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REDOX STATE, REACTIVE OXYGEN SPECIES AND ADAPTIVE GROWTH IN COLONIAL HYDROIDS

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Summary

Colonial metazoans often encrust surfaces over which the food supply varies in time or space. In such an environment, adaptive colony development entails adjusting the timing and spacing of feeding structures and gastrovascular connections to correspond to this variable food supply. To investigate the possibility of such adaptive growth, within-colony differential feeding experiments were carried out using the hydroid *Podocoryna carnea*. Indeed, such colonies strongly exhibited adaptive growth, developing dense arrays of polyps (feeding structures) and gastrovascular connections in areas that were fed relative to areas that were starved, and this effect became more consistent over time. To investigate mechanisms of signaling between the food supply and colony development, measurements were taken of metabolic parameters that have been implicated in signal transduction in other systems, particularly redox state and levels of reactive oxygen species. Utilizing fluorescence microscopy of *P. carnea* cells *in vivo*, simultaneous measurements of redox state [using NAD(P)H] and hydrogen peroxide (using 2′,7′-dichlorofluorescin diacetate) were taken. Both measures focused on polyp epitheliomuscular cells, since these exhibit the greatest metabolic activity. Colonies 3–5 h after feeding were relatively oxidized, with low levels of peroxide, while colonies 24 h after feeding were relatively reduced, with high levels of peroxide. The functional role of polyps in feeding and generating gastrovascular flow probably produced this dichotomy. Polyps 3–5 h after feeding contract maximally, and this metabolic demand probably shifts the redox state in the direction of oxidation and diminishes levels of reactive oxygen species. In contrast, 24 h after feeding, polyps are quiescent, and this lack of metabolic demand probably shifts the redox state in the direction of reduction and increases levels of reactive oxygen species. Within-colony differential feeding experiments were carried out on colonies 24 h after the usual, colony-wide feeding. At this time, a single polyp was fed, and this polyp was compared with an otherwise similar polyp from the same colony. A pattern similar to the whole-colony experiments was obtained: the just-fed polyp, as it begins contracting shortly after feeding, appears to be relatively oxidized, with low levels of peroxide compared with the polyp that was not fed. These data are consistent with the hypothesis that adaptive colony development in response to a variable food supply is mediated by redox state or reactive oxygen species or both, although alternative hypotheses are also discussed.

Key words: adaptation, clonal, colony, evolutionary morphology, hydroid, NAD(P)H fluorescence, *Podocoryna carnea*, *Podocoryne carnea*, reactive oxygen species, redox control.

Introduction

In contrast to the ‘worms, flies, mice’ more frequently studied by biologists, individual colonial animals are relatively persistent and long-lived, occupying a given habitat for long periods. Such longevity presents environmental challenges not faced by more ephemeral organisms. Food supply, for instance, may vary in space and time 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; Buss and Blackstone, 1991). Closely spaced polyps with short vascular connections can enhance food-gathering ability in a particular location, while widely spaced polyps with long gastrovascular connections can efficiently ‘sample’ a barren microhabitat that may, in the short 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. Natural selection will favor those mechanisms of development that link environmental cues to the timing of the initiation of polyps and stolon tips relative to rates of stolon elongation (Buss and Blackstone, 1991).

Redox control is a reliable mechanism by which an environmental signal can be transduced into gene activity (Allen, 1993). In eukaryotic redox control, perturbations of the redox state of electron carriers in chloroplasts or mitochondria are transduced into gene activity, and an adaptive response ensues (Escobar Galvis et al., 1998; Pfannschmidt et al., 1999; Dai et al., 2000). Such a mechanism might also 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, and the colony's local redox state may be altered relative to that outside the food-rich area (Blackstone, 1998a; Blackstone, 1999; Wagner et al., 1998; Dudgeon et al., 1999). If such a metabolic gradient can differentially affect the timing of polyp and stolon tip development, adaptive changes in the local pattern of colony development can result.

In fact, links between metabolic gradients and metazoan development were once widely accepted, particularly by workers on clonal and colonial organisms (Child, 1941; Tardent, 1963; Rose, 1970). The outlook of Child and co-workers, who studied primarily hydroids and planarians, contrasted sharply with that of contemporaries such as T. H. Morgan and colleagues, who, of course, studied *Drosophila melanogaster* (Mitman and Fausto-Sterling, 1992). While the views of Child and like-minded colleagues have fallen into disrepute, legitimate scientific grounds for their advocacy of metabolic regulation of development in clonal and colonial organisms may yet be found (Blackstone, 2000), and a considerable amount of recent work provides a general framework for this view (e.g. Eto et al., 1999; Dai et al., 2000; Nishikawa et al., 2000; Smith et al., 2000).

