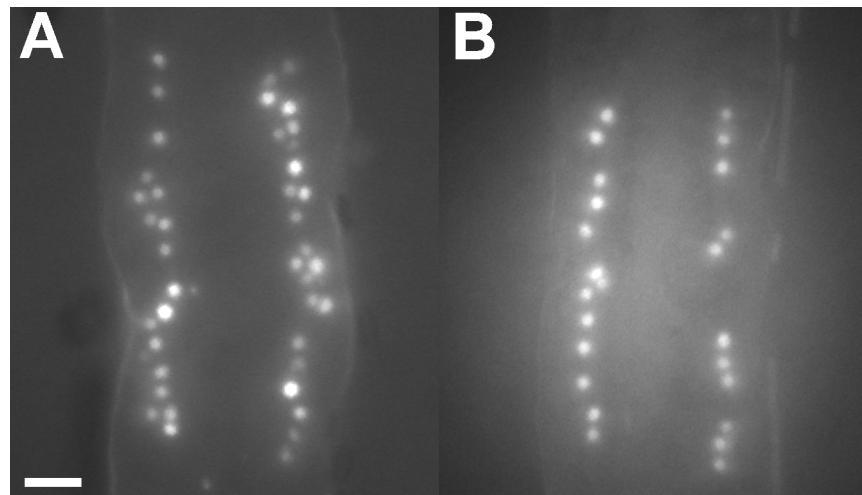


Figure 2. Images of fluorescent emissions (excitation, 450–490 nm; emission, 515–565 nm) at polyp-stolon junctions of a noncompeting (A) and competing (B) *Podocoryna carnea* colony treated with CM-H₂DCFDA, a probe for reactive oxygen species. Colonies are viewed from beneath, using an inverted microscope. The circular high-luminance objects are the mitochondrion-rich cells (Blackstone et al. 2004). Earlier interpretations of these objects as connected to the longitudinal muscles of the polyps (Blackstone 1999, 2003) are not supported by ultrastructural data (Blackstone et al. 2004). In A, the luminance of these cells (mean ± SE) is 2,454 ± 91, while the luminance of an equivalent area of their immediate background is 1,829 ± 56 and the relative luminance (foreground minus background) is 624 ± 44. In B, the corresponding luminance measures are 2,909 ± 132, 2,287 ± 102, and 621 ± 39, respectively. While the probe is expected to remain localized in the mitochondrion-rich cells, quantities of peroxide have likely diffused into the background area, and hence the competing colony has brighter foreground and background luminances. Scale, 15 μm.

