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Redox signaling in the growth and development of colonial hydroids

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Summary

Redox signaling provides a quick and efficient mechanism for clonal or colonial organisms to adapt their growth and development to aspects of the environment, e.g. the food supply. A 'signature' of mitochondrial redox signaling, particularly as mediated by reactive oxygen species (ROS), can be elucidated by experimental manipulation of the electron transport chain. The major sites of ROS formation are found at NADH dehydrogenase of complex I and at the interface between coenzyme Q and complex III. Inhibitors of complex III should thus upregulate ROS from both sites; inhibitors of complex I should upregulate ROS from the first but not the second site, while uncouplers of oxidative phosphorylation should downregulate ROS from both sites. To investigate the possibility of such redox signaling, perturbations of colony growth and development were carried out using the hydroid *Podocoryna carnea*. Oxygen uptake of colonies was measured to determine comparable physiological doses of antimycin A_1 (an inhibitor of complex III), rotenone (an inhibitor of complex I) and carbonyl cyanide m-chlorophenylhydrazone (CCCP; an uncoupler of oxidative phosphorylation). Using these doses, clear effects on colony growth and development were obtained. Treatment with antimycin A_1 results in 'runner-like' colony growth, with widely spaced polyps and stolon branches, while treatment with CCCP results in 'sheet-like' growth, with closely spaced polyps and stolon branches. Parallel results have been obtained previously with azide, an inhibitor of complex IV, and dinitrophenol, another uncoupler of oxidative phosphorylation. Perhaps surprisingly, rotenone produced effects on colony development similar to those of CCCP. Assays of peroxides using 2′,7′-dichlorofluorescin diacetate and fluorescent microscopy suggest a moderate difference in ROS formation between the antimycin and rotenone treatments. The second site of ROS formation (the interface between coenzyme Q and complex III) may thus predominate in the signaling that regulates colony development. The fat-rich, brine shrimp diet of these hydroids may be relevant in this context. Acyl CoA dehydrogenase, which catalyzes the first step in the mitochondrial β-oxidation of fatty acids, carries electrons to coenzyme Q, thus bypassing complex I. These results support a role for redox signaling, mediated by ROS, in colony development. Nevertheless, other redox sensors between complexes I and III may yet be found.

Key words: clonal, colony development, evolutionary morphology, hydroid, *Podocoryna*, *Podocoryne*, reactive oxygen species, redox signaling.

Introduction

Individual colonial animals are relatively persistent and long-lived, occupying a given habitat for long periods of time. Such longevity presents environmental challenges not faced by more ephemeral organisms. Food supply, for instance, may vary in time and space in such habitats, and adjusting the timing and spacing of feeding structures and gastrovascular connections may represent a major challenge for colonial animals. Consider a colonial cnidarian: the arrangements of polyps (feeding structures) and stolons (vascular connections) are major determinants of a colony's ability to acquire food (Larwood and Rosen, 1979; Jackson et al., 1985; Buss, 1990). 'Sheet-like' growth – closely spaced polyps with short vascular connections – can enhance food-gathering ability in a particular location, while 'runner-like' growth – widely spaced polyps with long gastrovascular connections – can efficiently 'sample' a barren microhabitat that may in the near term become food rich. In an environment where food supply is locally variable, a colony's fitness can thus be enhanced by developmental mechanisms that are locally sensitive to the food supply.

Redox signaling is a reliable mechanism by which an environmental signal can be transduced into gene activity (Allen, 1993). In animals, redox signaling typically occurs when the redox states of electron carriers of the mitochondria are perturbed by substrate (Nishikawa et al., 2000; Blackstone, 2001; Brownlee, 2001; Rutter et al., 2001) or related factors (Bürkle, 2000; Coffman and Davidson, 2001; Larsen and Clarke, 2002). Such perturbations can alter the rate of formation of reactive oxygen species (ROS; reduced electron carriers are more likely to donate electrons to oxygen, while

oxidized electron carriers are less likely to do so). ROS are frequently, but not always, a key intermediary in metabolic and redox signaling (Nishikawa et al., 2000; Brownlee, 2001; Echtay et al., 2002; Larsen and Clarke, 2002; Nemoto and Finkel, 2002). Such a mechanism may function in colonial animals. For instance, if a growing hydroid colony encounters an area locally rich in food, polyps in the food-rich area will experience a surfeit of nutrients. These nutrients will trigger contractions of polyp epitheliomuscular cells and resulting gastrovascular flow (Wagner et al., 1998; Dudgeon et al., 1999). Because of this metabolic demand, fed polyps will be more oxidized, with lower levels of peroxide, than unfed polyps (Blackstone, 2001). If such a redox gradient can differentially affect the timing of polyp and stolon tip development, adaptive changes in the local pattern of colony development can result.

