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## Redox signaling in colonial hydroids: many pathways for peroxide

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### Summary

Studies of mitochondrial redox signaling predict that the colonial hydroids *Eirene viridula* and *Podocoryna carnea* should respond to manipulations of reactive oxygen species (ROS). Both species encrust surfaces with feeding polyps connected by networks of stolons; *P. carnea* is more 'sheet-like' with closely spaced polyps and short stolons, while *E. viridula* is more 'runner-like' with widely spaced polyps and long stolons. Treatment with the chemical anti-oxidant vitamin C diminishes ROS in mitochondrion-rich epitheliomuscular cells (EMCs) and produces phenotypic effects (sheet-like growth) similar to uncouplers of oxidative phosphorylation. In peripheral stolon tips, treatment with vitamin C triggers a dramatic increase of ROS that is followed by tissue death and stolon regression. The enzymatic anti-oxidant catalase is probably not taken up by the colony but, rather, converts hydrogen peroxide in the medium to water and oxygen. Exogenous catalase does not affect ROS in mitochondrion-rich EMCs, but

does increase the amounts of ROS emitted from peripheral stolons, resulting in rapid, runner-like growth. Treatment with exogenous hydrogen peroxide increases ROS levels in stolon tips and results in somewhat faster colony growth. Finally, untreated colonies of *E. viridula* exhibit higher levels of ROS in stolon tips than untreated colonies of *P. carnea*. ROS may participate in a number of putative signaling pathways: (1) high levels of ROS may trigger cell and tissue death in peripheral stolon tips; (2) more moderate levels of ROS in stolon tips may trigger outward growth, inhibit branching and, possibly, mediate the redox signaling of mitochondrion-rich EMCs; and (3) ROS may have an extra-colony function, perhaps in suppressing the growth of bacteria.

Key words: anti-oxidant, clonal, cnidarian, colony development, *Eirene*, evolutionary morphology, hydroid, *Podocoryna*, *Podocoryne*, reactive oxygen species, redox signalling.

### Introduction

Exemplifying the broad scope for redox signaling in bacteria, Oh and Kaplan's (2000) study of the electron transport chain (ETC) in *Rhodobacter sphaeroides* concludes: 'The advantage of redox sensing through the ETC, as demonstrated here, appears to be the ability to respond rapidly and precisely to environmental stimuli as well as to provide a mechanism to integrate all cellular and metabolic activities.' While animals and plants have been investigated in this context (e.g. Pfannschmidt et al., 1999; Brownlee, 2001), some of the most fertile ground for such studies – and for applying Oh and Kaplan's insight – may be found in early evolving animals. These animals typically exhibit several features – agametic, asexual reproduction, active stem cells, and potentially long life spans (Blackstone and Jasker, 2003) – that render them particularly responsive to environmental and metabolic signals. Indeed, studies of hydractiniid hydroids (colonial cnidarians that consist of feeding polyps connected by gastrovascular stolons) implicate metabolic and redox signaling as a basic feature of colony growth (Blackstone, 1999; 2003). For instance, shortly after a polyp in a colony feeds, contractions of epitheliomuscular cells (EMCs) begin to

circulate substrate-rich gastrovascular fluid throughout the colony (Dudgeon et al., 1999). The metabolic demand imposed by these contractions shifts the redox state of the EMCs mitochondria in the direction of oxidation. As a result the electron carriers of these mitochondria become relatively oxidized and are less likely to donate electrons to molecular oxygen. Formation of reactive oxygen species (ROS; e.g. superoxide, hydrogen peroxide and hydroxyl radicals) is thus diminished. Low levels of ROS seem to inhibit the outward growth of stolons; consequently, diminished ROS lead to increased polyp initiation and stolon branching in the area of the fed polyp. The colony thus responds adaptively to the environmental stimulus of feeding.

In the mitochondrial ETC, there are two sites of ROS formation, site 1 on complex I and site 2 at the interface between coenzyme Q and complex III (Nishikawa et al., 2000; Armstrong et al., 2003). Experimental manipulations of mitochondrial function in hydractiniid hydroids suggest that it is site 2 that produces the ROS that affect colony growth and development (Blackstone, 2003). At comparable physiological doses (determined by measures of oxygen uptake), blocking

the mitochondrial electron transport chain at complex III with antimycin A<sub>1</sub> produces the same phenotypic effects as blocking at complex IV with azide. This phenotypic effect is similar to that observed in areas of colonies that are only indirectly supplied with food from polyps elsewhere in the colony (Blackstone, 2001). In each case, ROS are increased, and the resulting phenotype consists of 'runner-like' growth with widely spaced polyps and stolon branches. Conversely, at appropriate physiological doses the uncoupler of oxidative phosphorylation, carbonyl cyanide m-chlorophenylhydrazone, has the same phenotypic effect as another uncoupler, 2,4-dinitrophenol. This effect is similar to that observed in areas of colonies that are well fed – 'sheet-like growth', with closely spaced polyps and stolon branches – and correlates with low levels of mitochondrial ROS. Rotenone was used to inhibit electron transport 'downstream' of site 1 of ROS formation and 'upstream' of site 2. The resulting phenotypic effects were very similar to those produced by uncouplers and strikingly different from those produced by antimycin or azide. This suggests that signal transduction is initiated at or near site 2, and this role of site 2 has been found in other studies (Nishikawa et al., 2000; Armstrong et al., 2003). While the effects of antimycin are sometimes difficult to interpret because of the intricate interaction between coenzyme Q and complex III (Armstrong et al., 2003; Osyczka et al., 2004), in this case the similarities between the effects of azide and antimycin suggest that blocking the ETC anywhere downstream of site 2 will produce similar effects. A lesser role for site 1 may be due to differences between sites 1 and 2 in electron flux in colonies subject to a fat-rich diet.

