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REDOX CONTROL IN DEVELOPMENT AND EVOLUTION: EVIDENCE FROM COLONIAL HYDROIDS

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Summary

Redox chemistry, involving the transfer of electrons and hydrogen atoms, is central to energy conversion in respiration, and the control of gene expression by redox state commonly occurs in bacteria, allowing rapid responses to environmental changes, for instance, in the food supply. Colonial metazoans often encrust surfaces over which the food supply varies in time or space; hence, in these organisms, redox control of the development of feeding structures and gastrovascular connections could be similarly adaptive, allowing colonies to adjust the timing and spacing of structures in response to a variable food supply. To investigate the possibility of redox control of colony development, the redox states of hydractiniid hydroid colonies were manipulated experimentally. As in many colonial animals, hydractiniid hydroids display a range of morphological variation from sheet-like forms (i.e. closely spaced polyps with high rates of stolon branching) to runner-like forms (i.e. widely spaced polyps with low rates of stolon branching). In the runner-like *Podocoryna carnea*, azide, a blocker of the electron transport chain, and dinitrophenol, an uncoupler of oxidative phosphorylation, diminished the largely polyp-driven gastrovascular flow to a similar extent. Measures of the redox state of the polyp epitheliomuscular cells using the fluorescence of NAD(P)H suggest that azide shifts the redox state in the direction of reduction, while dinitrophenol shifts the redox state in the direction of oxidation. Colony development corresponds to redox state in that azide-treated colonies were more runner-like, while dinitrophenol-treated colonies were more sheet-like. Nevertheless, the functional role of polyps in feeding and generating gastrovascular flow probably contributed to a trade-off between polyp number and size such that azide-treated colonies had few large polyps, while dinitrophenol-treated colonies had many small polyps. Regardless of the treatment, *P. carnea* colonies developed to maturity and produced swimming medusae in the normal fashion. In the sheet-like *Hydractinia symbiolongicarpus*, treatment with azide resulted in complete suppression of the development of both the stolonal mat and the blastostyles, the reproductive polyps. Azide-treated *H. symbiolongicarpus* colonies therefore developed in a juvenilized, runner-like manner and much resembled colonies of *P. carnea*. Following cessation of azide treatment in *H. symbiolongicarpus*, normal colony development ensued, and both a stolonal mat and blastostyles formed. In both hydroid species, relative oxidization favors sheet-like growth, while relative reduction favors runner-like growth. Since feeding triggers strong contractions of polyp epitheliomuscular cells and results in relative oxidation, this experimental evidence supports the hypothesis of adaptive redox control of colony development and evolution.

Key words: *Hydractinia symbiolongicarpus*, hydroid, NAD(P)H fluorometry, *Podocoryna carnea* (*Podocoryne carnea*), redox control.

Introduction

During photosynthesis and respiration, environmental oxidants and reductants are linked by a series of electron carriers operating close to their mid-point potentials. There is therefore a compelling logic to redox control of gene expression (Allen, 1993): perturbations of these redox potentials by environmental changes in energy and electron sources and sinks lead directly to rapid and appropriate organismal responses (Pfannschmidt et al., 1999). While such redox control is commonly found in bacteria (Allen, 1993), the same logic can be applied to multicellular eukaryotes as well. 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 and to compete for space (Larwood and Rosen, 1979; Jackson et al., 1985; Buss, 1990; Buss and Blackstone, 1991). For instance, closely spaced polyps with short vascular connections will clearly enhance food-gathering ability in a particular location. In an environment where food supply is locally variable, a colony's fitness can thus be enhanced by developmental mechanisms that produce morphological patterns that are locally sensitive to the food supply. Natural selection will therefore 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).

In such a system, redox control is a reliable mechanism by which an environmental signal can be transduced into the gene activity that underlies polyp and stolon tip morphogenesis. 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; 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). For largely sociological reasons (i.e. because of social conflicts between groups of scientists who championed different model systems; see Mitman and Fausto-Sterling, 1992), the views of Child and like-minded colleagues have fallen into disrepute. Nevertheless, legitimate scientific grounds for their advocacy of metabolic regulation of development in clonal and colonial organisms may yet be found (Blackstone, 1997a), and a considerable amount of recent work provides a general framework for this view (e.g. Allen and Balin, 1989; Adair et al., 1990; Nijhout, 1990; Fanburg et al., 1992; Poyton and McEwen, 1996; Polyak et al., 1997; Wiesner, 1997; Eto et al., 1999).