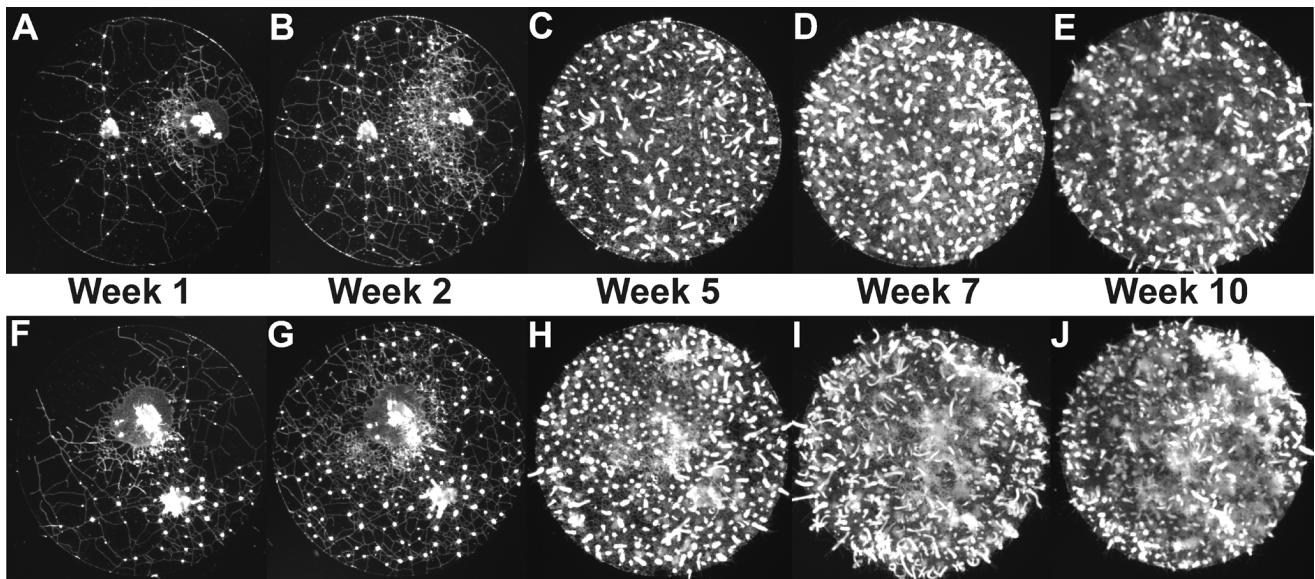


Figure 3. Control and azide-treated colonies competing on 15-mm round coverglass over a 10-wk period. A–E represent control replicates, and F–J represent replicates treated with 1 mmol L^{-1} sodium azide for 6–8 h d^{-1} . On the control coverglasses, the colony of *Podocoryna carnea* is to the left of the colony of *Hydractinia symbiolongicarpus*, while on the azide-treated coverglasses, the colony of *P. carnea* is below the colony of *H. symbiolongicarpus*. In replicates of both, the colony of *P. carnea* defeated the colony of *H. symbiolongicarpus* at week 7. Initially, hyperplastic stolons were produced by both colonies in the area of contact (A–C, F–H). In this control replicate, the colony of *P. carnea* started to produce reproductive polyps at week 7 and production of hyperplastic stolons ceased by week 8 (image not shown). By week 10, hyperplastic stolons were not present. In contrast, the azide-treated colony of *P. carnea* started to produce reproductive polyps at week 5 (H) and continued to produce hyperplastic stolons following the death of the colony of *H. symbiolongicarpus* (I, J).

with azide. Treated colonies ($n = 5$) were incubated in a 2-mmol L^{-1} solution of azide and seawater for ~ 3 h while controls ($n = 5$) were similarly incubated in seawater. In the second experiment, noncompeting colonies of *P. carnea* ($n = 5$) were compared with colonies competing with *H. symbiolongicarpus* ($n = 5$). For both experiments, the gastrovascular cavity of *P. carnea* was imaged near two polyp-stolon junctions per replicate. In competing colonies, junctions were imaged outside of the hyperplastic area. Previous studies using azide and other inhibitors have imaged the gastrovascular cavity near the stolon tip (Blackstone and Buss 1992). However, this was not feasible for competing colonies, because the majority of stolon tips were either hyperplastic or touching the edge of the coverglass. Thus, for all replicates, the gastrovascular cavity was imaged shortly after feeding, and lumen widths were measured at the polyp-stolon junctions. Live-feed images were taken at 1-s intervals for 150 s. Subsequently, lumen widths (endoderm to endoderm) and total stolon widths (perisarc to perisarc) were measured. Each data set was analyzed using a nested ANOVA (lumen widths within junctions, junctions within replicates, replicates within treatments).

Effects of Competition on ROS

Competing and noncompeting replicates were used. Three hours after feeding, replicates were placed in the dark and incubated in a $10\text{-}\mu\text{mol L}^{-1}$ solution of H_2DCFDA and seawater.

After ~ 1 h, the mitochondrion-rich cells of *P. carnea* were imaged at three polyp-stolon junctions per replicate, and images were analyzed as described above. Data from four competing and four noncompeting replicates were analyzed using a nested ANOVA (mitochondrion-rich cells nested within junctions, junctions nested within replicates, replicates nested within treatments). A similar experiment was performed with $\text{CM-H}_2\text{DCFDA}$ (final concentration, $2 \mu\text{mol L}^{-1}$), which is better retained by cells because of the chloromethyl group (Kirkland and Franklin 2001; Kirkland et al. 2007). Data from eight competing and eight noncompeting replicates were analyzed using a nested ANOVA.

Results

Effects of Azide on Stolon Proliferation in Competing Colonies

In the 12 control replicates in which *Podocoryna carnea* prevailed, the production of hyperplastic stolons ceased before or shortly after the death of *Hydractinia symbiolongicarpus*, and normal stolon production resumed. However, in the 13 azide-treated replicates in which *P. carnea* prevailed, hyperplastic stolon production continued subsequent to the death of *H. symbiolongicarpus*. Out of these 25 replicates, two representative ones are shown in Figures 3 and 4. Overall, for the week that *P. carnea* prevailed, the mean \pm SE of total hyperplastic area divided by total colony area was 0.089 ± 0.038 for control replicates and 0.474 ± 0.036 for azide-treated replicates. The mean