In particular, there is considerable evidence that reactive oxygen species (ROS), abundantly produced by mitochondria, are a key intermediary in metabolic and redox signaling, either by themselves or in conjunction with nitric oxide (Wells, 1999; Chiueh, 2000; Nishikawa et al., 2000). Generally, when metabolic demand is low and substrate is still available, mitochondria will enter the resting state. In this state, phosphorylation is minimal, electron carriers are highly reduced and these carriers can act like a poorly insulated wire (Wells, 1999), readily donating electrons to oxygen (Chance et al., 1979; Poyton and McEwen, 1996; Nishikawa et al., 2000). However, when metabolic demand is high and sufficient substrate is available, mitochondria will enter 'state 3' (Scheffler, 1999). In this state, mitochondria are phosphorylating maximally, electron carriers are oxidized and ROS formation is low (Chance and Baltscheffsky, 1958; Chance et al., 1979). Finally, when there is metabolic demand but insufficient substrate, mitochondria will enter 'state 2' (Scheffler, 1999). In this state, phosphorylation is substrate-limited, electron carriers are highly oxidized and ROS formation is at a minimum (Chance and Baltscheffsky, 1958; Chance et al., 1979). While these generalities may appear counter to the widely held notion that a high metabolic rate correlates with high levels of ROS formation, in fact, no such contradiction exists. Cells with a high metabolic rate develop many mitochondria and many electron carriers per mitochondrion; in such cells, the presence of many metal-containing macromolecules invariably leads to ROS formation (Allen, 1996; Chiueh, 2000). Nevertheless, the same cells will emit more ROS when their mitochondria are in the resting state than when their mitochondria are in state 2 or 3.

When considering the effects of food supply on a hydroid colony, a key feature of these (and other) colonies is that substrate is shared among polyps. Thus, if a single polyp feeds, it will subsequently pump food-containing fluid throughout the colony. Colony-wide gradients of substrate are thus of short duration. However, gradients of metabolic demand may be more persistent. Contractions of polyp epitheliomuscular cells largely drive the gastrovascular flow, and these contractions probably constitute a major metabolic cost to the colony. For some time after feeding, a fed polyp may contract more actively than unfed polyps within the same colony (i.e. stage 2 of Dudgeon et al., 1999). Thus, gradients in metabolic demand may potentially occur in a differentially fed colony.

To investigate such metabolic gradients and their relationship to adaptive colony development, *Podocoryna carnea* colonies were used in a series of experimental manipulations. Within-colony differential feeding experiments were carried out, and polyp and stolon development were measured. Subsequently, redox state and ROS levels were assessed in whole colonies both 3–5 h and 24 h after feeding. Finally, in colonies 24 h after feeding, a single polyp was fed, and measurements of redox state and ROS of the fed polyp were compared with measurements of another similar, but unfed, polyp of the same colony. The data obtained from these experiments are compatible with the hypothesis that redox state and ROS mediate adaptive colony development, although alternative hypotheses are also discussed.

Materials and methods

Culture conditions

Podocoryna (=*Podocoryne*) *carnea* colonies of a single clone were cultured using standard methods (see Blackstone, 1996; Blackstone, 1998a). For measurements of polyp and stolon development, clonal replicates were grown from small explants on 18 mm diameter round glass coverslips; for measurements of redox state and ROS, clonal replicates were grown on 15 mm diameter round glass coverslips. All experiments were carried out at 20.5 °C.

Within-colony differential feeding and colony development

Ten small clonal replicates were assigned at random to two treatments: controls, in which all polyps were equally fed brine shrimp, and treated, in which only a single peripheral polyp was fed. A 10 µl mouth pipette was used to feed all polyps, and colonies of both treatments received approximately the same total number of brine shrimp as food each day. Initially, the single fed polyp of each treated colony was fed continuously

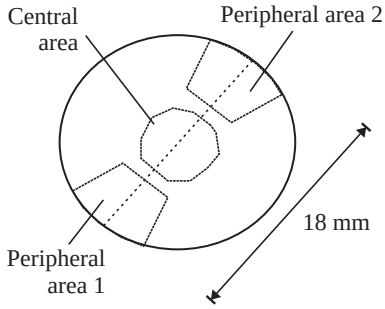

Fig. 1. Schematic diagram of a circular coverslip showing the sampling of differentially fed and control colonies. The transect (dotted line bisecting the overslip) determines three 30 mm^2 areas (the central area and peripheral areas 1 and 2) in which the development of the colony was quantified as described in the text. In differentially fed colonies, peripheral area 1 contains the area that was fed.

throughout each day to supply the colony with sufficient food. As the treated colonies developed, those polyps that were initiated distal to the original fed polyp on the same stolon system were also fed, and thus the feeding each day was accomplished in less and less time as the experiment progressed.

In both treatments, colony development was measured using image analysis with Optimas software. Two images were taken for each colony, after both 30 and 80 days of treatment. For each image, a linear transect was applied to the colony, and three 30 mm^2 areas along this transect were measured: peripheral area 1, the central area and peripheral area 2 (Fig. 1). In the differentially fed colonies, the fed area was always designated peripheral area 1, and the transect was defined as the line between this area and the center of the colony. In the controls, a random-numbers generator was used to define the angle from the horizontal for this transect line, and the peripheral area at the beginning of this line was designated peripheral area 1. For each of the three areas in all colonies, three measurements were taken: the total area, the total area of polyps and the total area of empty, unencrusted coverslip. Both polyp area and empty, unencrusted inner area were expressed as a fraction of the total area (note that the total area of stolons can be calculated as 1 minus this combined fraction, although this third variable was not used in the analyses). Polyp area is clearly a measure of polyp development; empty, unencrusted inner area is largely a measure of stolon branching and anastomosis (as these aspects of stolon development increase, inner area decreases). Although polyps can shield empty inner area from observation and measurement, in practice this is a minor source of error because stolon development is generally most extensive at the base of the polyps. Thus, polyp area and unencrusted inner area behave as largely independent measures of two different aspects of colony development (for further discussion of methods, see Blackstone, 1996).