To investigate the possibility of redox control of colony development, hydractiniid hydroid colonies were used in a series of experimental manipulations. As in many colonial animals, hydractiniid hydroids display a range of morphological variation from sheet-like forms (i.e. closely spaced polyps with high rates of stolon branching) to runner-like forms (i.e. widely spaced polyps with low rates of stolon branching). These differences in form are attributable to heterochrony, an evolutionary change in the timing of development (McNamara, 1997; Reilly et al., 1997; Schlichting and Pigliucci, 1998). In the hydractiniid clade, a sheet-like ground pattern (*sensu* Ax, 1996) appears to be basal, as represented by *Hydractinia* species. *Podocoryna* (=*Podocoryne*, see Calder, 1988; but see also Boero et al., 1998) species seem to have secondarily derived a runner-like form by paedomorphosis and progenesis (the free-living medusae of *Podocoryna* are nevertheless considered plesiomorphic; Cunningham and Buss, 1993). As emphasized by Reilly et al. (1997), these heterochronic terms should be used narrowly (i.e. paedomorphosis='child-shaped' relative to a sister group, and progenesis=precocial sexual maturity relative to a sister group). Nevertheless, parallels to this between-species variation can and should be investigated by experimental, within-species approaches (Stebbins and Basile, 1986; Sinervo and Basalo, 1996; Blackstone, 1997b, 1998a,b; Schlichting and Pigliucci, 1998).

Here, the redox states of runner-like colonies of *Podocoryna carnea* are manipulated using azide, a blocker of the electron transport chain, and 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation. Both these treatments reduce the rate of ATP formation (Heytler, 1981; Erecinska and Wilson, 1981; Scheffler, 1999) and both are expected to similarly reduce ATP-requiring physiological processes such as the largely polyp-driven gastrovascular flow (Schierwater et al., 1992). Thus, the effects of gastrovascular flow on colony morphology (Blackstone and Buss, 1993; Dudgeon and Buss, 1996; Blackstone, 1996, 1997b) can be effectively controlled at least during initial treatments. At the same time, azide and dinitrophenol are expected differentially to perturb the cellular redox state; dinitrophenol shifts the redox state in the direction of oxidation (Heytler, 1981), while azide shifts the redox state in the direction of reduction (Erecinska and Wilson, 1981). To emphasize further the trans-specific implications of this work, additional experiments using azide were carried out on sheet-like colonies of *Hydractinia symbiolongicarpus*.

Materials and methods

Study species

Life cycles, colony development and laboratory culture conditions of *Podocoryna carnea* (Sars) and *Hydractinia symbiolongicarpus* (Buss and Yund) are discussed in detail elsewhere (e.g. Blackstone and Buss, 1991; Blackstone, 1996). As small colonies (10–50 polyps; Blackstone, 1996) of *H. symbiolongicarpus* develop, stolons branch and fuse at a high rate to form a continuous stolonal mat, a closely knit complex of stolons capped by a continuous layer of ectoderm, which shows sheet-like growth and from which extend relatively few peripheral stolons. Once the stolonal mat forms in a developing colony, subsequent clonal explants of this colony will exhibit this structure from their first attachment. While *H. symbiolongicarpus* always forms a stolonal mat prior to maturity, *P. carnea* never forms this structure, and *P. carnea* generally has a lower rate of production of polyps and stolon tips relative to rates of stolon growth and colony maturation.

In both species, stolons encase fluid-filled canals that are continuous with the gastrovascular cavity of the polyp. Gastrovascular fluid circulates in the lumen of the stolons and carries food from the feeding polyp to other parts of the colony; contractions of the polyp largely propel this fluid (Schierwater et al., 1992). Polyp contractions commence upon feeding and continue actively for 12–24 h; waste material is subsequently regurgitated, and the polyp becomes quiescent until the next feeding (Wagner et al., 1998; Dudgeon et al., 1999). NAD(P)H fluorescence is concentrated in the polyp epitheliomuscular cells that drive these contractions, and the intensity of this

fluorescence at rest may indicate the metabolic capacity of these cells (Blackstone, 1998a). In response to feeding, colony oxygen uptake increases and the redox state of the epitheliomuscular cells shifts in the direction of oxidation; when polyps become quiescent, the redox state of these cells shifts in the direction of reduction (Blackstone, 1997b, 1998a).