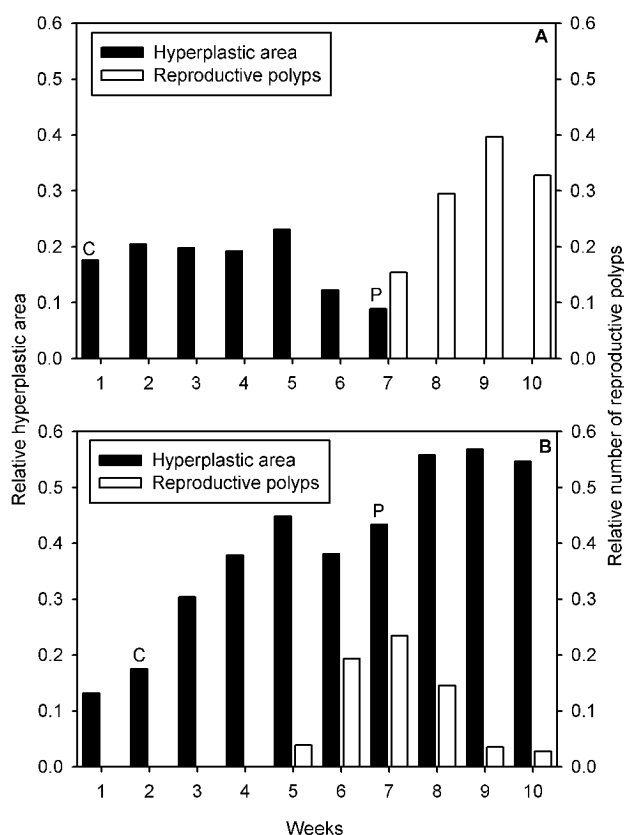


Figure 4. Morphology of control and azide-treated colonies. Graphs correspond to colonies in Figure 3 (A = control replicate, B = replicate treated with 1 mmol L⁻¹ sodium azide for 6–8 h d⁻¹). Relative hyperplastic area (total hyperplastic area divided by total colony area; black bars) and relative number of reproductive polyps (total number of reproductive polyps divided by total number of polyps; white bars) are shown for each week. C indicates the week at which the total area of the coverglass was covered by the stolons of the two colonies together, and P indicates the week at which the colony of *Hydractinia symbiolongicarpus* was killed.

of the angular arcsine-transformed ratio of total hyperplastic area divided by total colony area was significantly different between controls and azide-treated replicates (*t*-test for unequal variances; $n = 25$, $df = 16.4$, $t = -6.80$, $P < 0.0001$). In the two control and the three azide-treated replicates in which *H. symbiolongicarpus* prevailed, there were no hyperplastic stolons (i.e., total hyperplastic area divided by total colony area = 0) for the week that *H. symbiolongicarpus* prevailed, and no hyperplastic stolons were subsequently produced.

Effects of Azide on ROS in Competing Colonies

These experiments were done on replicates that were beginning to cover the available surface (indicated by C in Fig. 4). The relative luminance (foreground luminance minus background luminance) of mitochondrion-rich cells of competing colonies treated with azide was not significantly different from that of

untreated competing colonies (nested ANOVA: $F = 0.72$, $df = 1, 36$, $P = 0.40$). Similarly, the foreground or background luminances did not differ significantly between azide-treated and untreated colonies (foreground: $F = 0.26$, $df = 1, 36$, $P = 0.6105$; background: $F = 1.29$, $df = 1, 36$, $P = 0.26$). Although the ANOVAs were slightly unbalanced (numbers of mitochondrion-rich cells ranged from 15 to 82), examination of the coefficients of the variance components suggests that the *F* ratios are only slightly conservative. Thus, there is no evidence that mitochondrion-rich cells in azide-treated competing colonies produce more ROS than untreated competing colonies. This result cannot be rationalized by suggesting that the ROS or the probe leak out of the mitochondrion-rich cells because the fluorescence of both the foreground and the background areas (the areas immediately surrounding the cells) showed no difference. These results were surprising, and inclusion of some noncompeting colonies of *P. carnea* is instructive. For a group of replicates measured on the same day, the foreground luminance of the azide-treated competing colonies (mean \pm SE: $3,457 \pm 31$ for 292 cells in three replicates) was only slightly larger than the foreground luminance of control competing colonies ($3,205 \pm 37$ for 314 cells in three replicates), while the foreground luminance of the azide-treated noncompeting colonies ($3,461 \pm 31$ for 265 cells in two replicates) was considerably greater than the foreground luminance of control noncompeting colonies ($2,799 \pm 43$ for 262 cells in two replicates). The data thus suggest that competing colonies produce high levels of ROS even in the absence of azide. These high levels of ROS may make the effects of azide too weak to be detected (see "Discussion").