Analysis of these data focused on within-colony variation. In the controls, comparisons between the two peripheral areas assess within-colony variation due to unknown causes, while comparisons between the central and peripheral areas may indicate more systematic aspects of colony variation (e.g. the center contains the original explant, it represents the oldest part of the colony and it provides the nexus for the movement of substrate). In the treated colonies, in addition to variation due to unknown causes, comparisons between peripheral areas assess the treatment effects, i.e. direct feeding *versus* direct starvation (albeit indirect feeding because of food exchange between the fed polyps and the rest of the colony). Comparisons between peripheral area 1 and the central area assess treatment effects and, again, possibly more systematic aspects of colony organization and variation. Hence, differences in polyp area and unencrusted inner area were calculated by two paired comparisons, i.e. subtracting the values of peripheral area 2 and the central area from those of peripheral area 1 for each colony. Using PC-SAS software for each of the two differences, paired-comparison *t*-tests were used to test whether the mean for the five replicates of each treatment was significantly different from zero. Data approximately met the assumptions of parametric statistics, and arcsine transformations provided similar results overall (and no better fit to these assumptions).

Whole-colony feeding, redox state and ROS

Polyps in these colonies are contracting maximally 2–8 h after feeding but are largely quiescent 24 h after feeding (Schierwater et al., 1992; Dudgeon et al., 1999). Previous studies using spectrofluorometry and fluorescence microscopy have shown that colony-wide feeding shifts the redox state in the direction of oxidation, whereas 24 h of starvation shifts the redox state in the direction of reduction (Blackstone, 1997; Blackstone, 1998a). To assess the effects of these treatments on levels of ROS, simultaneous measurements of redox state and ROS were carried out. Two treatments (3–5 h and more than 24 h after feeding) of four replicate colonies each were used. Two polyps from each replicate were each measured for 10 epitheliomuscular cell fibers (as described below). Comparisons at the level of the muscle fibers were carried out using least-squared regression and analysis of covariance (ANCOVA). These exploratory analyses were intended to examine the general trends of ROS and redox state and the concordance of these trends with other studies of these variables. Between-treatment effects were more precisely measured using a mixed-model analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA), which took into account the nested structure of the data (i.e. fibers within polyps, polyps within replicates and replicates within treatments). Generally, these data fitted the assumptions of parametric statistics, although for some calculated variables (e.g. integrated area of peroxide), natural logarithmic transformations were used because they provided a better fit to these assumptions.

The characteristic fluorescence of NADH and NADPH compared with the oxidized forms of these molecules can be used to measure cellular redox state (Chance, 1991; Hajnóczky

et al., 1995; Eto et al., 1999). NAD(P)H fluorescence includes both mitochondrial and cytosolic compartments. Under physiological conditions, these compartments are in a slowly equilibrated steady state, and the redox states show corresponding behavior when subject to perturbation (Scholz et al., 1969; Hajnóczky et al., 1995). Localized measurements of NAD(P)H fluorescence were obtained as described elsewhere (Blackstone, 1998a; Blackstone, 1999). Briefly, with a Zeiss Axiovert 135 and ultraviolet light (excitation at 365 nm, barrier filter at 420 nm), images were recorded on film (10 s exposure, ASA 160 balanced for tungsten filaments), digitized and quantified with densitometry in Optimas software. In such images, stolons appear dark, except for a weak signal from the chitinous perisarc (stolons lack the muscular fibers characteristic of polyp epitheliomuscular cells; Schierwater et al., 1992), whereas polyps show a much stronger signal. Because polyps are highly contractile *in vivo*, only the base can be used in precise between-polyp comparisons. In cross-sectional images of the base of a living polyp, the fluorescence of the base of the polyp epitheliomuscular cell fibers or myonemes can be clearly identified (Blackstone, 1998a). These fibers form a longitudinal network in a polyp, and their contractions drive the gastrovascular flow. Often, 50 or more fibers are visible at the base of each polyp. Using a random-numbers generator, 10 fibers were selected for each polyp. Measurements were taken of the total cross-sectional area of these 10 fibers and their relative luminance. The relative luminance was calculated as the ratio of the average luminance of the fiber throughout its entire cross-sectional area to the average luminance of an equivalent-sized area immediately surrounding that fiber.

Hydrogen peroxide represents a major component of ROS under physiological conditions (Chance et al., 1979), and simultaneous measurements 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). Living cells are freely permeable to this non-fluorescent dye. 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 and for secondary and tertiary peroxides. Nevertheless, H_2O_2 is usually the major peroxide within cells and is the major peroxide measured by this method. A 10 mmol l^{-1} stock solution of H_2DCFDA was prepared in anhydrous dimethylsulfoxide (DMSO); colonies were incubated in sea water containing 10 μmol l^{-1} H_2DCFDA in the dark for 1 h prior to measurement. Negative controls were treated with the same concentration of DMSO. Again, using the Zeiss Axiovert 135, immediately following the imaging of NAD(P)H, peroxide (as indicated by H_2DCFDA-derived 2′,7′-dichlorofluorescein) was imaged for the identical region (excitation 450–490 nm, barrier filter 515–565 nm), and images were analyzed using the same protocols. The same 10 fibers were analyzed for the relative luminance of both NAD(P)H and peroxide.