This hypothesis allows a series of predictions about the effects of commonly used experimental manipulations of the mitochondrial electron transport chain with uncouplers of oxidative phosphorylation or inhibitors of the individual complexes of this chain (Scheffler, 1999). The major sites of ROS formation are found at NADH dehydrogenase of complex I and at the interface between coenzyme Q and complex III (Nishikawa et al., 2000). Inhibitors of complexes III and IV should thus upregulate ROS from both sites; inhibitors of complex I that act 'downstream' of NADH dehydrogenase should upregulate ROS from the first but not the second site, while uncouplers of oxidative phosphorylation should downregulate ROS from both sites (Fig. 1). If ROS mediate colony development, inhibitors of complexes III and IV should produce similar phenotypic effects, and these effects should differ from those of uncouplers of oxidative phosphorylation. Other things being equal, inhibitors of complex I should have intermediate phenotypic effects. Treatments of colonial hydroids with azide (an inhibitor of complex IV) and dinitrophenol (an uncoupler of oxidative phosphorylation) support this hypothesis; the former leads to relatively reduced redox states and runner-like growth, while the latter leads to relative oxidation and sheet-like growth (Blackstone, 1999).

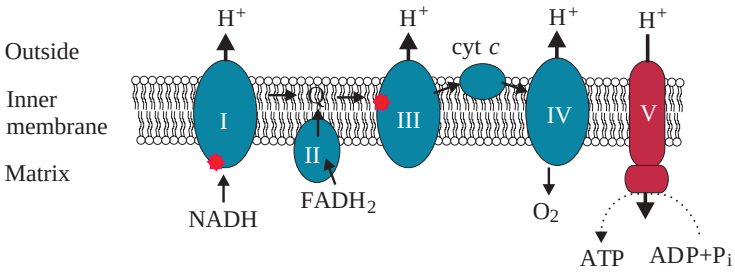

Fig. 1. Schemata of the mitochondrial electron transport chain, showing complexes I–V, coenzyme Q and cytochrome *c*. Small arrows trace the flow of electrons from NADH and $FADH_2$ to oxygen. Large arrows show the extrusion of protons (H^+) by complexes I, III and IV and the return of protons to the matrix *via* complex V, triggering the assembly of ATP (dashed arrow). Red stars indicate the two major sites of reactive oxygen formation. Uncouplers of oxidative phosphorylation allow electrons to return to the matrix without passing through complex V (after Blackstone and Kirkwood, 2003).

To further investigate such redox signaling, perturbations of colony growth and development were carried out using the hydroid *Podocoryna carnea*. Oxygen uptake of colonies was measured to determine comparable physiological doses of antimycin A_1 (an inhibitor of complex III), rotenone (an inhibitor of complex I downstream of NADH dehydrogenase) and carbonyl cyanide m-chlorophenylhydrazone (CCCP; an uncoupler of oxidative phosphorylation). Genetically identical replicate colonies were grown at appropriate physiological doses, and colony development was measured. Using fluorescent microscopy of colonies treated with antimycin and rotenone, assays of peroxides were carried out with 2′,7′-dichlorofluorescin diacetate. The data obtained from these experiments support the hypothesis that redox state and ROS are factors that mediate adaptive colony development, although alternative hypotheses are also discussed.

Materials and methods

Culture conditions

Podocoryna (= *Podocoryne*) *carnea* Sars colonies of a single clone were cultured using standard methods (e.g. Blackstone, 1999; the same clone, P3, has been used extensively in previous investigations). For measures of oxygen uptake, colonies were grown from small explants on 12 mm diameter circular glass cover slips; for various measures of polyp and stolon development, colonies were grown on 12 mm, 15 mm or 18 mm diameter circular glass cover slips; for measures of peroxides, colonies were grown on 15 mm diameter circular glass cover slips. Growth of the colonies was confined to one side of the cover slip by daily scraping with a razor blade. All experiments were carried out at 20.5°C.

Measures of oxygen uptake

The actions of inhibitors and uncouplers can be gauged by the effects on oxygen uptake; the former inhibit uptake, while the latter stimulate it. Comparable physiological doses can thus be determined. For instance, if two inhibitors at different concentrations produce the same effect on oxygen uptake, a comparable physiological dose has been obtained. Similarly, if an uncoupler at a particular concentration produces a similar, but inverse, effect as these inhibitors, again a comparable physiological dose has been obtained. Ideally, in all cases the perturbation of oxygen uptake should be mild to moderate, i.e. physiologically appropriate, so as not to introduce any pathological responses.

To determine comparable and appropriate physiological doses, standard concentrations of inhibitors and uncouplers were used: 1 μmol l^{-1} CCCP, 1 μmol l^{-1} antimycin A_1 and 10 μmol l^{-1} rotenone (e.g. Erecinska

and Wilson, 1981; Nishikawa et al., 2000). Stock solutions of the inhibitors were prepared in ethanol, while the stock of CCCP was prepared in seawater. For each compound, five assays were carried out; five assays were also performed for each of the two controls (ethanol at the appropriate concentration in seawater and plain seawater). For each of these assays, a *P. carnea* colony on a 12 mm diameter cover slip was attached with a drop of silicone grease to a cover slip cemented to a small magnet. This assembly was contained in a 13 mm diameter sealed glass chamber (RC300; Strathkelvin, Glasgow, UK) with 0.7 ml of seawater (filtered to 0.2 μm). Chamber temperature was held constant (20.5±0.02°C) using an external circulation water bath (RTE-100D; Neslab, Newington, USA), and the rate of decline in oxygen concentration over a 30 min period was measured (using a Strathkelvin 1302 electrode and a 781 oxygen meter) with stirring (by slowly spinning the magnet, cover slips and colony). The chamber was then opened, a small volume of seawater removed, an equivalent amount of the appropriate stock solution added to achieve the target concentration, the solution mixed and aerated thoroughly with a small pipette, and the chamber resealed (this procedure took approximately 7 min). The rate of decline in oxygen concentration was then measured over another 30 min period. These assays were performed 3–5 h after the feeding of the subject colony as part of the normal feeding schedule. For each colony, the before/after difference in the rate of decline in oxygen concentration over a 30 min period was calculated, where this decline was measured by the least-squared slope of oxygen concentration *versus* time. An overall trend in these differences for the five colonies was analyzed using a paired-comparison *t*-test. Furthermore, as the controls showed slight changes (the ethanol control showed a slight decrease in oxygen consumption, while the seawater control showed a slight increase), the change in slope for each treatment was tested against the change in slope of the appropriate control using a standard *t*-test.