Effects of Competition on Gastrovascular Flow

Noncompeting control colonies exhibited significantly greater relative lumen widths (i.e., lumen width divided by total width) at polyp-stolon junctions than those treated with azide (Fig. 5; $F = 11.08$, $df = 1, 8$, $P = 0.01$). In a separate experiment, noncompeting colonies exhibited significantly greater relative lumen widths at polyp-stolon junctions than did competing colonies (Fig. 6; $F = 25.2$, $df = 1, 8$, $P = 0.001$). These results likely provide insight into the functions of mitochondrion-rich cells, which are found at polyp-stolon junctions. Nevertheless, these results are far from a complete characterization of the gastrovascular system during competition.

Effects of Competition on ROS

Using H₂DCFDA as a probe, there was no significant difference in the relative luminance (foreground luminance minus background luminance) of competing and noncompeting colonies ($F = 0.36$, $df = 1, 6$, $P = 0.56$). However, both the foreground and the background luminance of the mitochondrion-rich cells are significantly greater in competing colonies than in noncompeting ones (foreground: $F = 13.32$, $df = 1, 6$, $P = 0.01$; background: $F = 11.05$, $df = 1, 6$, $P = 0.01$). Again, examination of the coefficients of the variance components suggests

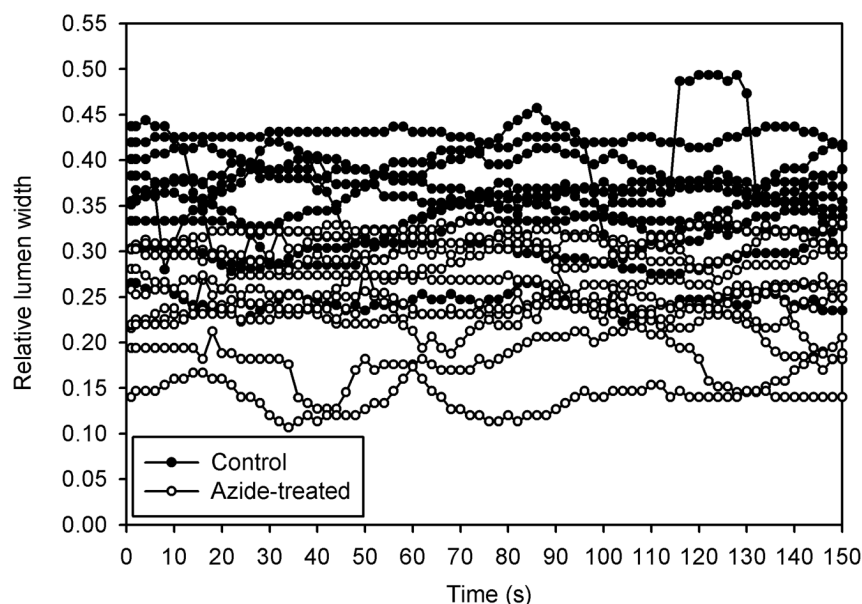


Figure 5. Relative lumen widths of contracting polyp-stolon junctions in control and azide-treated colonies of *Podocoryna carnea*. All of these colonies were not competing. Changes in relative lumen width (lumen width divided by total stolon width) are presented over the course of 150 s for two polyp-stolon junctions per control colony (filled circles) and colonies treated with 2 mmol L⁻¹ azide for 3 h (open circles). Five control and five azide-treated colonies were used. The relative lumen widths of azide-treated colonies are less than those of control colonies.

that the F ratios are slightly conservative. The greater foreground luminance is likely an indication of greater ROS production by mitochondrion-rich cells in competing colonies. The greater background luminance may be due to either the ROS diffusing out of the cells or the fluorescent probe (DCF) diffusing out (or both). Using CM-H₂DCFDA, the relative luminance (foreground luminance minus background luminance) of competing colonies of *P. carnea* was significantly greater than that of noncompeting ones (Fig. 7; $F = 8.32$, $df = 1, 14$, $P = 0.01$). The absolute foreground and background luminance did not differ significantly between competing and noncompeting colonies (foreground: $F = 0.01$, $df = 1, 14$, $P = 0.93$; background: $F = 0.71$, $df = 1, 14$, $P = 0.41$). Again, examination of the coefficients of the variance components suggests that the F ratios are slightly conservative.