Within-colony differential feeding, redox state and ROS

Within-colony measures of redox state and peroxide levels are somewhat more complex and have the disadvantage of exposing the colony to considerable amounts of light prior to measurements of fluorescence. A small colony (10–15 polyps) 24 h after feeding was incubated in sea water containing 10 μmol l^{-1} H_2DCFDA in the dark for 30 min. At this point, the colony was removed and examined under a dissecting microscope. Two similar-sized polyps from opposite sides of the colony were identified; one of these polyps was fed a single brine shrimp. Once the polyp had successfully engulfed the brine shrimp, the colony was returned to incubation in H_2DCFDA. After 15–30 min, the colony was examined again for indications of active gastrovascular flow emanating from the fed polyp. Once this flow had been identified, the fed polyp and the unfed polyp were imaged for NAD(P)H and peroxide, as indicated by H_2DCFDA-derived 2′,7′-dichlorofluorescein. In this way, 12 replicate colonies were imaged. During image analysis, 10 epitheliomuscular cell fibers were again selected for each polyp using a random-numbers generator, and these fibers were imaged as described above. Data were analyzed in two ways. First, the mean values for the 10 fibers from each polyp were taken, and between-polyp, within-colony differences were calculated and analyzed using paired-comparison *t*-tests for the 12 replicates. Second, a nested ANOVA (fibers within polyps, polyps within replicates) was used. In this latter analysis, the effect of feeding is apparent from the between-polyp, within-replicate effect. Compared with the *t*-test, the ANOVA has the advantage of including the between-fiber, within-polyp variance in the analysis.

Results

Within-colony differential feeding and colony development

Differential feeding had a noticeable and sometimes striking effect on the development of polyps and stolons in a colony (Fig. 2). In control colonies at 30 days (Fig. 3A), peripheral area 1 has significantly less polyp area (paired comparison *t*-test, $t=-5.5$, $P<0.01$) and significantly more unencrusted inner area ($t=5.47$, $P<0.01$) than the central area. At the same time, peripheral area 1 is not significantly different from peripheral area 2 for these measures ($t=-0.58$, $P>0.5$; $t=0.79$, $P>0.45$, respectively). In contrast, in differentially fed colonies after 30 days (Fig. 3B), peripheral area 1 (the fed area) has more polyp area and less unencrusted inner area than the other two areas. These differences are nearly significant for the central area ($t=2.17$, $P=0.09$; $t=-2.19$, $P=0.09$, respectively) and are significant for peripheral area 2 ($t=3.17$, $P<0.05$; $t=-5.13$, $P<0.01$, respectively). In control colonies after 80 days (Fig. 3C), peripheral area 1 is not significantly different in polyp area ($t=-1.37$, $P>0.2$) but has significantly more inner area ($t=4.74$, $P<0.01$) than the central area. At the same time, peripheral area 1 is not significantly different from peripheral area 2 for these measures ($t=0.4$, $P>0.7$; $t=-0.24$, $P>0.8$, respectively). Finally, in differentially fed colonies after 80 days (Fig. 3D), peripheral area 1 (the fed area) has more polyp