Comparisons of colony growth and development

A series of experiments was carried out to investigate the effects of these compounds on colony growth. Generally, the same target concentrations were used as in the oxygen uptake experiments. Several preliminary experiments were performed to determine the optimal time of exposure and to test the effects of different solvents [ethanol and dimethyl sulfoxide (DMSO)]. Stocks of the inhibitors and uncouplers were prepared at concentrations that allowed the same solvent concentrations in all treatments. Controls were carried out at these solvent concentrations (ethanol, 0.17%; DMSO, 0.08%). Although each of these initial trials showed roughly similar trends (data not shown), observations suggested that ethanol had a greater deleterious effect on the colonies than DMSO. In part, this may be because the latter dissolved higher stock concentrations of rotenone and thus allowed lower concentrations of solvent in all treatments.

An in-depth follow-up experiment was thus performed using DMSO as the solvent. Twenty-two replicates were explanted on 15 mm cover slips, with five each assigned to control (0.08% DMSO) and to antimycin A_1 treatments and six each assigned to rotenone and to CCCP treatments. Each group was treated with the appropriate solution for approximately 4–6 h day^{-1}. As previously (e.g. Blackstone, 1999), inhibitors and uncouplers seemed to be best tolerated by colonies when treatment was intermittent. As each colony initiated medusae production (up to 60 days after explanting), that colony was imaged. Images were processed to facilitate automatic measurement in Image-Pro Plus software (Mediacybernetics, Silver Spring, USA). The gray level of some image objects (i.e. background, stolons or polyps) was adjusted using Corel Photo-Paint software (background gray level = 10, stolon = 201, polyp = 255). Processed images were checked against the original images to ensure accuracy. Processed images were measured in Image-Pro for the following parameters: total colony area and perimeter, total cover slip area outside the colony, total unencrusted cover slip area within the colony, and total polyp area.

Using PC-SAS software (SAS Institute, Cary, USA), the four treatments of this experiment were compared using univariate analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA) for the relationship between the total area of polyps and the total area of empty, unencrusted cover slip both inside and outside the colony. Both polyp area and empty, unencrusted area were expressed as a fraction of the total surface area of the cover slip (176.71 mm^2). To better meet the assumptions of parametric statistics, these data were arcsine transformed before analysis. Polyp area is clearly a measure of polyp development; empty, unencrusted cover slip area is a measure of how the colony covers and monopolizes space. This latter trait largely depends on stolon branching and anastomosis (i.e. as stolon development increases, empty cover slip area decreases). A second measure of stolon branching and anastomosis was also used: the mean size of the empty, unencrusted areas within the colony. These latter data were natural logarithm transformed before analysis with ANOVA.

Comparisons of ROS

Hydrogen peroxide represents a major component of ROS under physiological conditions (Chance et al., 1979), and measures of H_2O_2 were taken using 2′,7′-dichlorofluorescin diacetate (H_2DCFDA; 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, H_2DCFDA is deacetylated to H_2DCF, which in turn interacts with peroxides to form 2′,7′-dichlorofluorescein, which can then be visualized with fluorescent microscopy. The activation of H_2DCF is relatively specific for the detection of H_2O_2 as well as secondary and tertiary peroxides. Nevertheless, H_2O_2 is usually the major peroxide within cells and is primarily measured by this method. A 10 mmol l^{-1} stock solution of H_2DCFDA was prepared in anhydrous DMSO. One day after feeding, five colonies each were incubated in antimycin A_1 and rotenone (from stocks in DMSO) at the same