At least in hydractiniid hydroids, the bulk of the mitochondria in a colony are concentrated in narrow regions of contractile epitheliomuscular cells (EMCs) located in polyp–stolon junctions (Blackstone et al., 2004). Within a colony, polyp–stolon junctions tend to be more centrally located as compared with peripheral stolon tips. Both are similar in basic structure, for instance, exhibiting a layer of endoderm and ectoderm covered by a protective perisarc. Both are also connected by a continuous lumen through which gastrovascular fluid circulates at a high rate. Nevertheless, peripheral stolons are devoid of these muscular, mitochondrion-rich cells (Schierwater et al., 1992; Blackstone et al., 2004). Mitochondrion-rich EMCs may be the locus of colony-wide redox signaling (Blackstone et al., 2005a). Since the outward growth of a colony and its form are ultimately determined by the behavior of peripheral stolon tips, signals from mitochondrion-rich EMCs at polyp–stolon junctions may be conveyed to these peripheral tips. ROS from mitochondrion-rich contractile regions can be considered a potential candidate to provide stolons with signals influencing elongation, branching and regression, leading to the emergence of colony growth form. To investigate further the possible role of ROS in such redox signaling, perturbations of colony growth and development were carried out using the hydroid *Podocoryna carnea*. Some experiments also used *Eirene viridula*. Chemical (vitamin C) and enzymatic (catalase) anti-

oxidants were used to attempt to diminish ROS, and these results were compared with those that have been obtained previously using uncouplers of oxidative phosphorylation to diminish mitochondrial ROS. ROS were also manipulated using exogenous peroxide. Using fluorescent microscopy of both stolon tips and mitochondrion-rich contractile regions, assays of ROS were carried out with 2',7'-dichlorofluorescein diacetate. The data obtained from these experiments suggest that ROS in general and hydrogen peroxide in particular are involved in a number of as-yet-uncharacterized signaling pathways in colonial hydroids.

## Materials and methods

### *Study species and culture conditions*

Most of this work was done with the anthoathecate hydroid *Podocoryna* (= *Podocoryne*) *carnea* Sars 1846 using colonies of a single clone, which were cultured using standard methods (e.g. Blackstone, 1999; the same clone, P3, has been used extensively in previous investigations). Some key experiments were repeated with the leptothecate hydroid *Eirene viridula* Peron and Lesueur 1809, again using a single clone. Comparable results from both species provide some assurance that the mechanisms observed may have some generality. For measures of polyp and stolon development, colonies were grown on 18 mm diameter round glass cover slips. For measures of peroxides, colonies were grown on 15 mm diameter round glass cover slips. Growth of the colonies was confined to one side of the cover slips by daily scraping with a razor blade. All experiments were carried out at 20.5°C.

Even though genetically identical stocks were used, colony growth may differ between experiments because of environmental and perhaps epigenetic effects (Ponczek and Blackstone, 2001). Seasonal effects are particularly common with more sheet-like and slow-growing colonies occurring in the winter (Ponczek and Blackstone, 2001). Hence, control colonies were part of each experiment, and all control and treated explants for an experiment were always made from the same source colony. Nevertheless, some variation can be found even within a group of explants made from the same colony. Typically, the slowest growing explants (which are assigned the highest numbers in the figures) are also the more sheet-like.

### *Treatment with vitamin C, catalase and peroxide*

To investigate the pathways by which lithium ions affect development, Jantzen et al. (1998), treated *Hydra vulgaris* and *Hydra magnipapillata* with vitamin C, vitamin E, and catalase. We have largely adopted their protocols. Vitamin E ( $\alpha$ -tocopherol), however, requires a solvent for treatment in aqueous media, and in this regard ethanol is unsatisfactory for treatments of these hydroids (Blackstone, 2003). While dimethyl sulfoxide (DMSO) can be used, experiments suggest that DMSO may stimulate oxygen uptake (data not shown), perhaps because it is permeabilizing the mitochondrial inner membrane. To simplify the interpretation of the experiments, only vitamin C (ascorbic acid) was used to investigate the

effects of chemical anti-oxidants. For all experiments, vitamin C was prepared in a 10 mmol l<sup>-1</sup> stock solution and adjusted to a pH 8 with NaOH. This stock was prepared afresh each day, immediately prior to use. Treatment of hydroid colonies was carried out at 100 µmol l<sup>-1</sup>. While vitamin C is generally considered an anti-oxidant, under some conditions it can interact with catalytically active metals such as iron or copper and produce ROS (Carr and Frei, 1999). Hydroids are highly sensitive to such metals in their culture medium (Lenhoff, 1983). The seawater medium used (Reef Crystals, Aquarium Systems, Mentor, Ohio, USA) contains chelators that probably keep concentrations of such metals very low, particularly when reverse osmosis (RO) water is used to mix up the medium. Catalase treatments were carried out at 0.1 mg ml<sup>-1</sup>. Follow-up experiments show that similar effects are obtained using 0.033 mg ml<sup>-1</sup> (data not shown). Hydrogen peroxide treatments were carried out at nominal concentrations of ~20–50 µmol l<sup>-1</sup>. Because peroxide is reactive, the actual concentrations may have been somewhat less than this. Nevertheless, combining catalase and peroxide at considerably more dilute solutions than those used in the experiments quickly saturated an oxygen electrode, so considerable activity of both the catalase and peroxide is thus assured.