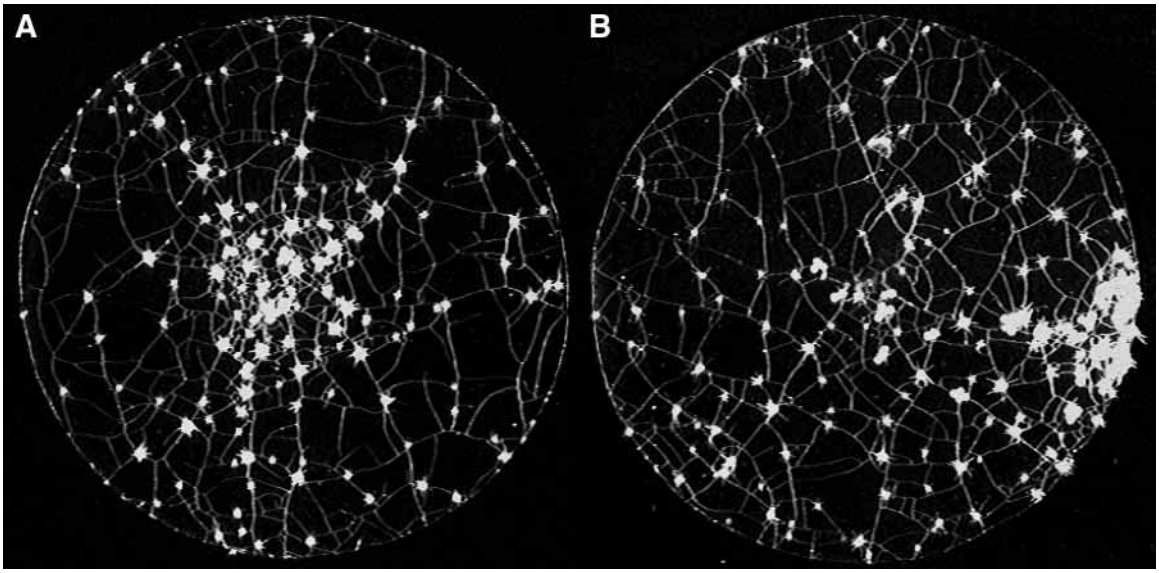

Fig. 2. Images of genetically identical colonies of *Podocoryna carnea* growing on 18 mm diameter glass coverslips after 30 days of differential feeding (A, control; B, differentially fed). Polyps are bright and circular; stolons are darker and web-like; unencrusted 'inner' areas of coverslip appear dark. The triangular patch of very dense polyp and stolon growth on the right of colony in B is the 'fed' area; the remainder of this colony was not directly fed.

area and less unencrusted inner area than the other two areas. These differences are significant both for the central area (t=16, $P \ll 0.001$; t=–7.2, P<0.01, respectively) and for peripheral area 2 (t=13.7, P<0.001; t=–11.1, P<0.001, respectively).

Whole-colony feeding, redox state and ROS

The native fluorescence of NAD(P)H is a relatively weak, but nonetheless quantifiable, signal (Blackstone, 1998a; Blackstone, 1999). Peroxide, as indicated by H_2DCFDA-derived 2′,7′-dichlorofluorescein, provides a considerably stronger signal. At the fluorescein excitation and emission wavelengths, negative controls show that there is very little native fluorescence (Fig. 4). Fluorescence at fluorescein wavelengths can thus be attributed to H_2DCFDA.

At the level of the epitheliomuscular cell fiber, simple exploratory analyses suggest that these data conform with other studies of redox state and ROS. As expected, there is a correlation between these two variables. For fibers from colonies 24 h after feeding, there is a moderate correlation (Fig. 5; r^2=0.44) between the relative luminance of NAD(P)H and peroxide. For fibers from colonies 3–5 h after feeding, this correlation is considerably weaker (Fig. 5; r^2=0.13), although still statistically significant (F=12, d.f.=1,78, P<0.001). The correlations for the two

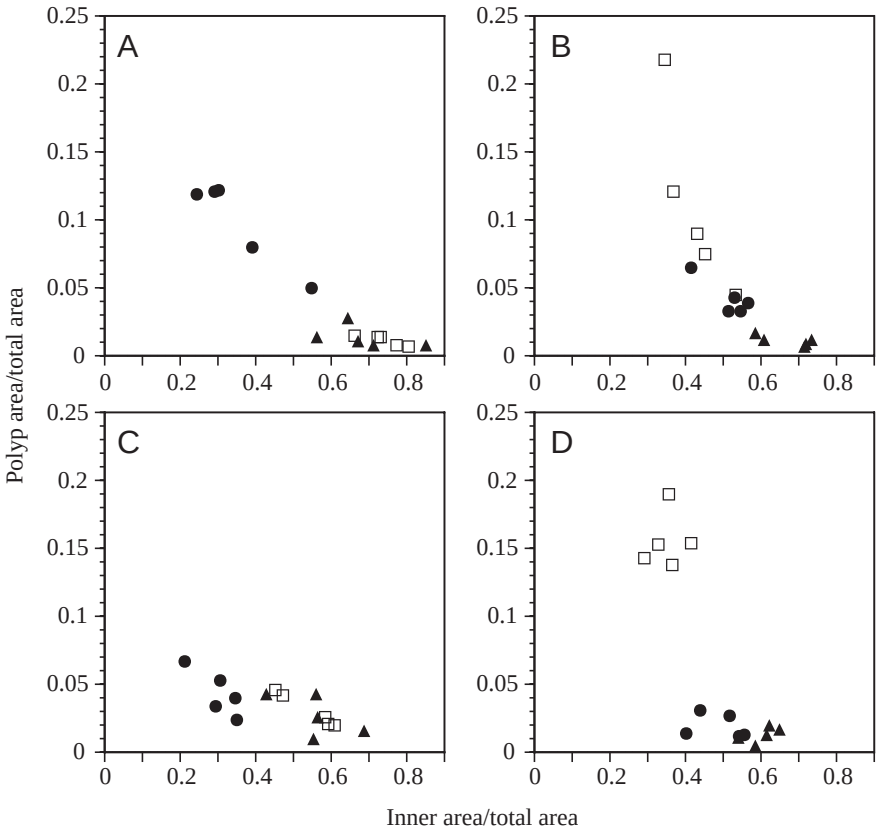

Fig. 3. Bivariate scatterplots of the amount of stolon development (inversely correlated to inner area/total colony area) and the amount of polyp development (polyp area/total area) for peripheral area 1 (open squares), the central area (circles) and peripheral area 2 (triangles) of five genetically identical *Podocoryna carnea* colonies (A, controls at 30 days; B, differentially fed colonies at 30 days; C, controls at 80 days; D, differentially fed colonies at 80 days). In differentially fed colonies, peripheral area 1 was fed, but the other two areas were not directly fed.