Discussion

As described above, hyperplastic stolons are thought to form when approaching stolon tips from one hydroid colony induce the formation of new tips in another colony (Müller et al. 1987). In histoincompatible colonies, these tips then grow, contact additional stolons, and induce more tips (Müller et al. 1987). Via some combination of proliferation and migration, nematocytes and interstitial cells accumulate in the epidermis of the proliferating stolons (Buss et al. 1984; Lange et al. 1989). A tangled mass of raised stolons, packed with nematocytes, thus emerges in the zone of contact. A variety of signaling pathways are likely involved in the hyperproliferation of cells and stolons.

The results from these experiments suggest that ROS may

be involved in these signaling pathways. During competition, colonies of *Podocoryna carnea* usually stop producing hyperplastic stolons shortly after the death of the competing colony, and normal stolon growth then resumes. However, when treated with azide, a colony of *P. carnea* continues producing hyperplastic stolons even after the death of the competing colony. Azide mimics molecular oxygen and inhibits cytochrome *c* oxidase, the terminal electron carrier in the mitochondrial electron transport chain (Erecinska and Wilson 1981; Scheffler 1999). When treated with azide, electrons back up on the electron transport chain, and partially reduced forms of oxygen (i.e., ROS) form at high rates. Azide (N₃⁻) and its active inhibitory form (HN₃) are both very simple molecules. In the absence of any other information, it seems reasonable to interpret the effects of azide in terms of its inhibition of cytochrome *c* oxidase.

Nevertheless, the mitochondrion-rich cells in competing colonies treated with azide did not exhibit higher amounts of ROS than those in competing control colonies. Using a fluorescent ROS probe, neither foreground nor background luminance (nor foreground minus background) showed any effect of treatment despite a substantial sample size ($n = 38$). Given previous work (Blackstone 1999) and some data from noncompeting colonies in this study, it is unlikely that azide is not effective under the concentrations and conditions used. Rather, our result can be rationalized if competition itself is altering metabolic state and increasing ROS. Previous work has also shown that the role of these mitochondrion-rich cells in regulating gastrovascular flow affects their redox state and ROS production

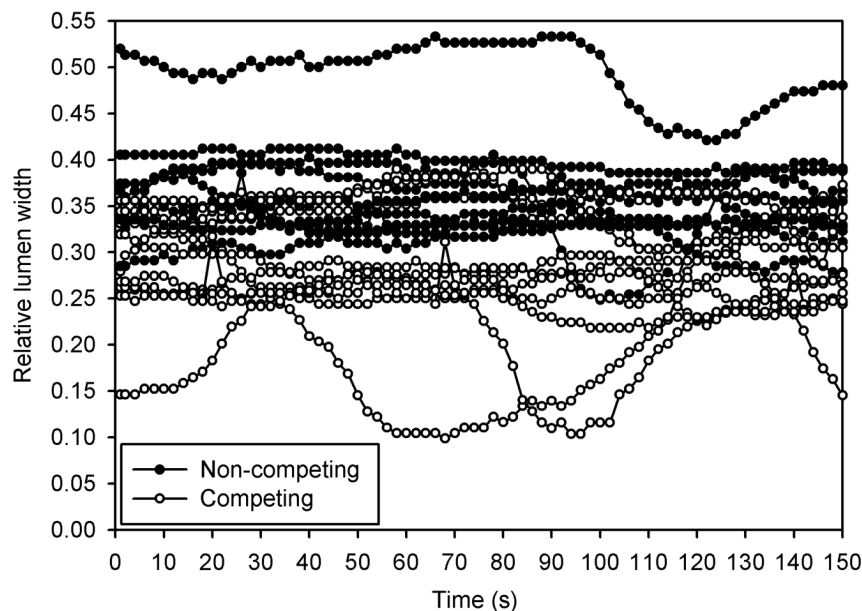


Figure 6. Relative lumen widths of contracting polyp-stolon junctions in noncompeting and competing colonies of *Podocoryna carnea*. Changes in relative lumen width (lumen width divided by total stolon width) are presented over the course of 150 s for two polyp-stolon junctions, per noncompeting colony (filled circles) and competing colony (unfilled circles). Five noncompeting and five competing colonies were used. The relative lumen widths of competing colonies are less than those of noncompeting colonies.

(Blackstone 2003; Doolen et al. 2007). When gastrovascular flow is maximal, these cells are relatively oxidized and ROS formation is minimal. On the other hand, when gastrovascular flow is minimal, the mitochondria enter the resting state and cellular redox state is shifted in the direction of reduction with maximal ROS formation. If competition is altering metabolic state, the sensitivity of mitochondrion-rich cells to azide may be diminished. When studied in isolation, mitochondria that are in the resting state are less sensitive to azide than actively respiring mitochondria (Erecinska and Wilson 1981).