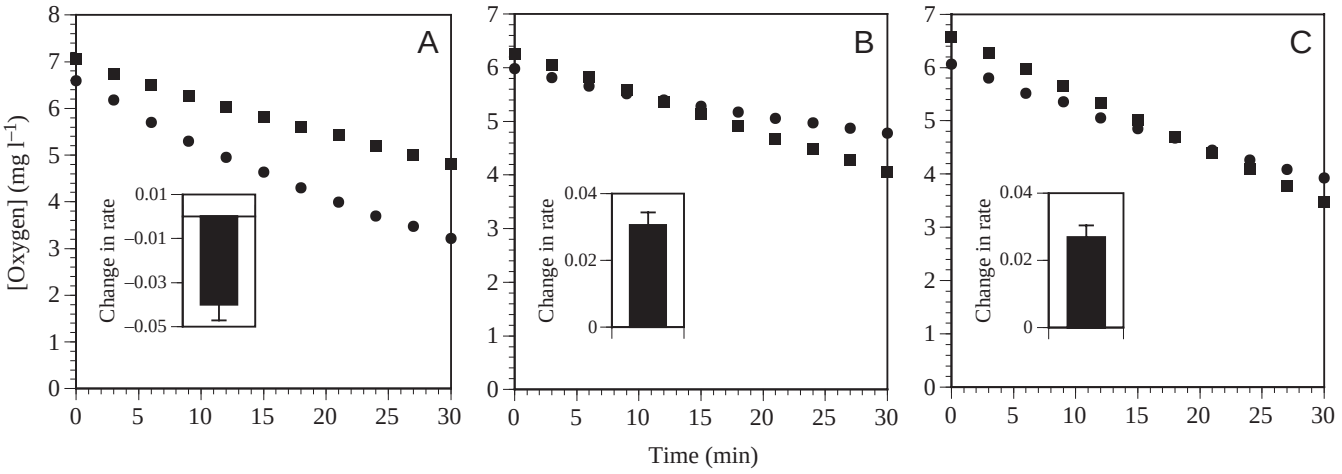

Fig. 2. Rate of decline in oxygen concentration for *P. carnea* colonies before (squares) and after (circles) treatment with (A) 1 μmol l^{-1} CCCP, (B) 1 μmol l^{-1} antimycin A_1 and (C) 10 μmol l^{-1} rotenone. For five colonies of each treatment, inset plots show the mean ± S.E.M. of the before/after difference in the rate of decline in oxygen concentration, where this decline is measured by the least-squared slope of oxygen concentration *versus* time. This difference in rate was significantly negative for (A), i.e. the oxygen uptake increased after treatment, and significantly positive for (B) and (C), i.e. the oxygen uptake decreased after treatment.

concentrations as in the oxygen uptake experiments. Within 1 h, H_2DCFDA was added to a concentration of 10 μmol l^{-1}, and colonies were incubated for an additional hour in the dark prior to measurement. Using a Hamamatsu Orca-100 cooled-CCD camera and a Zeiss Axiovert 135, peroxide (as indicated by H_2DCFDA-derived 2′,7′-dichlorofluorescein) was imaged for an approximately 50 μm×150 μm region at the base of three polyps per colony (excitation 450–490 nm, emission 515–565 nm). The major metabolic signals from these colonies emanate from polyps, particularly the epitheliomuscle cells near the junction of polyps and stolons. These cells exhibit clusters of mitochondria surrounding their muscle fibers, and these mitochondria provide strong metabolic signals relative to the remainder of the colony (Blackstone, 1999, 2001). Images with 12-bit depth (4096 gray levels) were thus obtained and were analyzed in Image-Pro. A major conceptual difficulty in such an analysis is determining where to draw the boundary between the foreground, bright areas (which constitute the area of fluorescent signal) and the background (see for instance Fig. 6 and Blackstone, 2001). This consideration is particularly relevant here because H_2O_2 easily diffuses, and a concentration gradient is expected. Fortunately, advances in image analysis technology allow an objective resolution of this methodological difficulty. For each image, after the stolon area was outlined as the region of interest, the software automatically identified the ‘bright areas’ (i.e. the area of signal) and the ‘dark areas’ (i.e. the background), using the same algorithm for each image. Total areas and average luminance of both bright and dark areas were measured. Bright area luminance was corrected for background luminance by subtraction, and the data were analyzed by a nested ANOVA (polyp nested within replicate colony, replicate colony nested within treatment).

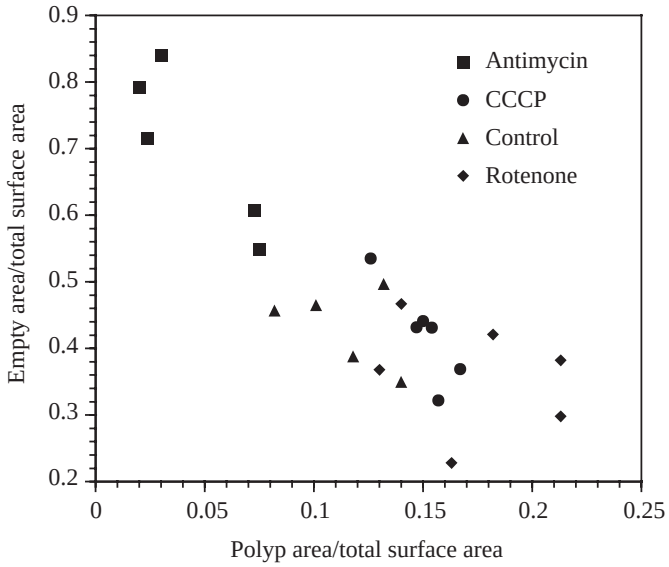

Fig. 3. Bivariate scatterplots of the amount of stolon development (inversely correlated to empty area/total surface area) and the amount of polyp development (polyp area/total surface area) for genetically identical *P. carnea* colonies at the initiation of medusa production. Squares, treated with 1 μmol l^{-1} antimycin; circles, treated with 1 μmol l^{-1} m-chlorophenylhydrazone (CCCP); triangles, treated with 0.08% dimethyl sulfoxide; diamonds, treated with 10 μmol l^{-1} rotenone.