#### Comparisons of colony growth and development

Experiments were conducted separately over a period of several years. For each experiment, 14 replicates were explanted on 18 mm cover slips, with seven each assigned to control and to the appropriate treatment. Occasionally, broken cover slips resulted in smaller sample sizes. Each group was treated with the appropriate solution in finger bowls for ~6 h day<sup>-1</sup>. As previously (e.g. Blackstone, 2003), intermittent treatments seemed to be best tolerated by colonies. As each colony covered the surface of the cover slip, that colony was imaged. A colony was considered to be covering the surface when stolons were contacting the edge of the cover slip throughout ~60% of its circumference. Images were processed to facilitate automatic measurement in Image-Pro Plus software (Media Cybernetics, Silver Spring, Maryland, USA). The gray level of some image objects (i.e. background, stolons or polyps) was adjusted using Corel Photo-Paint software (Corel, Ottawa, Canada; background gray level = 10, stolon = 201, polyp = 255). Processed images were checked against the

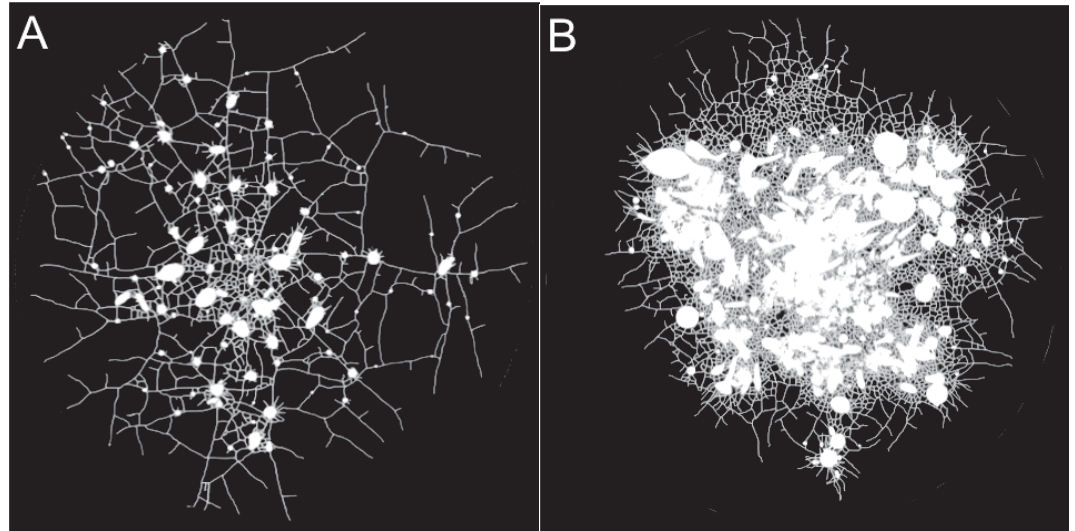


Fig. 1. Images of genetically identical colonies of *P. carnea* growing on 18 mm diameter glass cover slips near the time of covering the surface. (A) Control; (B) treated with 100 µmol l<sup>-1</sup> vitamin C. Polyps are bright and circular, while stolons are darker and web-like.

original images to insure accuracy. Processed images were measured in Image-Pro for total colony area, total polyp area, and empty, unencrusted areas within the colony ('inner' areas). Analyses focused on the mean size of these inner areas, which largely depends on stolon branching and anastomosis (i.e. as stolon development increases, mean inner area decreases). These data were natural logarithm transformed before analysis of variance (ANOVA) with PC-SAS software (SAS Institute, Cary, North Carolina, USA). Total polyp area adjusted for total colony area was analyzed in the same way. Other parameters (e.g. total colony area and number of days for a colony to cover the surface) are also reported and compared as mean ± S.E.M. (twice the S.E.M. provides a 95% confidence interval).

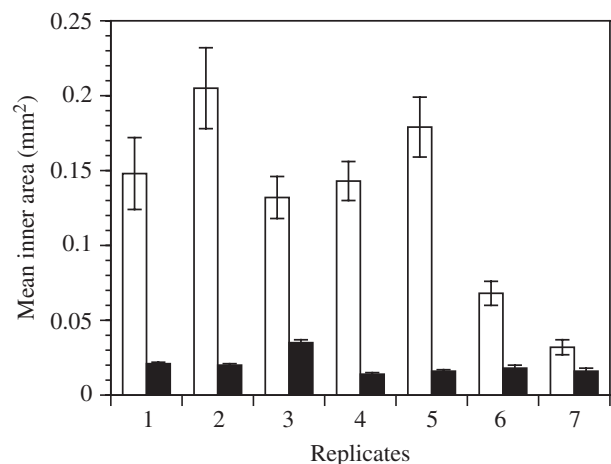


Fig. 2. Mean ± S.E.M. of the average size of the areas of empty cover slip within the colonies ('inner area') for the control colonies (unfilled bars) and colonies treated with 100 µmol l<sup>-1</sup> vitamin C (filled bars).