In previous work, gastrovascular flow was examined in peripheral stolon tips. This could not be done in competing colonies because of the hyperplastic stolon formation and tissue death caused by competition. Nevertheless, examination of lumen widths at polyp-stolon junctions supports this hypothesis: competing colonies have smaller lumen widths relative to total stolon width than do noncompeting colonies. Azide-treated noncompeting colonies exhibited similarly smaller lumen widths as noncompeting controls. These data suggest that at least part of the function of mitochondrion-rich cells is to pull open the junction between polyps and stolons. When energy conversion is inhibited (i.e., with azide treatment) or the gastrovascular system is perturbed (i.e., during competition), the function of these cells diminishes and the stolon lumen is not pulled open fully. While the gastrovascular system of competing colonies remains incompletely characterized (indeed, the same could be said for noncompeting colonies), the localization of mitochondrion-rich cells to polyp-stolon junctions suggests that this is a reasonable interpretation of the data.

With H_2DCFDA , a probe for ROS, both foreground and background luminance was higher in competing colonies than in noncompeting colonies, despite a small sample size ($n = 8$). This suggests that mitochondrion-rich cells are producing more ROS and either ROS or the fluorescent probe (DCF) is leaking out of these cells, yielding both the brighter foreground and the brighter background. $CM-H_2DCFDA$ is better retained by the cells (Kirkland and Franklin 2001; Kirkland et al. 2007). Indeed, in this case, the mitochondrion-rich cells of competing colonies exhibited greater relative fluorescence (foreground minus background). While the difference in ROS between competing and noncompeting colonies is likely small, the cumulative effect of such small differences may be important. For instance, ROS are known to have a variety of effects on signaling pathways (see below).

In sum, these data suggest the following scenario (Fig. 8). When competition ensues, a colony of *P. carnea* alters its normal pattern of gastrovascular flow. Polyps in areas of the colony that are not directly subjected to competition to some extent diminish their active contractions, which drive the gastrovascular flow. Mitochondrion-rich cells, which regulate this flow by pulling open polyp-stolon junctions, enter the resting state, and the formation of ROS increases. Via mechanisms that remain as yet uncharacterized, these ROS contribute to the formation of hyperplastic stolons. When competition ceases, normal flow resumes, mitochondrion-rich cells exhibit high levels of metabolic demand, and ROS formation diminishes. Hyperplastic stolons return to the normal morphology. Lange et al. (1989) observed that nematocyst discharge into

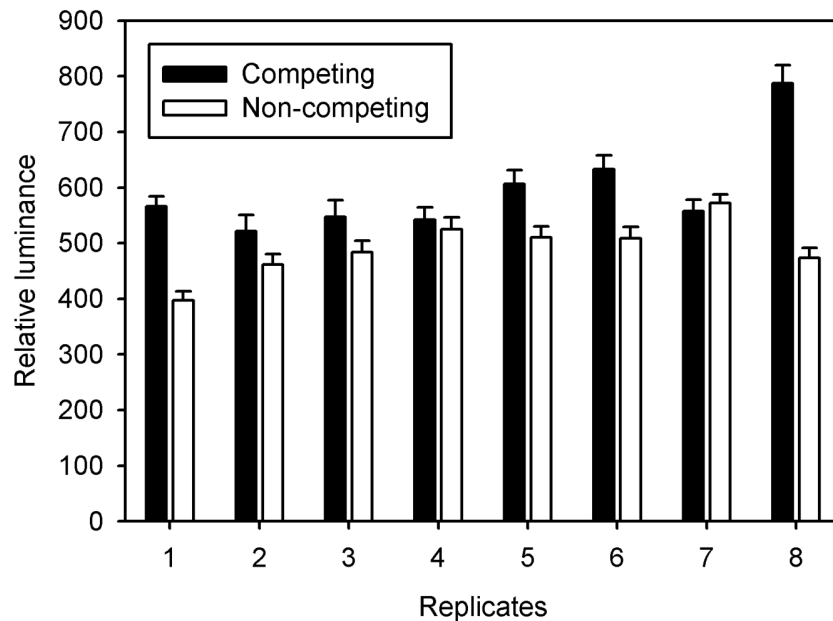


Figure 7. Levels of endogenous reactive oxygen species (ROS) in competing and noncompeting colonies of *Podocoryna carnea*. The mean \pm SE of relative luminance (foreground fluorescence minus background fluorescence) for mitochondrion-rich cells from three polyp-stolon junctions per replicate colony when CM- H_2 DCFDA (final concentration, $2 \mu\text{mol L}^{-1}$) was used (black bars, competing; white bars, noncompeting). Mitochondrion-rich cells from competing colonies of *P. carnea* produce more ROS than do those from noncompeting colonies.

a stolon halted gastrovascular flow for a considerable distance along the length of the stolon. Such an effect could contribute to diminished activity of mitochondrion-rich cells.