Measures of oxygen uptake

To determine an appropriate dose for the azide experiments, the rate of oxygen uptake of a single *P. carnea* colony growing on a 12 mm round coverslip was tested every other day, 3–5 h after feeding, at four concentrations of sodium azide [100, 200, 400 and 800 $\mu mol\,l^{-1}$; note that the binding of azide to cytochrome oxidase is pH-dependent (Erecinska and Wilson, 1981), so azide is relatively ineffective at the high pH of sea water]. For each of these four trials, the colony and its coverslip was attached using a drop of silicone grease to a coverslip cemented to a small magnet. This assembly was contained in a 13 mm diameter sealed glass chamber (Strathkelvin RC300) with 0.7 ml of sea water (filtered to 0.2 μm). Chamber temperature was held constant (20.5±0.02 °C) using an external circulation water bath (Neslab RTE-100D), and the rate of decline in oxygen concentration over a 30 min period was measured (using a Strathkelvin 1302 electrode and 781 oxygen meter) with stirring (by slowly spinning the magnet, coverslips and colony). The chamber was then opened, a small volume of sea water was removed, an equivalent amount of 25 $mmol\,l^{-1}$ sodium azide solution in sea water was added to achieve the target concentration, the solution was mixed and aerated thoroughly with a small pipette, and the chamber was resealed (this procedure took approximately 7 min). The rate of decline in oxygen concentration was then measured over another 30 min period.

These preliminary experiments indicated that all concentrations of sodium azide significantly diminished oxygen uptake, but that the effect only became large as concentrations approached 800 $\mu mol\,l^{-1}$. An additional experiment was then carried out to quantify the effect of 800 $\mu mol\,l^{-1}$ sodium azide. Five clonal replicates of the same *P. carnea* colony were grown for 1 week on 12 mm coverslips. At this point, the oxygen uptake of each colony was assayed as described above. These experiments 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 azide difference in the rate of decline in oxygen concentration over a 30 min period was calculated; 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 (see Blackstone, 1997b, for further discussion of methodology).

Comparisons of colony development and morphology

Eighteen clonal replicates of the same *P. carnea* colony were explanted onto 18 mm coverslips and randomly assigned to three treatments in sea water (800 $\mu mol\,l^{-1}$ azide, control and 60 $\mu mol\,l^{-1}$ dinitrophenol; the same treatment concentrations were used in all experiments described below). Each group was treated with the appropriate solution for approximately 12 h per day using standard methods (Blackstone, 1997b, 1998b) and was measured three times: at the time the surface of the coverslip was covered, when medusa production was initiated, and when the first medusae were released. Of these stages of colony development, the first is somewhat arbitrary, while the latter two are more precise. Measurements were taken using image-analysis technology (e.g. Marcus et al., 1996) as described elsewhere (e.g. Blackstone and Buss, 1992).

Using PC-SAS software, the three treatments were compared using univariate (ANOVA) and multivariate (MANOVA) analysis of variance for the relationship between the total area of polyps and the total area of empty, unencrusted coverslip enclosed within the colony. Both polyp area and empty, unencrusted inner area were expressed as a fraction of the total colony 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 present analyses). Polyp area is clearly a measure of polyp development; empty, unencrusted inner area is largely a measure of stolon branching and anastomosis (i.e. as these aspects of stolon development increase, inner area decreases). While 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 (see further discussion of methods in Blackstone, 1996). While some heterogeneity of variances was apparent in some of the measures used (e.g. polyp counts), generally all of these data approximately meet the assumptions of parametric statistics. Both natural logarithm and arcsine transformations provided a poorer fit to these assumptions.

Subsequently, 27 clonal replicates of the same *P. carnea* colony were explanted onto 15 mm coverslips, randomly assigned to the three treatments (azide treatment, control and dinitrophenol treatment), measured for morphology at the time of the initiation of medusa production and analyzed as described above. These colonies were also used in the video and fluorescent microscopy experiments described below.