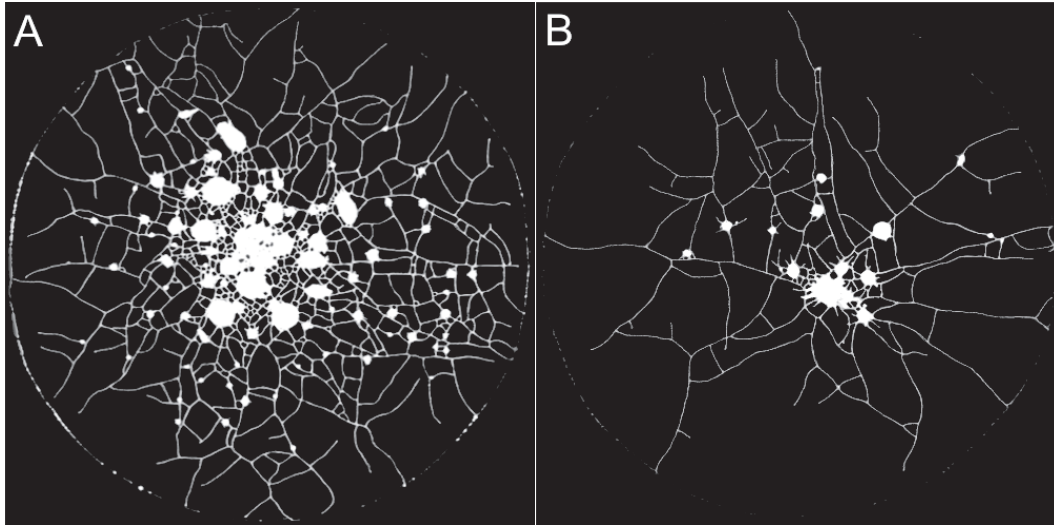


Fig. 3. Images of genetically identical colonies of *P. carnea* growing on 18 mm diameter glass cover slips near the time of covering the surface. (A) Control; (B) treated with  $0.1 \text{ mg ml}^{-1}$  catalase.

#### Comparisons of reactive oxygen species

Hydrogen peroxide represents a major component of ROS under physiological conditions (Chance et al., 1979), and 2',7'-dichlorofluorescein diacetate (H<sub>2</sub>DCFDA; Molecular Probes, Eugene, Oregon, USA) is usually used to assay H<sub>2</sub>O<sub>2</sub> (Jantzen et al., 1998; Nishikawa et al., 2000; Pei et al., 2000). This non-fluorescent dye is freely permeable to living cells. Once inside a cell, the acetate groups are removed by intracellular esterases. In turn, H<sub>2</sub>DCF is usually oxidized by peroxides in the presence of peroxidase, cytochrome c, or Fe<sup>2+</sup> to form 2',7'-dichlorofluorescein which can then be visualized with fluorescent microscopy. There is some debate as to whether the activation of H<sub>2</sub>DCF is specific for the detection of H<sub>2</sub>O<sub>2</sub> (Finkel, 2001). Conservatively, this assay should be regarded as a semi-quantitative measure of general ROS activity. A  $10 \text{ mmol l}^{-1}$  stock solution of H<sub>2</sub>DCFDA was prepared in anhydrous DMSO. Twenty-four hours after feeding, 5–7 naïve

colonies (i.e. colonies previously untreated) were incubated in the appropriate treatment with an equivalent number of control colonies. After 1 h, H<sub>2</sub>DCFDA was added to a concentration of  $10 \text{ } \mu\text{mol l}^{-1}$ , and colonies were incubated an additional hour in the dark prior to measurement. Colonies were imaged in a RC-16 chamber (Warner Instruments, Hamden, USA) in plain seawater immediately after being removed from the treatment solution. Using a Orca-100 cooled-CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan) and a Axiovert 135 (Carl Zeiss, Jena, Germany), peroxide (as indicated by H<sub>2</sub>DCFDA-derived 2',7'-dichlorofluorescein) was imaged for a  $\sim 50 \times 150 \text{ } \mu\text{m}$  region at the base of three polyps per colony (excitation 450–490 nm, emission 515–565 nm). At these wavelengths, negative controls show that there is little native

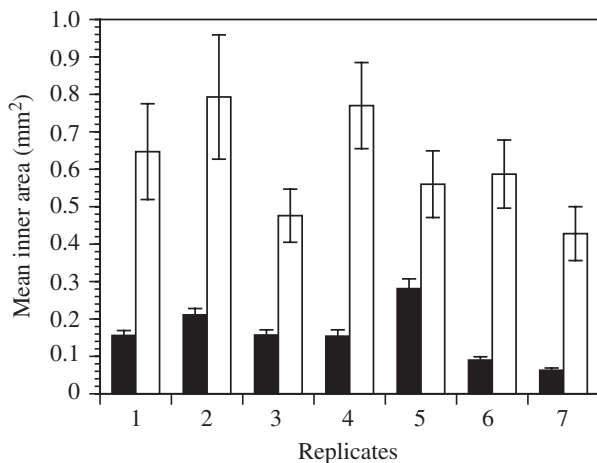


Fig. 4. Mean  $\pm$  S.E.M. of the average size of the areas of empty cover slip within the colonies ('inner area') for the control colonies (filled bars) and colonies treated with  $0.1 \text{ mg ml}^{-1}$  catalase (unfilled bars).

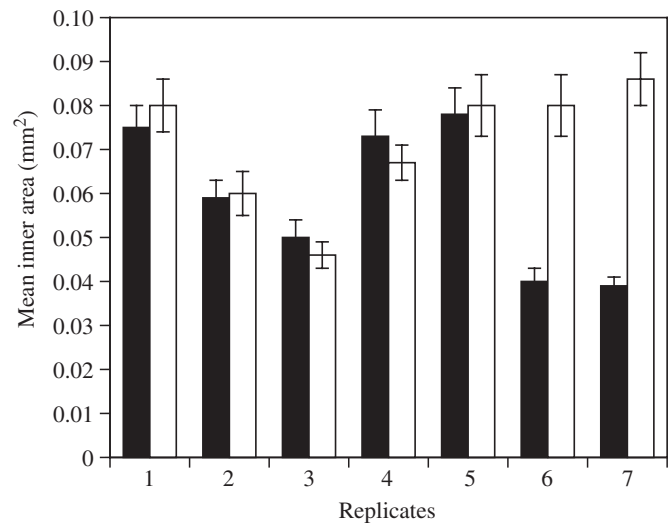


Fig. 5. Mean  $\pm$  S.E.M. of the average size of the areas of empty cover slip within the colonies ('inner area') for the control colonies (filled bars) and colonies treated with  $20\text{--}50 \text{ } \mu\text{mol l}^{-1}$  hydrogen peroxide (unfilled bars).