Results

Measures of oxygen uptake

At appropriate physiological doses, uncouplers are expected to increase oxygen uptake, while inhibitors are expected to decrease uptake. Such results are obtained in the present study (Fig. 2). CCCP significantly increased oxygen uptake after treatment (Fig. 2A; paired-comparison *t*-test, t=–5.56, P<0.01,

N=5). While seawater controls also showed a slight increase, the increase triggered by CCCP treatment was significantly greater (*t*-test, *t*=4.9, *P*<0.01, *N*=10). On the other hand, antimycin significantly decreased oxygen uptake after treatment (Fig. 2B; paired-comparison *t*-test, *t*=7.99, *P*<0.01, *N*=5). While ethanol controls also showed a slight decrease, the decrease triggered by antimycin was significantly greater (*t*-test, *t*=3.36, *P*<0.01, *N*=10). Similarly, rotenone significantly decreased oxygen uptake after treatment (Fig. 2C; paired-comparison *t*-test, *t*=7.7, *P*<0.01, *N*=5), and this decrease was significantly greater than the controls (*t*-test, *t*=2.82, *P*<0.05, *N*=10). Generally, these results suggest that the treatment concentrations of inhibitors and uncouplers provide appropriate and roughly comparable physiological doses.

Comparisons of colony growth and development

Each colony was imaged at the initiation of medusa production (Fig. 3). A significant treatment effect is apparent (MANOVA, *F*=9.3, d.f.=6, 34, *P*<0.001), and this effect derives from an effect on both polyp development (ANOVA, *F*=24.4, d.f.=3, 18, *P*<0.001) and stolon development (ANOVA, *F*=15.6, d.f.=3, 18, *P*<0.001). In essence, antimycin produces less polyp and stolon development and thus more runner-like phenotypes than CCCP or rotenone. Strikingly, the most divergent colony morphologies are produced by treatment with antimycin and rotenone (Fig. 4).

An analysis of the average size of the areas of empty cover slip within the colonies shows a similar pattern (Fig. 5). This measure reflects the branching and anastomosis of stolons within a colony, and there is a significant treatment effect (ANOVA, *F*=42.8, d.f.=3, 18, *P*<0.001). Indicative of more runner-like growth, colonies treated with antimycin have larger inner areas than the controls. Indicative of more sheet-like growth, colonies treated with CCCP and rotenone have smaller inner areas. Again, there is a clear difference between colonies treated with antimycin and rotenone, although in this case colonies treated with CCCP are slightly more divergent.

Comparisons of reactive oxygen species

Peroxide, as indicated by H_2DCFDA-derived 2′,7′-dichlorofluorescein, provides a strong signal in the epitheliomuscle cells at the base of polyps (Fig. 6). At the fluorescein excitation and emission wavelengths, negative controls show that there is little native fluorescence under these conditions (Blackstone, 2001). Fluorescence at fluorescein wavelengths can thus be attributed to H_2DCFDA. There is a