Finally, 14 clonal replicates of the same *H. symbiolongicarpus* colony were explanted onto 15 mm coverslips and randomly assigned to two treatments (azide treatment and control). These colonies were measured at three different times and analyzed as described above. Because of the profound effects of azide on *H. symbiolongicarpus* (e.g. complete suppression of both the development of the stolonal mat and even immature blastostyles, the reproductive polyps), it was not possible to take these measurements at any precise developmental landmarks, so measurements were taken every 10 days. The third measure (30 days) nevertheless corresponds roughly to the time that the surface was covered. After an additional several weeks of treatment, the azide-treated *H. symbiolongicarpus* colonies were returned to the normal culture conditions to observe whether normal colony

development resumed. The *H. symbiolongicarpus* colony used in these studies of explants was also used as the male parent in crosses with another field-collected colony to produce three colonies which were raised from embryos and metamorphosed into primary polyps using standard methods (Blackstone, 1996). The goal here was not genetic analysis, but rather to determine whether stolonal mat development could be completely suppressed; these three colonies were treated with azide prior to developing a stolonal mat and measured at the time of covering of the surface.

Video microscopic measurements of peripheral gastrovascular flow

The *P. carnea* colonies on 15 mm coverslips were first exposed to the treatment solutions during and after feeding for 3–5 h. Immediately following this initial exposure, each colony was measured for gastrovascular flow to two peripheral stolons using standard methods (e.g. Blackstone, 1996). The colony was placed in a flow-through chamber maintained at 20.5±0.3 °C. Colonies were viewed at 400× on an inverted light microscope (Zeiss Axiovert 135). Using the MTI CCD camera, stolon tips were videotaped for 10 min each. To control for time effects, one replicate from each treatment was measured in each trial, and the order in which the different treatments were given was varied for each trial. While previous studies (Blackstone, 1997b, 1998a,b) have measured three stolon tips per replicate, for three replicates per trial sufficient time was available only to measure two stolon tips per replicate colony.

Statistical analyses focused on the three measured outcomes: net change in lumen width per cycle, contraction cycle period and stolon width. These measures can be combined into a biologically meaningful measure of gastrovascular flow rate for each stolon contraction cycle: net change in lumen width divided by cycle period and stolon width (µm of lumen width expansion and contraction per total µm of stolon width per second). Biologically, this rate measure describes the ‘rate of supply’ of food to the tissues of the stolon tip. Both this rate measure and the individual flow variables generally meet the assumptions of parametric statistics. While some heterogeneity of variances is apparent in measures of cycle period, neither arcsine nor natural logarithm transformations provide a better fit. To compare treatments, a mixed-model ANOVA was used with cycles nested within stolons, stolons nested within replicates and replicates nested within treatments.

Assays of cellular redox state using fluorescent microscopic measures of NAD(P)H

The characteristic fluorescence of NADH and NADPH compared with the oxidized forms of these molecules has been used extensively to measure cellular redox state (for a review, see Chance, 1991). Currently, this technique is widely used (e.g. Pralong et al., 1992, 1994; Heineman and Balaban, 1993; Hajnóczky et al., 1995; Rohács et al., 1997; 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 measures of NAD(P)H fluorescence were obtained with a Zeiss Axiovert 135 microscope and ultraviolet light (excitation at 365 nm, barrier filter at 420 nm). Brief exposures were used, since hydroids are sensitive to ultraviolet light. A colony was contained in the flow-through chamber at 20.5±0.3 °C. Images were recorded on film (10 s exposure, ASA 160 balanced for tungsten filaments), digitized and quantified with densitometry in OPTIMAS (brighter values relative to the dark background signal greater reduction). In such images of hydractiniids, 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), while 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. Measurements were taken of the number of fibers fluorescing visibly above the dark background, the total cross-sectional area of these fibers and of their relative luminance. The relative luminance was calculated as the ratio of the mean luminance of the fiber throughout its entire cross-sectional area divided by the mean luminance of an equivalent-sized area immediately surrounding that fiber. Since all the fibers of a polyp are part of the same epitheliomuscular cell network, it is not clear that these individual fibers can be considered statistically independent. Therefore, the relative luminance of all visible fibers of a polyp was averaged, and this mean value was used in statistical comparisons.

Because these measures of NAD(P)H fluorescence were somewhat time-consuming, only five of the *P. carnea* colonies on 15 mm coverslips were used from each treatment. Assays were conducted 2 days after each colony had been measured for gastrovascular flow and immediately after 3–5 h of being fed and treated in the appropriate solution (azide treatment, control or dinitrophenol treatment). Two polyps were measured from each replicate, and between-treatment effects were assessed using a mixed-model ANOVA (polyps within replicates, replicates within treatments).