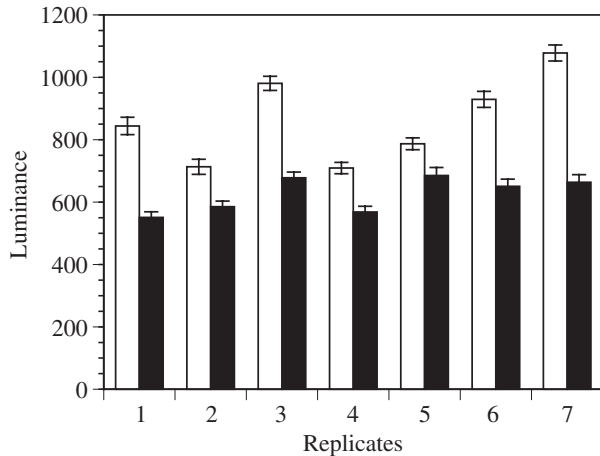


Fig. 6. Mean  $\pm$  S.E.M. luminance (grayscale from 0–4095) for three polyp–stolon junctions per replicate colony treated with H<sub>2</sub>DCFDA (unfilled bars, controls; filled bars, 100  $\mu$ mol l<sup>-1</sup> vitamin C).

fluorescence. Images with 12-bit depth (4096 gray levels) were thus obtained and were analyzed using Image-Pro Plus software. In such images, fluorescence is visible from many  $\sim$ 10 mm<sup>2</sup>-sized clusters of mitochondria from EMCs at polyp–stolon junctions (Blackstone et al., 2004). The luminance and area for each of these fluorescent objects was measured in Image Pro Plus software by: (1) selecting the object and an equivalent area of its immediate surroundings (background) as a circular region of interest; (2) allowing the software to identify the area and luminance of the foreground ‘bright’ region (i.e. the area of fluorescent signal); (3) exporting these measures to file; (4) automatically identifying the area and luminance of the complementary background ‘dark’ region and exporting these measures to file. The area of each cluster was thus calculated, and the luminance of the cluster was adjusted for the background luminance by subtraction. These measures were analyzed by a nested ANOVA, clusters nested within polyps, polyps nested within clonal replicates and replicates within treatments. In separate experiments with similarly treated naïve colonies, three peripheral stolon tips were measured per colony. Images of stolon tips were analyzed similarly, except the entire stolon tip was measured and compared with an equivalent area of the background fluorescence outside the colony.

## Results

### Comparisons of colony growth and development

In *P. carnea*, vitamin C strongly inhibits the outward growth of stolons, and the result is a slow-growing and extremely sheet-like colony with many, closely packed polyps and short stolon connections (Fig. 1). Only three treated colonies even approached covering the surface of the cover slip; the remaining four were imaged at 60 days. The controls thus achieved larger total areas (mean  $\pm$  S.E.M.; 131.05 $\pm$ 8.32 mm<sup>2</sup>) than the treated colonies (85.05 $\pm$ 16.75 mm<sup>2</sup>) over a shorter time period (controls, 33.3 $\pm$ 4.9 days; treated, 57.5 $\pm$ 1.2 days).

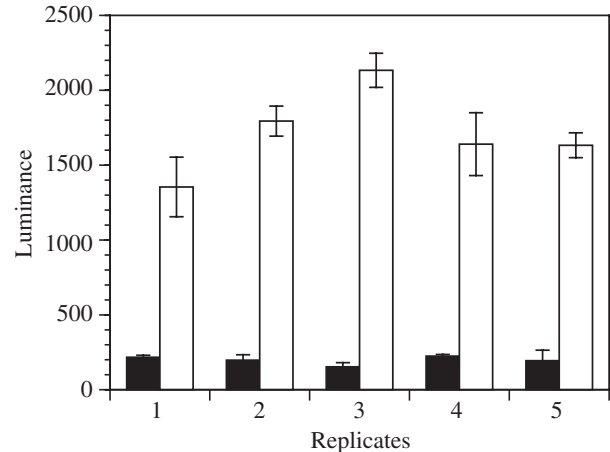


Fig. 7. Mean  $\pm$  S.E.M. luminance (grayscale from 0–4095) for three peripheral stolon tips per replicate colony treated with H<sub>2</sub>DCFDA (filled bars, controls; unfilled bars, 100  $\mu$ mol l<sup>-1</sup> vitamin C).

Treated colonies exhibited greater branching and anastomosis of stolons as indicated by the mean size of unencrusted areas within the colony (Fig. 2;  $F=42.2$ , d.f.=1, 12,  $P\ll 0.001$ ). Treated colonies also exhibited a greater percent of the total area devoted to polyp growth ( $F=21.5$ , d.f.=1, 12,  $P<0.001$ ). In *E. viridula*, vitamin C had similar effects. Treated colonies covered the surface more slowly than controls (controls, 22.3 $\pm$ 2.7 days; treated, 34.3 $\pm$ 1.3 days) and exhibited greater branching of stolons as indicated by the mean size of unencrusted areas within the colony ( $F=12.6$ , d.f.=1, 12,  $P<0.01$ ).