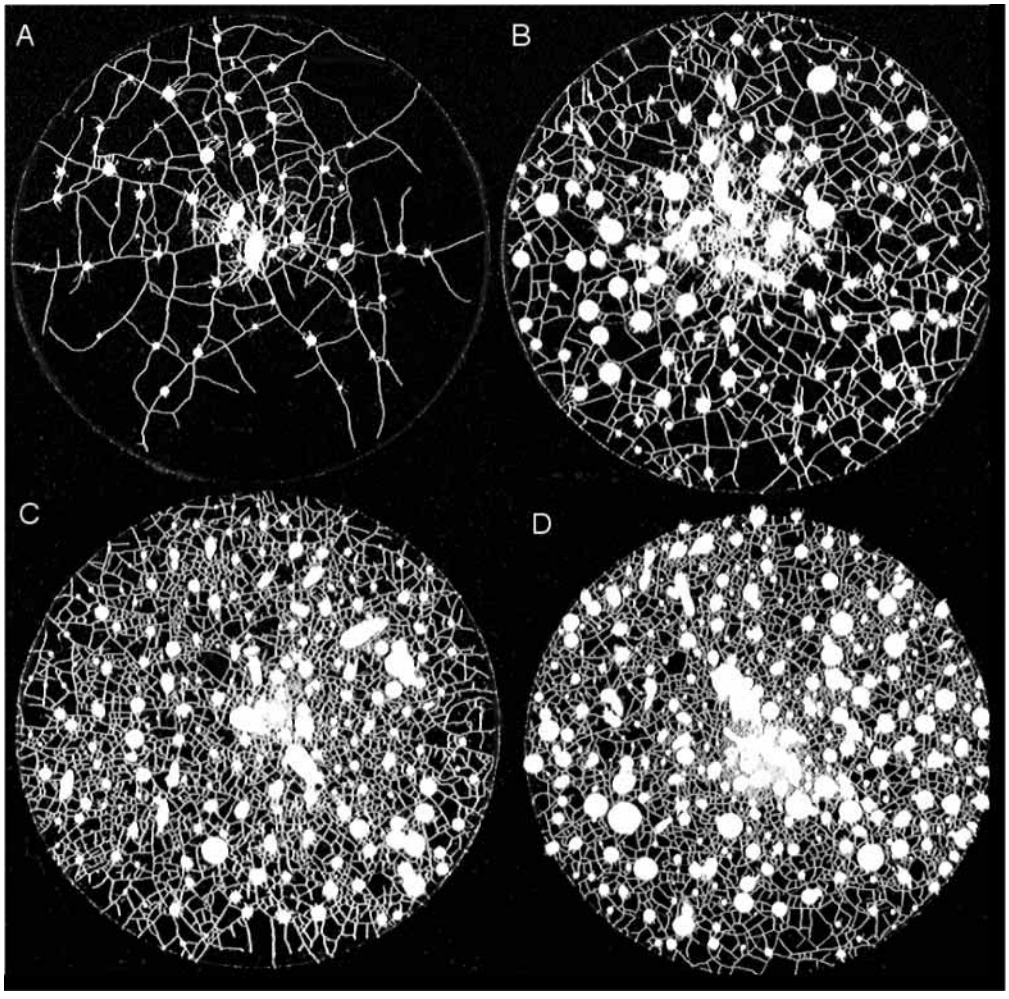

Fig. 4. Images of genetically identical colonies of *P. carnea* growing on 15 mm diameter glass cover slips at the initiation of medusa production. Treatments are as in Fig. 3: (A) antimycin, (B) DMSO control, (C) CCCP and (D) rotenone.

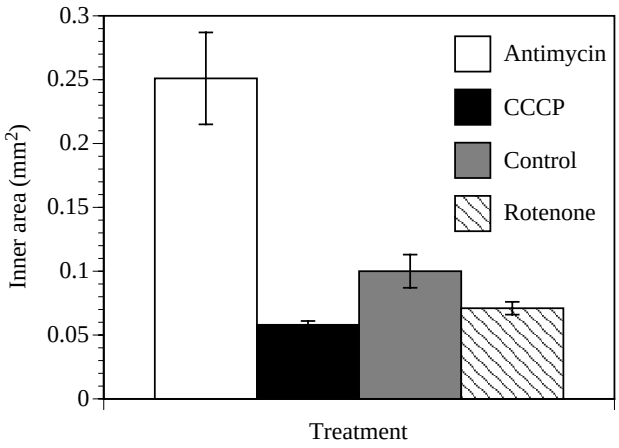

Fig. 5. Mean ± S.E.M. of the average size of the areas of empty cover slip within the colonies ('inner area') for the colonies treated as in Fig. 3.

slight, but statistically significant, difference between the fluorescence emitted by polyps treated with antimycin and those treated with rotenone (Fig. 7; nested ANOVA, F=6.03, d.f.=1, 8, P<0.05).

Discussion

Generally, these results support the hypothesis that redox signaling is a factor in determining the patterns of colony growth and development of these hydroids. At comparable physiological doses, blocking the mitochondrial electron transport chain at complex III with antimycin produces the same phenotypic effects as blocking at complex IV with azide (Blackstone, 1999). 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, the resulting phenotype consists of runner-like growth with widely spaced polyps and stolon branches. On the other hand, at appropriate physiological doses, the uncoupler CCCP has the same phenotypic effect as another uncoupler, dinitrophenol (Blackstone, 1999). This phenotypic effect is similar to that observed in areas of colonies that are well fed: sheet-like growth, with closely spaced polyps and stolon branches (Blackstone, 2001).

Interpreting these results depends critically on the observation that direct feeding triggers high amplitude contractions of polyps (Wagner et al., 1998; Dudgeon et al., 1999). In view of these studies, the data consistently suggest that relative oxidation of mitochondrial electron transport chains in polyp epitheliomuscular cells has a permissive effect on polyp and stolon branch initiation. Relative oxidation can be achieved either by the strong metabolic demand associated with feeding-related contractions of these muscle cells or by treatment with uncouplers of oxidative phosphorylation. On the other hand, relative reduction of mitochondrial electron transport chains in polyps seems to have an inhibitory effect on polyp and stolon branch initiation. Relative reduction can be achieved either by the low metabolic demand associated with only indirect feeding or by treatment with inhibitors of complexes III and IV.

Patterns of colony development thus correspond to distinct mitochondrial redox states. Sheet-like growth corresponds to 'state 3' (plentiful substrate and high metabolic demand). Runner-like growth corresponds to 'state 4' (sufficient substrate and low metabolic demand; Scheffler, 1999). Uncouplers mimic state 3, while inhibitors of complexes III and IV mimic state 4. This correlation suggests that signaling occurs between mitochondrial redox state and pattern-forming genes located in the nucleus (cf. Coffman and Davidson,