Results

Measurements of oxygen uptake

Dinitrophenol has been shown to increase the rate of oxygen uptake of hydroid colonies (Blackstone, 1997b). This result is consistent with the general observation that dinitrophenol and other uncouplers activate the oxidation of substrate and electron transport (Heytler, 1981). Azide, however, binds to cytochrome oxidase and reduces the rate of oxygen uptake (Erecinska and Wilson, 1981), and this effect is apparent in

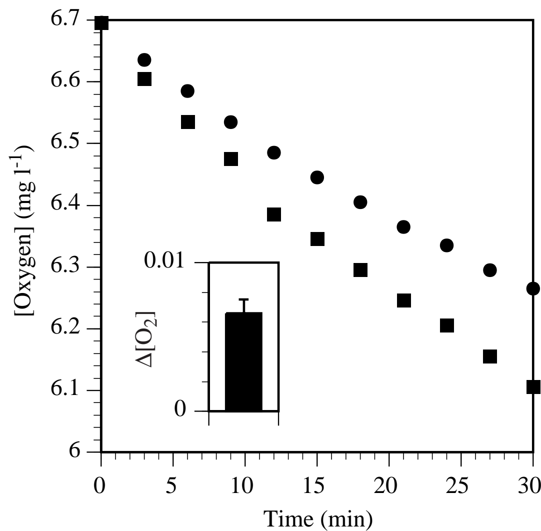

Fig. 1. Rate of decline in oxygen concentration for a *Podocoryna carnea* colony before (squares) and after (circles) treatment with 800 μmol l^{-1} azide. For five colonies, the inset plot shows the mean + S.E.M. of the before/after difference in the rate of decline in oxygen concentration ($\Delta[O_2]$; mg l^{-1} min^{-1}), where this decline is measured by the least-squared slope of oxygen concentration *versus* time. This difference in rate was significantly positive, i.e. the oxygen uptake of the colonies decreased subsequent to the treatment.

these hydroids (Fig. 1). Before/after differences in the least-squared slopes of oxygen concentration *versus* time (min) for the five colonies ranged from 0.004 to 0.009 mg l^{-1} min^{-1} with a mean of 0.0066±0.0009 mg l^{-1} min^{-1} (mean ± S.E.M.; paired-comparison *t*-test, t=7.12, P<0.002). Azide at 800 μmol l^{-1} causes a significant decrease in the rate of decline of oxygen concentration.

Comparisons of colony development and morphology

The uncoupler dinitrophenol has been shown to increase rates of polyp initiation and stolon branching, resulting in more sheet-like *P. carnea* (Blackstone and Buss, 1992; Blackstone, 1997b, 1998b). Azide has equally dramatic, although opposite, effects (Fig. 2), and the colonies grown on 18 mm coverslips and subject to the different treatments clearly show different developmental trajectories (Fig. 3). Since the first measurement (at the time of covering the surface) is relatively subjective, statistical analysis focused on the measurements taken at the initiation of medusa production (circles in Fig. 3) and at the release of the first medusae (triangles in Fig. 3). For both these times, there is a strong effect of treatment on the relationship between inner area and polyp area (MANOVA, F=5.2, d.f.=4,28, P<0.01 and F=12.8, d.f.=4,28, P≪0.001, respectively). This effect derives primarily from the strong treatment effect on inner area/total colony area (ANOVA, F=9.8, d.f.=2,15, P<0.01 and F=20.2, d.f.=2,15, P≪0.001, respectively) and from the considerably weaker effect on polyp area/total colony area (ANOVA, F=1.9, d.f.=2,15, P>0.15 and F=5.1, d.f.=2,15, P<0.05, respectively). Generally, in terms of stolon branching and anastomosis (which are inversely related to inner area), the azide-treated colonies are more runner-like than the controls, while the dinitrophenol-treated colonies are more sheet-like. Further, the relatively slight difference in overall polyp development obscures an interesting trade-off between the two components of polyp area, the number of polyps and mean polyp area (Fig. 4). For instance, at the time of the release of medusae (triangles in Fig. 4), there is a strong effect of treatment on the relationship between the number of polyps and mean polyp area (MANOVA, F=11.0, d.f.=4,28, P≪0.001), and this effect derives from the strong treatment effect on both mean polyp area (ANOVA, F=32.2, d.f.=2,15, P≪0.001) and the number of polyps (ANOVA, F=8.9, d.f.=2,15, P<0.01). Azide-treated colonies have few, large polyps, while dinitrophenol-treated colonies have many, small polyps. This observed trade-off between polyp number and size probably stems from two factors: the functional role of polyps in supplying the stolons with food, and the constraints on or enhancement of polyp initiation caused by the different treatments. Azide treatment inhibits polyp initiation, so the existing polyps must develop large sizes to feed and supply sufficient nutrient-rich gastrovascular fluid to the rest of the colony. Dinitrophenol treatment, in contrast, enhances polyp initiation, so many of the polyps initiated have a lesser role in