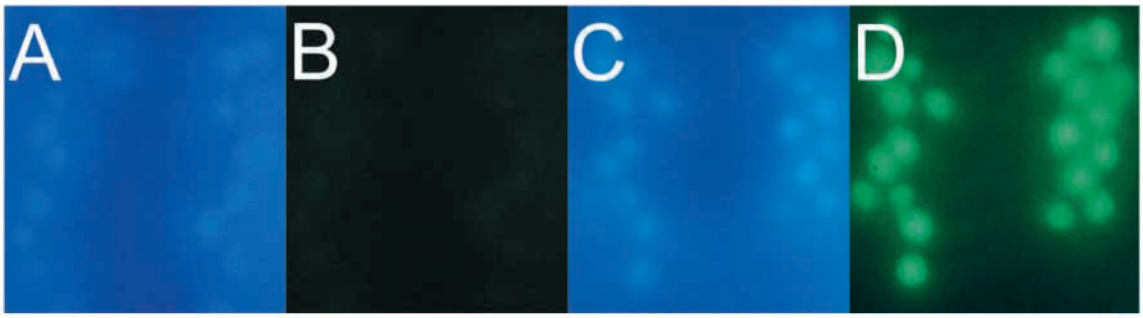

Fig. 4. Fluorescent photomicrographs showing the simultaneous imaging of redox state and reactive oxygen species in hydroid cells *in vivo*. Each pair of images is the same cross section of the epitheliomuscular cell fibers at the base of a living polyp shown at NAD(P)H (A,C) and fluorescein (B,D) wavelengths (each fiber is approximately 2 μm in diameter): A,B, negative control, 24 h after feeding, treated with DMSO but not H_2DCFDA; C,D, 24 h after feeding, treated with H_2DCFDA (fibers are relatively reduced; H_2O_2 is abundant).

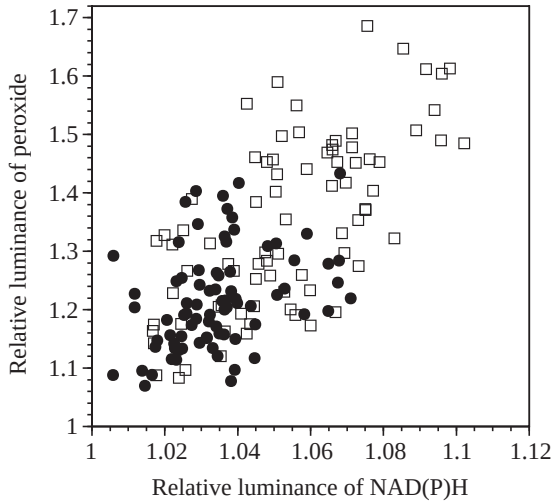

Fig. 5. Bivariate scatterplots for redox state, as indicated by the native fluorescence of NAD(P)H, and peroxide, as indicated by H_2DCFDA-derived 2′,7′-dichlorofluorescein. Each point represents the relative luminance of NAD(P)H and peroxide for a single epitheliomuscular cell fiber (circles, from colonies 3–5 h after feeding; open squares, from colonies 24 h after feeding).

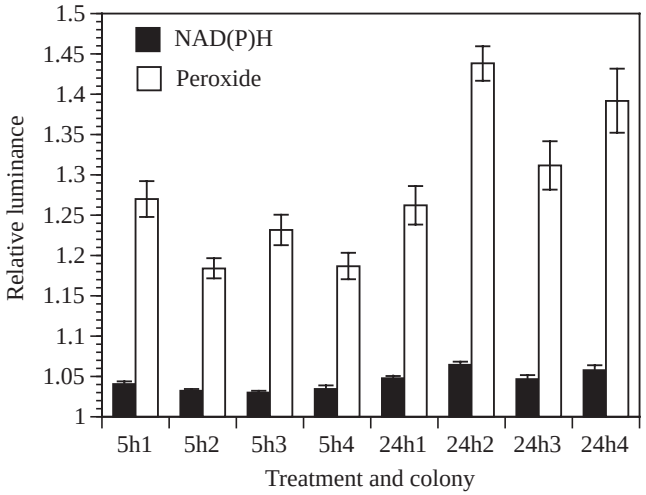

Fig. 6. For whole-colony experiments, means and standard errors are shown for the relative luminance of NAD(P)H (filled columns) and peroxide (open columns). For each colony (1–4) of each treatment (5 h and 24 h), means from two polyps, 10 fibers each, are shown.

treatments probably differ because of differences in the range of the outcome variable (a wider range provides a greater variance to be explained by the predictor variable and, hence, a higher correlation). A redox threshold for increased ROS formation (see Nishikawa et al., 2000) may also be suggested by the slight, but statistically significant, heterogeneity of slopes between the two treatments (F=6, d.f.=1,156, P<0.05), with the 24 h treatment exhibiting a steeper slope (slope ± S.E.M. for 3–5 h and 24 h respectively, 2.2±0.6 and 4.4±0.6).