Catalase, conversely, triggers rapid growth of peripheral stolons away from the center of the colony in *P. carnea*, and the result is a fast-growing and extremely runner-like colony with few, widely spaced polyps and long stolon connections (Fig. 3). While catalase-treated colonies were imaged at slightly smaller total areas than controls (controls, 135.86 $\pm$ 7.2 mm<sup>2</sup>; treated, 107.15 $\pm$ 10.82 mm<sup>2</sup>), this likely reflects their extremely runner-like growth form. In other words, when covering the surface the long, unbranched stolons of the treated colonies enclosed a smaller area than the more branched stolons of the controls. Catalase-treated colonies covered the surface more quickly than controls (controls, 30.4 $\pm$ 4 days; treated, 18.1 $\pm$ 2.3 days). Treated colonies exhibited less branching and anastomosis of stolons as indicated by the mean size of unencrusted areas within the colony (Fig. 4;  $F=46.8$ , d.f.=1, 12,  $P\ll 0.001$ ). Treated colonies also exhibited a smaller percent of the total area devoted to polyp growth ( $F=13.9$ , d.f.=1, 12,  $P<0.01$ ). In *E. viridula*, catalase had similar effects. Treated colonies covered the surface more quickly than controls (controls, 19.4 $\pm$ 1 days; treated, 16 $\pm$ 0.7 days). Since the time of covering is sometimes difficult to judge in *E. viridula*, the time that the first stolon touched the cover slip edge was also measured, with similar results (controls, 12.1 $\pm$ 1.2 days; treated, 7.2 $\pm$ 0.9 days). While treated colonies did not exhibit significantly greater branching of stolons as indicated by the mean size of unencrusted areas

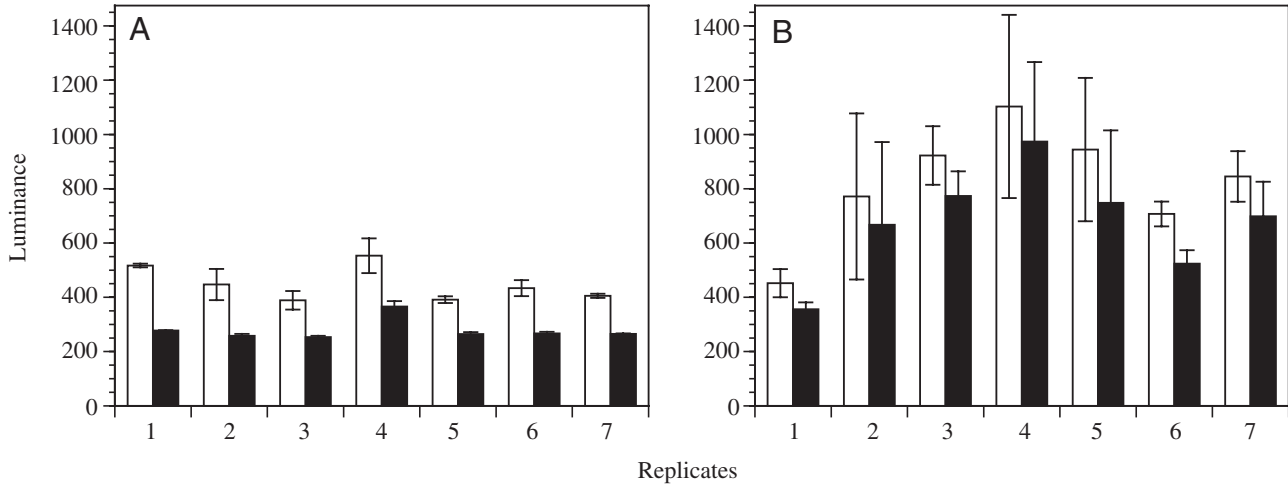


Fig. 8. Mean  $\pm$  S.E.M. luminance (grayscale from 0–4095) for three peripheral stolon tips per replicate colony treated with H<sub>2</sub>DCFDA. Unfilled bars represent the foreground luminance of the stolon tip; filled bars represent the background luminance of the surrounding area. (A) Controls; (B) 0.1 mg ml<sup>-1</sup> catalase. Colonies were imaged in a chamber containing plain seawater immediately after being removed from the treatment solution.

within the colony ( $F=2.8$ , d.f.=1, 12,  $P>0.1$ ), this is a less than ideal measure for the catalase-treated colonies of *E. viridula* because they branched so little that they did not form many inner areas. Other measures of growth form such as total colony perimeter divided by the square root of total colony area did show significant differences between catalase-treated and control colonies of *E. viridula* ( $F=19$ , d.f.=1, 12,  $P<0.001$ ), indicating that the treated colonies exhibited a more irregular, runner-like growth form (Blackstone and Buss, 1991).

Peroxide experiments were conducted in the winter; hence colonies of *P. carnea* were relatively slow-growing and sheet-like. Nevertheless, colonies treated with exogenous peroxide covered the surface faster than untreated colonies ( $56\pm 1.9$  days versus  $64\pm 2.6$  days). No significant effect was found of peroxide treatment on branching and anastomosis of stolons as indicated by the mean size of unencrusted areas within the

colony (Fig. 5;  $F=2.1$ , d.f.=1, 12,  $P>0.15$ ), nor did other measures of growth form show significant differences. Perhaps notably, the slowest growing treated and control colonies (replicates 6 and 7) did show a large difference in mean inner area and other measures. It may be that peroxide treatment has an effect under some circumstances, e.g. perhaps when endogenous levels of peroxide are low.