A number of additional experiments would support this scenario. Treatment with other inhibitors of the respiratory chain could rule out (or in) any side effects of azide (Blackstone 2003). Examination of the time course of ROS formation in mitochondrion-rich cells throughout a competitive encounter would also be illuminating because it would show at what point ROS increase relative to the formation of hyperplastic stolons and at what point ROS decrease relative to the end of competition. For example, if our scenario is correct, ROS might increase shortly after the onset of competition and possibly before the formation of hyperplastic stolons. Similarly, ROS might decrease following the cessation of competition and before the return of hyperplastic stolons to the normal morphology. However, in azide-treated colonies, we would expect ROS levels to remain elevated even after the cessation of competition and thus for the production of hyperplastic stolons to continue.

Nevertheless, this time-course experiment is not feasible for several reasons, and thus ROS measurements were taken using colonies that were in the early stages of competition (e.g., week 2 in Fig. 3 and stage C in Fig. 4). First, before competition, colonies are typically very small and contain few polyps. Second, by the resolution of competition, surviving colonies are often strewn with necrotic tissue and cells, which in this case produces artifactual fluorescence (Cherry Vogt et al. 2008). Finally, by the time competition ends, surviving

colonies thickly cover the entire coverglass and are typically initiating medusa production (see Fig. 3). Indeed, in these experiments, the week that surviving colonies initiated medusa production typically coincided with the week that the competing colony died. Covering the surface and initiating medusa production likely alters flow patterns and ROS formation (Blackstone 2009), thus confounding the effects of the cessation of competition.

In contrast to *P. carnea*, *Hydractinia symbiolongicarpus* was capable of controlling the production of hyperplastic stolons not only under normal physiological conditions but also when treated with azide. In both conditions, production of hyperplastic stolons was halted before or during the week in which *P. carnea* was defeated; at the same time, widespread formation of the stolonial mat occurred in areas previously covered by hyperplastic stolons. The stolonial mat of *H. symbiolongicarpus* inhibits the production of hyperplastic stolons (Buss et al. 1984; Buss and Grosberg 1990). A previously unrecognized advantage of the stolonial mat may thus be the regulation of hyperproliferation.

A connection between cnidarian competition and ROS has previously been suggested by Bartosz et al. (2008). In anemones, acrorhagial-derived nematocyst venom was found to be capable of inducing intracellular formation of ROS. The mechanism of ROS induction, however, remains completely obscure. These ROS may be entirely unrelated to mitochondria; for example, components of the venom may activate NADPH oxidases in the competing colony. Nevertheless, if such an effect is widespread in cnidarians, ROS could signal

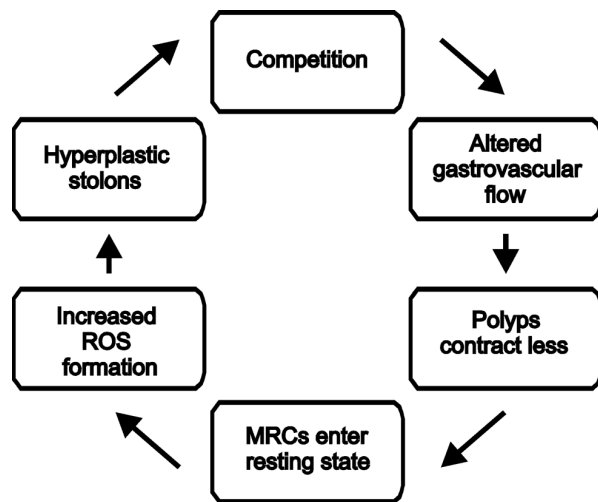


Figure 8. Schematic illustrating a hypothesis for the formation and regulation of hyperplastic stolons by *Podocoryna carnea* during competition. Competition alters the normal gastrovascular flow. Polyps that drive this flow contract less, and mitochondrion-rich cells (MRCs) enter the resting state. The formation of reactive oxygen species (ROS) increases, and these contribute to the formation of hyperplastic stolons. When competition ceases, normal flow and metabolic demand resume, ROS formation decreases, and hyperproliferation ceases.

an attack by a competitor. In this case, linking pathways that induce defensive structures to ROS makes a considerable amount of evolutionary sense.