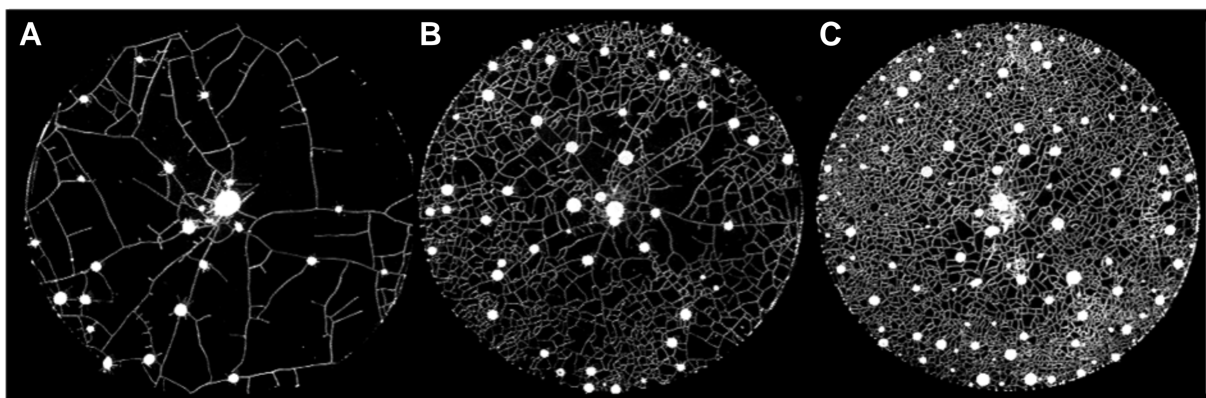

Fig. 2. Images of genetically identical colonies of *Podocoryna carnea* growing on 15 mm diameter glass coverslips at the initiation of medusa production. (A) Treated with 800 μmol l^{-1} azide for 12 h per day; (B) control; (C) treated with 60 μmol l^{-1} dinitrophenol for 12 h per day.