#### Comparisons of reactive oxygen species

In mitochondrion-rich polyp–stolon junctions in naïve colonies of *P. carnea*, vitamin C diminished levels of peroxide ~2 h after initiating treatment, as indicated by H<sub>2</sub>DCFDA-derived 2',7'-dichlorofluorescein (Fig. 6), and this difference is statistically significant ( $F=19$ , d.f.=1, 12,  $P<0.001$ ). In other naïve colonies after ~2 h, however, peripheral stolon tips in five colonies treated with vitamin C showed greatly increased

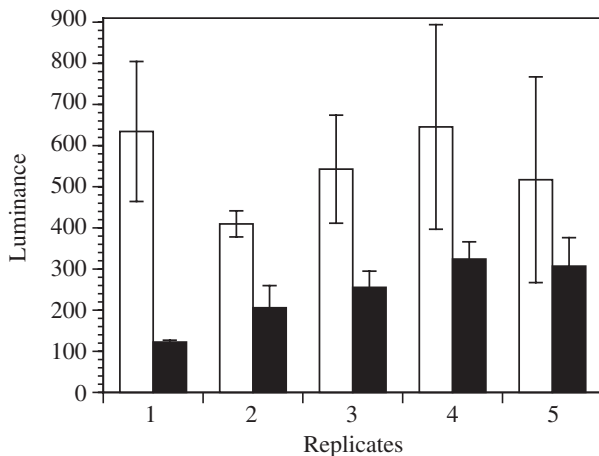


Fig. 9. Mean  $\pm$  S.E.M. luminance (grayscale from 0–4095) for three peripheral stolon tips per replicate colony treated with H<sub>2</sub>DCFDA (unfilled bars, treated with ~20–50  $\mu\text{mol l}^{-1}$  peroxide; filled bars, controls).

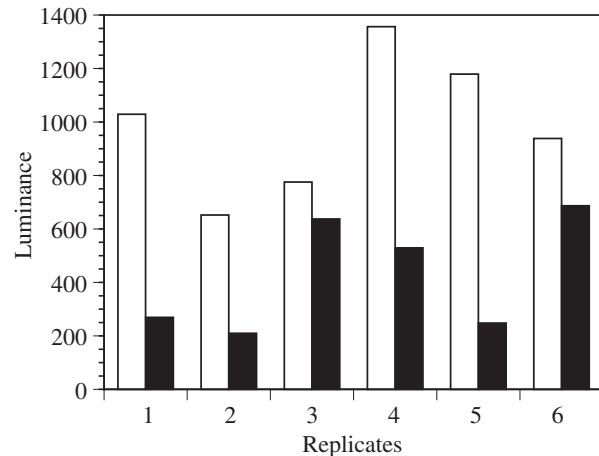


Fig. 10. Luminance (grayscale from 0–4095) for two stolon tips per replicate colony treated with H<sub>2</sub>DCFDA. A central stolon tip (unfilled bar) is compared with a peripheral stolon tip (filled bar) for each colony.

ROS levels compared with five controls (Fig. 7;  $F=140$ , d.f.=1, 8,  $P\leq 0.001$ ). This dramatic flux in peroxide occurs as these stolon tips are regressing (Blackstone et al., 2005a). In colonies treated repeatedly over many days, such stolon death does not occur; rather, stolons grow out very slowly with high rates of branching, i.e. there are not really any 'peripheral' stolons (e.g. Fig. 1). Conversely, in mitochondrion-rich polyp–stolon junctions in naïve colonies of *P. carnea*, catalase has no detectable effect on peroxide ~2 h after initiating treatment ( $F=2$ , d.f.=1, 8,  $P>0.2$ ). In other naïve colonies after ~2 h, peripheral stolon tips in colonies treated with catalase were again no different from those in the controls (Fig. 8; for the foreground – background difference;  $F=1.5$ , d.f.=1, 12,  $P>0.2$ ). While the naïve catalase-treated colonies showed no difference in relative luminance (foreground – background), they nevertheless did show an absolute difference such that treated stolon tips exhibit greater absolute levels of ROS when compared with controls (Fig. 8; for absolute foreground luminance;  $F=20.7$ , d.f.=1, 12,  $P<0.001$ ). For the latter measures, identical camera settings were used for all images to ensure that absolute measures of luminance were comparable.

In naïve colonies of *P. carnea*, those treated with exogenous peroxide for ~2 h show increased levels of ROS in stolon tips as compared with controls (Fig. 9;  $F=29.5$ , d.f.=1, 8,  $P<0.001$ ). In untreated colonies of *P. carnea*, peripheral and central stolon tips were examined for ROS, and a gradient was found such that central stolon tips exhibit greater amounts of ROS (Fig. 10; paired comparison *t*-test,  $t=4$ ,  $P<0.01$ ). Finally, colonies of *E. viridula* exhibit higher levels of ROS in stolon tips than colonies of *P. carnea* (Fig. 11;  $F=44.5$ , d.f.=1, 10,  $P<<0.001$ ).