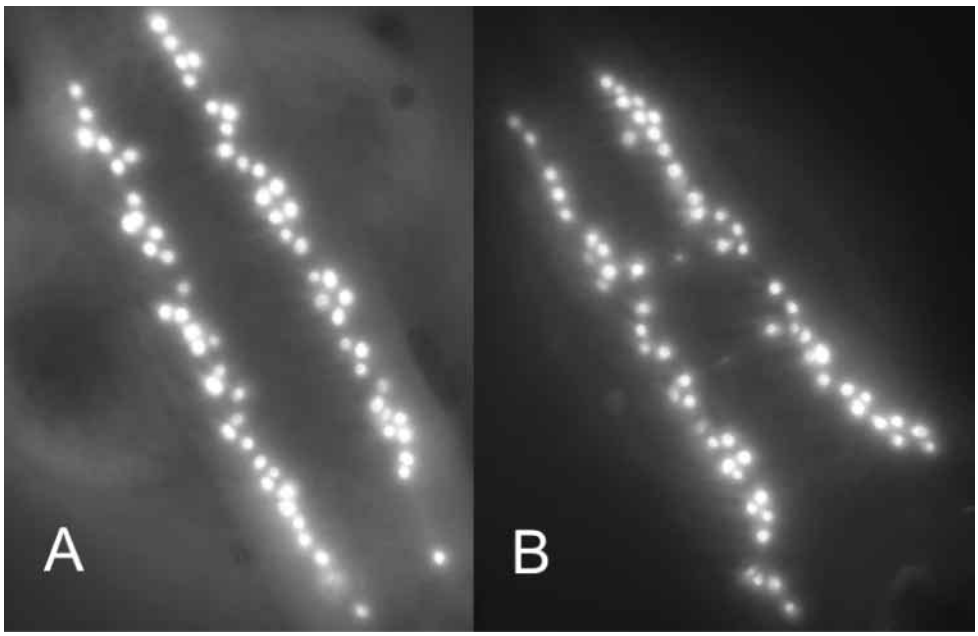

Fig. 6. Images of an approximately 50 μm×150 μm region at the base of two living polyps [treatments: (A) antimycin, (B) rotenone]. Each image shows emission at fluorescein wavelengths after treatment with 2′,7′-dichlorofluorescin diacetate (H_2DCFDA). The principal signal is from clusters of mitochondria surrounding muscle fibers in epitheliomuscular cells (approximately 3–4 μm in diameter). After background correction, there is a slight difference in the luminance (given in greyscale) of these objects in these two images; A=1485, B=1345 (grayscale from 0 to 4095; compare with Fig. 7).

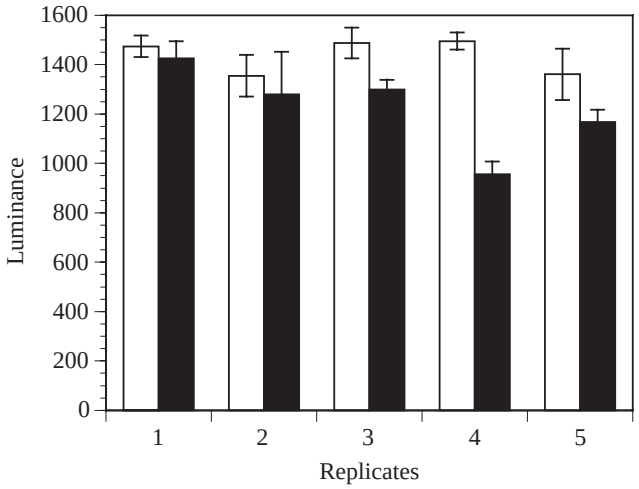

Fig. 7. Mean ± S.E.M. luminance (grayscale from 0 to 4095) for three polyps per replicate colony. Colonies were treated with either antimycin (open bars) or rotenone (filled bars).

2001). Often, mitochondrial signaling is mediated by ROS (Nishikawa et al., 2000; Brownlee, 2001; Echtay et al., 2002; Larsen and Clarke, 2002; Nemoto and Finkel, 2002). The correlation between redox state and peroxide levels in hydroid polyps is suggestive in this regard (Blackstone, 2001). More direct tests involve inhibiting electron transport at specific complexes of the electron transport chain relative to sites of ROS formation (Nishikawa et al., 2001). Here, rotenone was used to inhibit electron transport downstream of the first site of ROS formation and upstream of the second site (Fig. 1). 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 the second site of ROS generation. A similar pattern has also been found in other taxa, e.g. bacteria (Georgellis et al., 2001) and mammals (Nishikawa et al., 2001). The latter work implicates ROS in signal transduction, while the former implicates coenzyme Q. In hydroids, a slight but statistically significant difference was found in peroxide levels between polyps treated with rotenone and those treated with antimycin. ROS may thus have a role in signal transduction in these organisms as well.

Signaling from the second site of ROS generation may be more common than signaling from the first site because the former experiences greater electron flux. For instance, during typical metazoan aerobic metabolism, only electrons from NADH will pass through the first site. On the other hand, except for those few electrons donated to oxygen at the first site, electrons from both NADH and $FADH_2$ will pass through the second site (Fig. 1). Factors that enhance the formation of $FADH_2$ will exaggerate this effect. In particular, the relatively fat-rich diet of brine shrimp may explain why the second site of ROS formation is the principal locus of redox signaling in these hydroids. Acyl-CoA dehydrogenase, which catalyzes the first step in mitochondrial β-oxidation of fatty acids, has an FAD cofactor and carries electrons to coenzyme Q, thus bypassing complex I. Further oxidation of fatty acids does produce NADH and ultimately acetyl-CoA, which is then metabolized in the tricarboxylic acid (TCA) cycle. The TCA cycle yields principally NADH, which of course carries electrons to the electron transport chain *via* complex I. Overall, such fatty acid metabolism probably produces considerably greater electron flux at the second site of ROS generation than at the first. This may explain the observed effects of rotenone as compared with antimycin.

Some consideration of these experimental results in a natural context is perhaps warranted. Hydroids such as *Podocoryna carnea* usually encrust gastropod shells inhabited by hermit crabs. Colony feeding may be affected by various hermit crab-related factors (Van Winkle et al., 2000), e.g. how the hermit crab holds the shell and directs water currents over it. As hermit crabs molt, grow and switch shells over a period of days and weeks, these factors probably vary. Food-related perturbations of the growth of the hydroid colony would ensue, as generally suggested in the Introduction. While the fat content of the hydroid diet in nature is likely to be variable and, on average, less than that of brine shrimp, a greater flux of electrons at the second site of ROS generation is still expected (e.g. even with a carbohydrate diet). Thus, the second site will consistently produce the bulk of the ROS, and the signaling pathway suggested here will efficiently direct an adaptive response to feeding-related perturbations.