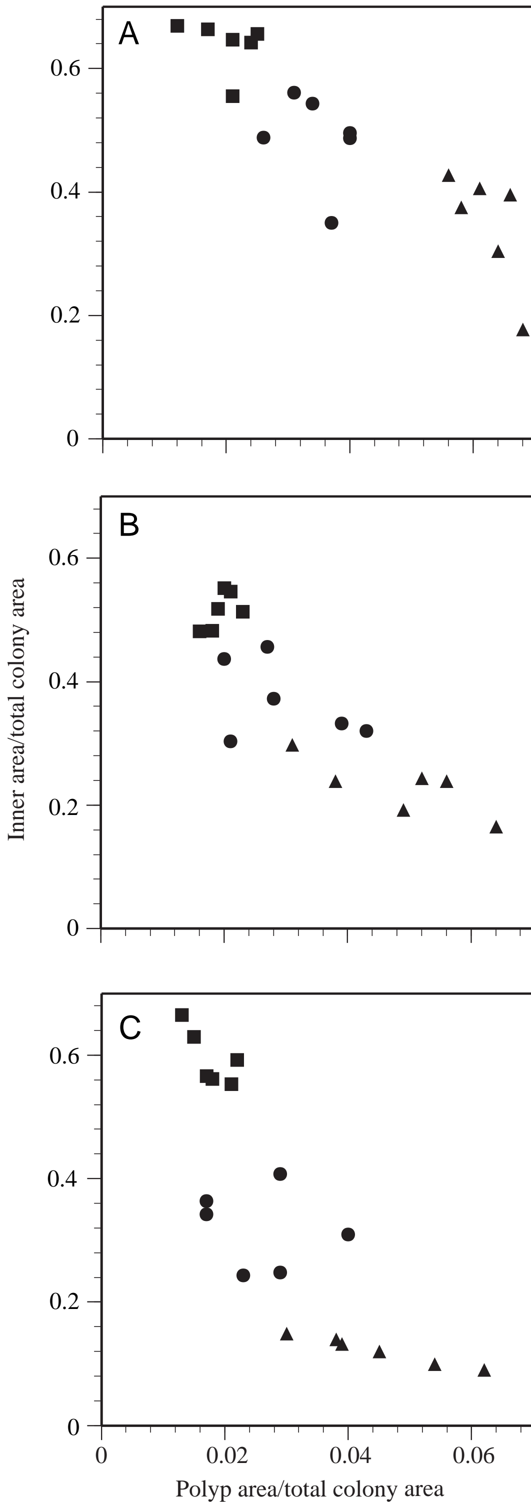

Fig. 3. Bivariate scatterplots of the amount of stolon development (inversely correlated to inner area/total colony area) and the amount of polyp development (polyp area/total colony area) for genetically identical *Podocoryna carnea* colonies at three stages of colony development: at the time the surface was covered (squares), when medusa production was initiated (circles) and when the first medusae were released (triangles). (A) Treated with 800 μmol l^{-1} azide for 12 h per day; (B) control; (C) treated with 60 μmol l^{-1} dinitrophenol for 12 h per day.

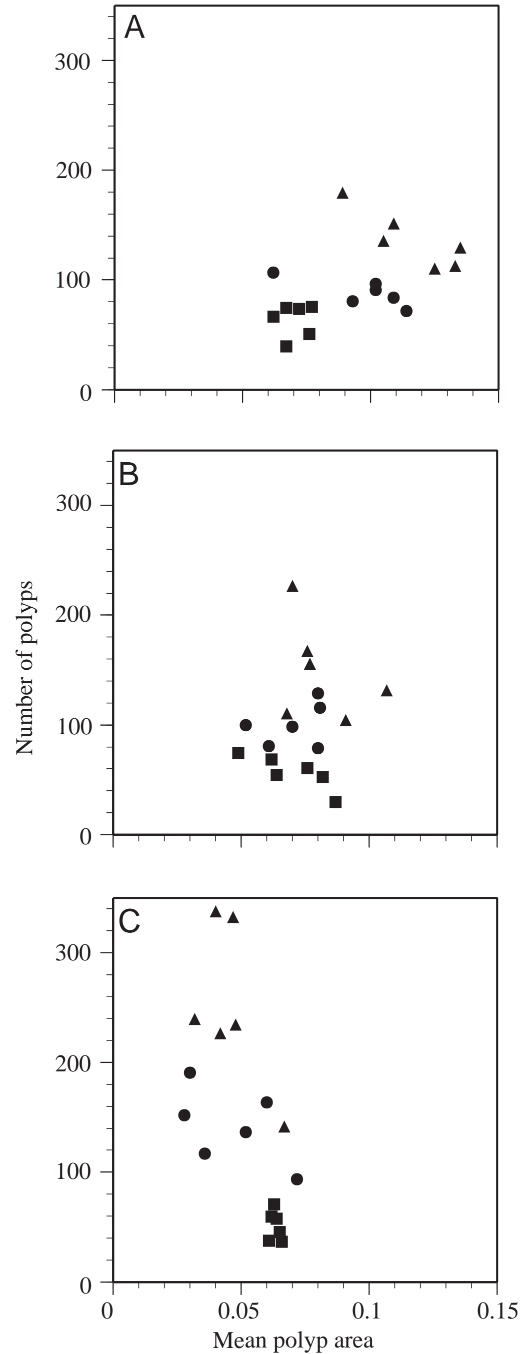

Fig. 4. Bivariate scatterplots showing the trade-offs between the number of polyps and mean polyp area (mm^2) for genetically identical *Podocoryna carnea* colonies at three stages of colony development: at the time the surface was covered (squares), when medusa production was initiated (circles) and when the first medusae were released (triangles). (A) Treated with 800 μmol l^{-1} azide for 12 h per day; (B) control; (C) treated with 60 μmol l^{-1} dinitrophenol for 12 h per day.

terms of either feeding or pumping gastrovascular fluid, and these polyps remain small.