### Discussion

In aggregate, the results lend support to the hypothesis that ROS in general and peroxide in particular are used by hydroid colonies in signaling and perhaps other processes. Nevertheless, while ROS may serve as an intermediary in mitochondrial redox signaling, treatment effects cannot necessarily be assumed in advance, nor can conclusions from one region of a colony at one time necessarily be extrapolated to the entire colony over a broader time period. With both *P. carnea* and *E. viridula*, colonies treated with vitamin C exhibit extremely sheet-like growth, much like colonies treated with uncouplers (Blackstone, 2003), yet colonies treated with catalase exhibit extremely runner-like growth. In *P. carnea*, treatment with vitamin C has the immediate effect of diminishing ROS at mitochondrion-rich polyp–stolon junctions, but also dramatically increasing ROS at stolon tips. A series of working hypotheses have been developed to explain these seemingly divergent results. The diminished ROS from mitochondrion-rich EMCs in vitamin C-treated colonies suggests that ascorbate-derived reducing capacity is transmitted into and across the plasma membrane of these cells (May, 1999). Diminished mitochondrial ROS emanating from polyp–stolon junctions generally inhibit the outgrowth of

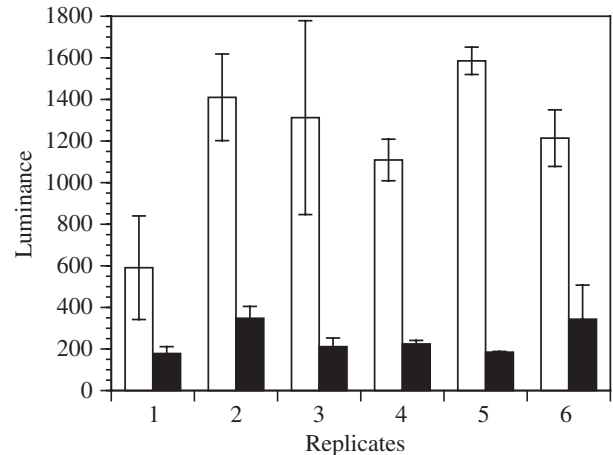


Fig. 11. Mean  $\pm$  S.E.M. luminance (grayscale from 0–4095) for three peripheral stolon tips per replicate colony treated with  $H_2DCFDA$  (unfilled bars, *E. viridula*; filled bars, *P. carnea*).

stolons and lead to sheet-like growth, and considerable research supports this hypothesis (e.g. Blackstone, 2003; Blackstone et al., 2004). In naïve colonies that have already assumed a more runner-like growth form, however, an acute response to diminished mitochondrial ROS ensues. This response may be mediated by an extreme and fleeting burst of ROS in peripheral stolon tips (possibly from non-mitochondrial sources, see Finkel, 2001; Hanna et al., 2002). Death and regression of these stolons may follow, possibly involving apoptosis (Cikala et al., 1999). In colonies treated with vitamin C over a long time period, subsequent to this initial acute response stolon tips remain healthy but in the presence of low amounts of mitochondrial ROS, they do not grow outward very quickly. Regional differences between central (i.e. polyp–stolon junctions) and peripheral (i.e. stolon tips) regions of a hydroid colony are suggested (Blackstone et al., 2005b).

Catalase, conversely, is a large (350 kDa) tetramer and is likely not taken up by a colony, nor can its enzymatic function be transferred across a plasma membrane. The lack of an effect on ROS levels of mitochondria-rich EMCs supports this hypothesis. Nevertheless, catalase probably does rapidly convert any peroxide released by the colony into water and oxygen. ROS emitted by colonies may serve a function (perhaps anti-bacterial, but see Bolm et al., 2004), hence the diminished amounts of ROS outside the colony may lead to compensatory formation and emission from within the colony. In treated colonies, stolon tissues thus have absolutely greater amounts of ROS, as suggested by the data. Such elevated levels of ROS are still considerably less than levels needed to provoke an acute response leading to cell death. Nevertheless, these elevated levels are sufficient to mimic mitochondrial redox signaling and result in rapid, runner-like growth. In support of this hypothesis, colonies treated with exogenous peroxide cover the surface of an 18 mm diameter cover slip faster than untreated colonies. Furthermore, there is a gradient in colonies of *P. carnea* with central stolon tips, which are



closer to the majority of the mitochondrion-rich EMCs, exhibiting higher levels of ROS than peripheral stolon tips. Finally, colonies of *E. viridula* exhibit higher levels of ROS in stolon tips than colonies of *P. carnea*, and this correlates with their extremely rapid, runner-like growth. It is not known what molecules may be the targets of such ROS. Nevertheless, cysteine-rich proteins involved in vascular development (e.g. vascular endothelial growth factors; Seipel et al., 2004) provide plausible candidates.

On the basis of these data, we hypothesize that in hydroid colonies ROS participate in a number of putative signaling pathways. High levels of ROS may be a factor in the cell and tissue death that seem to affect peripheral stolon tips when the environment is rapidly changing. Such a process would seem adaptive – if the colony becomes ‘overextended,’ stolons can retreat and the nutrients in the cells and tissues of the stolon may be taken up by the remainder of the colony. ROS emitted from the colony also seem to have an extra-colony function, perhaps in suppressing the growth of bacteria or other parasites. Hydractiniid hydroid colonies grow on snail shells that are crowded with epifauna and probably some of these can be rebuffed by peroxide. Notably, the foot region of *Hydra* is characterized by the activity of a peroxidase (Hoffmeister-Ullerich et al., 2002). *Hydra* may also emit peroxide and may use this peroxidase to protect its own tissue at the point of attachment to the substratum. More moderate levels of ROS in stolon tips seem to act as a growth factor, triggering outward growth, inhibiting branching and, possibly, mediating the redox signaling emanating from mitochondrion-rich EMCs. Treatment with exogenous peroxide suggests that stolon tips are capable of concentrating peroxide. Peroxide emitted from polyp–stolon junctions could be carried by gastrovascular flow to stolon tips. Nevertheless, because of the multiple pathways for peroxide, the particular phenotypic effects may depend on the spatial and temporal patterns of ROS formation within the colony. While the work reported here serves to outline the broad possibilities for signaling using ROS in colonial hydroids, considerable amounts of future research will be required to elucidate these spatial and temporal patterns, as well as the molecular targets of ROS.

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