More specific analyses focused on comparing the two treatments taking into account the nested structure of the data. The 24 h treatment exhibits significantly higher relative luminance of both NAD(P)H and peroxide than the 3–5 h treatment (Fig. 5, MANOVA, F=7.3, d.f.=2,5, P<0.05). This bivariate difference derives from significant univariate differences in both outcome variables [Fig. 6, ANOVA; for NAD(P)H, F=16.5, d.f.=1,6, P<0.01; for peroxide F=8.9, d.f.=1,6, P<0.05]. The integrated area of peroxide for each fiber (i.e. relative luminance times fiber area) also shows significant between-treatment differences (ANOVA of $\log_e$-transformed values; F=14, d.f.=1,6, P<0.01).

Within-colony differential feeding, redox state and ROS

Epitheliomuscular cell fibers from the differentially fed colonies show only weak correlations between the relative luminance of NAD(P)H and peroxide whether these fibers are from a fed polyp (r^2=0.07) or an unfed polyp (r^2=0.06). While both correlations are statistically significant (F=9.4, d.f.=1,118, P<0.01; F=7.7, d.f.=1,118, P<0.01, respectively), the low correlation coefficients are probably the result of exposure to light prior to measurement (light will exhaust the fluorescein emission). Some colonies were more difficult to manipulate than others, and this differential exposure to light weakens between-colony comparisons.

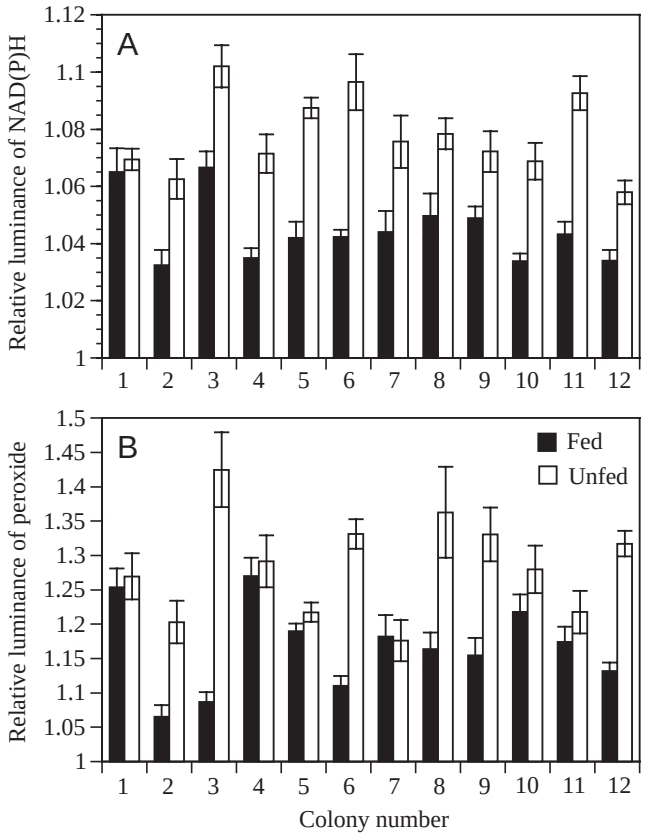

Fig. 7. For within-colony experiments, means and standard errors are shown for (A) the relative luminance of NAD(P)H and (B) the relative luminance of peroxide. For each colony (1–12), means of 10 fibers from both the fed polyp (filled columns) and the unfed polyp (open column) are shown.

Nevertheless, since each individual colony was necessarily exposed to the same amount of light, within-colony analyses can still be useful. Indeed, such analyses support the results from the between-colony comparisons (Fig. 7). Paired-comparisons *t*-tests of mean polyp values for 12 replicates show significant differences for NAD(P)H (t=8.6, $P \ll 0.001$), peroxide (t=3.9, $P<0.01$) and the integrated area of peroxide (t=2.4, $P<0.05$). Similarly, the nested ANOVA shows a strong polyp-within-replicates effect for NAD(P)H (Fig. 7A; F=17, d.f.=12,216, $P \ll 0.001$), peroxide (Fig. 7B; F=13.3, d.f.=12,216, $P \ll 0.001$) and integrated area of peroxide (F=14.4, d.f.=12,216, $P \ll 0.001$).

Discussion

Food supply has a strong effect on pattern formation in colonies of *P. carnea*. In differentially fed colonies, fed areas developed considerably greater amounts of polyps and stolons than areas that were starved, and this effect became more consistent over time. Adaptive colony development thus occurs in response to a spatially variable food supply. Questions remain, however, as to the mechanism or mechanisms governing this response, specifically how the signal is transduced from the ingestion of food to the activity of the pattern-forming genes that underlie polyp and stolon development.

My approach builds on previous studies of these hydroids which suggest that polyp and stolon development respond to perturbations of redox state (Blackstone, 1998b; Blackstone, 1999; Blackstone, 2000). When feeding regimes are identical, colonies that are relatively oxidized initiate greater polyp and stolon development than colonies that are relatively reduced. Feeding has an immediate effect on metabolic demand by stimulating contractions of epitheliomuscular cells (Dudgeon et al., 1999); this metabolic demand shifts the redox state in the direction of oxidation (Blackstone, 1998a). Redox signaling may thus mediate adaptive colony growth in response to a variable food supply. Because of the metabolic demands of polyp contractions, areas that are fed are relatively oxidized compared with areas that are not fed. This relative difference in oxidation subsequently triggers (or alternatively fails to suppress) the gene activity that leads to polyp and stolon development.