Our data, along with the results of previous studies, suggest that in cnidarians, ROS may act as a signaling molecule that is involved in a number of different pathways, including those that regulate colony growth, development, and stolon regression in hydroids (Doolen et al. 2007; Cherry Vogt et al. 2008), strobulation in scyphozoans (Berking et al. 2005), and coral bleaching in anthozoans (Perez and Weis 2006). For example, runnerlike growth in *P. carnea* is associated with moderate levels of ROS production from the mitochondria, whereas low levels of ROS are associated with sheetlike growth (Doolen et al. 2007). Also, moderate levels of ROS in stolon tips seem to act as a growth factor that triggers outward growth and inhibits branching, whereas high levels of ROS appear to be involved in stolon regression and cell and tissue death of peripheral stolon tips (Blackstone et al. 2005; Cherry Vogt et al. 2008).

Hypotheses about the role of ROS in competition can be guided by what we know about cnidarian and other systems. Presumably, a multistep signaling pathway is involved. For example, when treated with azide, *P. carnea* does not produce hyperplastic stolons in the absence of a competitor but will do so in the presence of a competitor. This along with observations from previous studies demonstrate that in hydroids, foreign tissue is usually necessary to induce the formation of hyperplastic stolons (Ivker 1972; Buss et al. 1984; McFadden 1986; Lange et al. 1989; but see Buss et al. 1985). In *H. symbiolongicarpus*, allelocognition is controlled by two

linked loci, *alr1* and *alr2*. The decision to fuse or reject is dependent on the number of alleles that are shared at each loci and the loci at which they are shared (Cadavid et al. 2004). These genes encode putative transmembrane receptors (Nicotra et al. 2009; Rosa et al. 2010). Subsequent to the decision to reject, stolons proliferate as described above (Müller et al. 1987). How ROS might affect this process remains unclear.

In this regard, the role of ROS in other systems may be instructive. ROS contribute to a number of signaling pathways, including those that are involved in immune and inflammatory responses, vascular function, cell proliferation and differentiation, and cell death (Vepa et al. 1999; Benhar et al. 2002; Taniyama and Griendling 2003; Zhang and Gutterman 2006; Veal et al. 2007; Xia et al. 2007). For example, endothelial cells release ROS in response to shear stress that in turn regulates vascular function. Peroxide has been shown to regulate shear-induced smooth muscle relaxation in mouse and human small arteries (Matoba et al. 2000) and to mediate flow-induced dilation in coronary arterioles from patients with coronary disease (Miura et al. 2003). The mechanism by which the mitochondria of endothelial cells produce ROS in response to shear stress is unclear. Endothelial cells may sense shear stress via integrins and other focal adhesion components (Jalali et al. 2001; Tzima et al. 2001), vascular endothelial growth factor receptor 2 (Shay-Salit et al. 2002), and/or platelet endothelial cell adhesion molecule 1 (Osawa et al. 2002). *Podocoryna carnea* has been shown to harbor integrin genes (Reber-Müller et al. 2001), which may be one way that external signals are recognized and transduced into gene activity. ROS are also produced in response to external stimuli such as peptide growth factors, cytokines, and insulin (Chiarugi and Cirri 2003; Rhee et al. 2003; Storz 2005). Some proteins, in particular protein tyrosine phosphatases, can be regulated by ROS because they contain a catalytic cysteine residue that can be reversibly oxidized by peroxide (Salmeen et al. 2003; van Montfort et al. 2003). ROS can also regulate the activity of transcription factors by oxidation of cysteine residues that regulate DNA binding activity. Alternatively, oxidative stress can promote increased phosphorylation by upstream kinases (Storz 2005). Particularly interesting in this context are studies of FoxO proteins. A number of organisms—including sponges, placozoans, cnidarians, *Drosophila*, *C. elegans*, and mammals—harbor FoxO transcription factors. In bilaterians, these transcription factors play a role in regulating cellular responses to stress and in particular increase resistance to oxidative stress by increasing the levels of antioxidant enzymes (Bridge et al. 2010). In mammals, FoxO proteins also regulate cell-cycle progression and arrest and apoptosis (Brunet et al. 1999; Burgering and Kops 2002). In early-evolving metazoans, the role of FoxO proteins is less clear. Recently, however, Bridge et al. (2010) identified a single FoxO gene in *Hydra magnipapillata* and provided data that suggest that like in bilaterians, FoxO proteins in *Hydra* also mediate cellular responses to stress. In summary, while the pathways connecting ROS to hyperplastic stolon formation

remain uncharacterized, there is no lack of interesting putative candidates to investigate with future research.

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