The results presented here thus strongly support the hypothesis of redox signaling in the growth and development of these hydroid colonies. Furthermore, these results strongly implicate a signal emanating from between complexes I and III of the mitochondrial electron transport chain. This signal may consist of ROS, and there is some support for this hypothesis. Nevertheless, signaling *via* other intermediaries in addition to or instead of ROS cannot be ruled out at this time. Experiments that perturb the appropriate electron carriers and simultaneously block the effects of ROS are likely to be informative.

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References

Allen, J. F. (1993). Control of gene expression by redox potential and the requirement for chloroplast and mitochondrial genomes. *J. Theor. Biol.* **165**, 609-631.

Blackstone, N. W. (1999). Redox control in development and evolution: evidence from colonial hydroids. *J. Exp. Biol.* **202**, 3541-3553.

Blackstone, N. W. (2001). Redox state, reactive oxygen species, and adaptive growth in colonial hydroids. *J. Exp. Biol.* **204**, 1845-1853.

Blackstone, N. W. and Kirkwood, T. B. L. (in press). Mitochondria and programmed cell death: "slave revolt" or community homeostasis? In

Genetic and Cultural Evolution of Cooperation. Dahlem Workshop Report (ed. P. Hammerstein). Cambridge, MA: MIT Press.

Brownlee, M. (2001). Biochemistry and molecular cell biology of diabetic complications. *Nature* **414**, 813-820.

Bürkle, A. (2000). Poly(ADP-ribosyl)ation, genomic instability, and longevity. *Ann. New York Acad. Sci.* **908**, 126-132.

Buss, L. W. (1990). Competition within and between encrusting colonial invertebrates. *Trends Ecol. Evol*. **5**, 352-356.

Chance, B., Sies, H. and Boveris, A. (1979). Hydroperoxide metabolism in mammalian organs. *Physiol. Rev.* **59**, 527-605.

Coffman, J. A. and Davidson, E. H. (2001). Oral-aboral axis specification in the sea urchin embryo. *Dev. Biol*. **230**, 18-28.

Dudgeon, S. R., Wagner, A., Vaisnys, J. R. and Buss, L. W. (1999). Dynamics of gastrovascular circulation in the hydrozoan *Podocoryne carnea*: the 1-polyp case. *Biol. Bull. Mar. Biol. Lab. Woods Hole* **196**, 1-17.

Echtay, K. S., Roussel, D., St-Pierre, J., Jekabsons, M. B., Cadenas, S., Stuart, J. A., Harper, J. A., Roebuck, S. J., Morrison, A., Pickering, S., Clapham, J. C. and Brand, M. D. (2002). Superoxide activates mitochondrial uncoupling proteins. *Nature* **415**, 96-99.

Georgellis, D., Kwon, O. and Lin, E. C. C. (2001). Quinones as the redox signal for the arc two-component system of bacteria. *Science* **292**, 2314-2316.

Jackson, J. B. C., Buss, L. W. and Cook, R. E. (ed.) (1985). *Population Biology and Evolution of Clonal Organisms*. New Haven, CT: Yale University Press.

Jantzen, H., Hassel, M. and Schulze, I. (1998). Hydroperoxides mediate lithium effects on regeneration in *Hydra*. *Comp. Biochem. Physiol. C* **119**, 165-175.

Larsen, P. L. and Clarke, C. F. (2002). Extension of life-span in *Caenorhabditis elegans* by a diet lacking coenzyme Q. *Science* **295**, 120-123.

Larwood, G. and Rosen, B. (ed.) (1979). *Biology and Systematics of Colonial Organisms*. London: Academic Press.

Nemoto, S. and Finkel, T. (2002). Redox regulation of forkhead proteins through a *p66shc*-dependent signaling pathway. *Science* **295**, 2450-2452.

Nishikawa, T., Edelstein, D., Du, X. L., Yamagishi, S.-I., Matsumura, T., Kaneda, Y., Yorek, M. A., Beebe, D., Oates, P. J., Hammes, H.-P., Giardino, I. and Brownlee, M. (2000). Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* **404**, 787-790.

Pei, Z.-M., Murata, Y., Benning, G., Thomine, S., Klüsener, B., Allen, G. J., Grill, E. and Schroeder, J. I. (2000). Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. *Nature* **406**, 731-734.

Rutter, J., Reick, M., Wu, L. C. and McKnight, S. L. (2001). Regulation of clock and NPAS2 DNA binding by the redox state of NAD cofactors. *Science* **293**, 510-514.

Scheffler, I. E. (1999). *Mitochondria*. New York: John Wiley.

Van Winkle, D. H., Longnecker, K. and Blackstone, N. W. (2000). The effects of hermit crabs on hydractiniid hydroids. *Mar. Ecol.* **21**, 55-67.

Wagner, A., Dudgeon, S. D., Vaisnys, R. J. and Buss, L. W. (1998). Nonlinear oscillations in polyps of the colonial hydroid *Podocoryne carnea*. *Naturwissenschaften* **85**, 1-5.