The transduction of redox signals into gene activity is an area of active and ongoing investigation in a number of fields (Escobar Galvis et al., 1998; Eto et al., 1999; Scheffler, 1999; Smith et al., 2000). In some cases, ROS may mediate redox signaling (Poyton and McEwen, 1996; Nishikawa et al., 2000). In these hydroids, levels of peroxide correlate with redox state in the expected manner. Relative reduction produces greater amounts of peroxide, while relative oxidation produces lesser amounts. No attempt was made to quantify the actual concentration of peroxide (e.g. by using a standard curve), since only the base of the polyp epitheliomuscular cell network could be imaged *in vivo*, and the details of this putative signal-transduction mechanism are by no means clear; for example, the target(s) of this signal are completely unknown, ROS other than peroxide may be involved, etc. Nevertheless, peroxide-mediated signaling has been observed in other systems (Pei et al., 2000), and generally peroxide levels should correlate with the amounts of other ROS (Chance et al., 1979). Since peroxide responds to feeding-related perturbations of redox state, a role for peroxide, or other ROS, in mediating the effects of redox state on pattern formation should be considered.

Pattern formation in hydroids has been extensively investigated (Javois, 1992). There has been considerable interest in the effects of moderate-sized molecules (sometimes termed morphogens) on pattern formation, e.g. head activator, a 1 kDa peptide (Schaller et al., 1989), stolon-inducing factor, a 20 kDa glycoconjugate (Lange and Müller, 1991), and other peptides (Takahashi et al., 1997). Models of the activity of such factors have also been developed (Meinhardt and Gierer, 2000). Much current work focuses on linking these factors and their effects to the activity of specific genes (Shenk et al., 1993; Weinziger et al., 1994; Hobmayer et al., 2000) and on the evolutionary implications of such data (Kuhn et al., 1996; Mokady et al., 1998; Cartwright and Buss, 1999). Nevertheless, small molecules also clearly have a role in hydroid pattern formation (Berking, 1991). In general, interest

in small molecules has increased as important roles for nitric oxide and ROS have been elucidated (Wells, 1999; Chiueh, 2000). Indeed, a role for ROS in hydroid pattern formation has already been suggested (Jantzen et al., 1998). The activity of pattern-forming genes (and thus the production of peptide and protein gene products) can potentially be triggered by small molecules (such as ROS) which, in turn, may be by-products of redox state.

While ROS-mediated redox signaling is consistent with the available evidence, much of this evidence is circumstantial. Other mechanisms of signaling between the food supply and pattern-forming genes must therefore be considered. For instance, differential feeding of a hydroid colony will clearly produce gradients of spatially distributed mechanical stresses on the constituent polyps, since fed polyps stretch more than unfed ones. Such stresses have been implicated in mechanisms of morphogenesis (see Beloussov, 1998). Simple tests of this hypothesis (such as feeding one area of a colony plastic beads) are complicated by the tendency of these hydroids immediately to regurgitate any non-living material that is consumed. Feeding-induced gastrovascular flow may also mediate gradients of mechanical stresses in differentially fed colonies, and there is convincing evidence that this flow can affect polyp and stolon development (Dudgeon and Buss, 1996; Blackstone, 1997; Blackstone, 1998a; Blackstone, 1998b). Further, fed polyps that are actively contracting will clearly generate greater flow than those that are quiescent (Dudgeon et al., 1999). The difficulty with this hypothesis is that the gradient of flow is the inverse of what seems necessary to trigger the observed effect. Previous experiments with *P. carnea* colonies indicate that lower flow rates trigger the development of polyps and stolons (Blackstone, 1997; Blackstone, 1998a; Blackstone, 1998b), while high flow rates suppress such development. Thus, higher flow rates in differentially fed areas would be expected to suppress, not to trigger, polyp and stolon development. At least two considerations may complicate this interpretation, however. First, flow could possibly exhibit a bimodal relationship to colony development (e.g. both very low and very high flow rates may trigger polyp and stolon development). Second, it is technically very difficult to measure polyp contractions and flow rates in all but the smallest colonies (Van Winkle and Blackstone, 1997; Dudgeon et al., 1999), so crucial data are lacking.

Finally, a particular chemical contained in the food may trigger pattern-forming gene activity. Retinoids, for instance, have been shown to have an effect on hydroid pattern formation (Müller, 1984). A high concentration of such a chemical in the food could produce gradients between fed and unfed areas in a colony, and such gradients could contribute to the observed effects on pattern formation. Nevertheless, the pumping of food from fed polyps to unfed polyps complicates this hypothesis. To be effective, such a chemical morphogen would have to have special properties to prevent its export to the unfed areas.

While there are several potential explanations for the observed pattern of adaptive colony development in response to a variable food supply, ROS-mediated redox signaling can still be considered a principal hypothesis. Not only would such a result be consistent with data from other systems, but levels of ROS have been quantified, and these data show that levels of ROS correlate with perturbations of redox state and with observed changes in pattern formation. The implication of a role for ROS in the process of adaptive colony growth leads to other testable hypotheses (such as the effects of anti-oxidants on pattern formation). Further investigations in this area will probably be informative.

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