The *P. carnea* explanted onto 15 mm coverslips and measured at the initiation of medusa production show similar

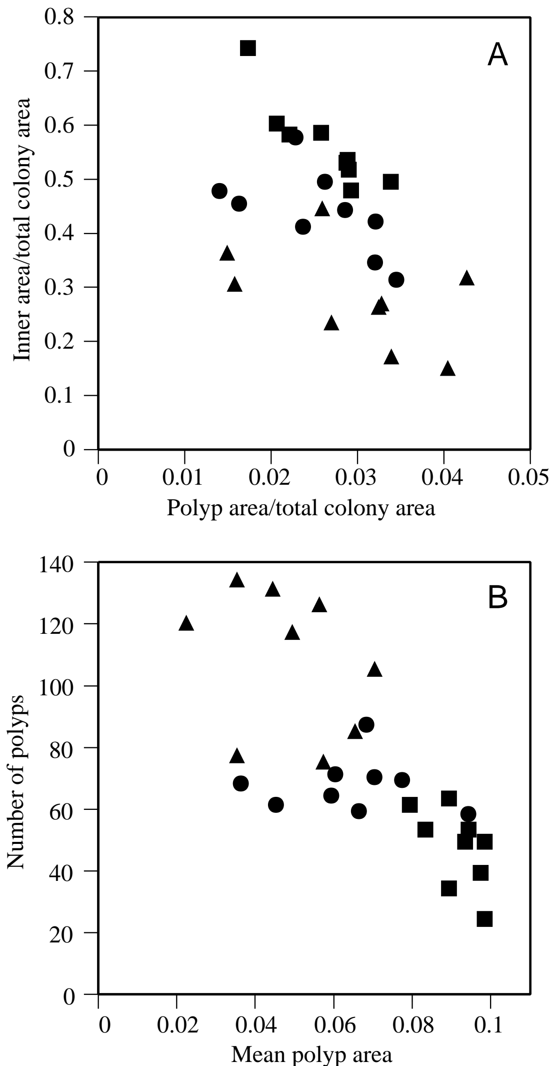

Fig. 5. Bivariate scatterplots for (A) the amount of stolon development (inversely correlated to inner area/total colony area) and the amount of polyp development (polyp area/total colony area) and (B) the number of polyps and mean polyp area (mm^2) for genetically identical *Podocoryna carnea* colonies at the time medusa production was initiated (squares, treated with 800 μmol l^{-1} azide for 12 h per day; circles, control; triangles, treated with 60 μmol l^{-1} dinitrophenol for 12 h per day).

trends in colony development (Fig. 5). There is a strong treatment effect on the relationship between inner area and polyp area (MANOVA, F=11.5, d.f.=4,46, P≪0.001), because of the strong effect on inner area/total area (ANOVA, F=25.9, d.f.=2,24, P≪0.001), with no significant effect on polyp area/total area (ANOVA, F=0.7, d.f.=2,24, P>0.5). Treatment also mediates a trade-off between polyp number and mean polyp area (MANOVA, F=15.1, d.f.=4,46, P≪0.001) with strong effects on both polyp number (ANOVA, F=32.8, d.f.=2,24, P≪0.001) and mean polyp area (ANOVA, F=22.1, d.f.=2,24, P≪0.001).

The uncoupler dinitrophenol has been shown to have complex effects on *H. symbiolongicarpus* (Blackstone and Buss, 1993) mediated by the need for relatively uniform gastrovascular flow rates in the developing stolonal mat (Dudgeon and Buss, 1996). Nevertheless, in colonies treated with dinitrophenol, the stolonal mat readily forms and grows, and these colonies mature normally (Blackstone and Buss, 1993). Azide-treated *H. symbiolongicarpus*, however, never develop a stolonal mat and never produce even immature blastostyles, the reproductive polyps (Fig. 6). In fact, the stolonal mat present in the original explants regressed and quickly disappeared once treatment with azide was begun. In terms of the quantitative aspects of colony development and focusing on the final measurements taken (triangles in Fig. 7), there is a strong treatment effect on the relationship between inner area and polyp area (MANOVA, F=231, d.f.=2,11, P≪0.001), because of the strong effect on inner area/total area (ANOVA, F=418, d.f.=1,12, P≪0.001), with no significant effect on polyp area/total area (ANOVA, F=1.6, d.f.=1,12, P>0.2). Again, treatment mediates a trade-off between polyp number and mean polyp area (Fig. 8; MANOVA, F=3,13, d.f.=2,11, P≪0.001) with strong effects on both polyp number (ANOVA, F=665, d.f.=1,12, P≪0.001) and mean polyp area (ANOVA, F=23.8, d.f.=1,12, P<0.001).

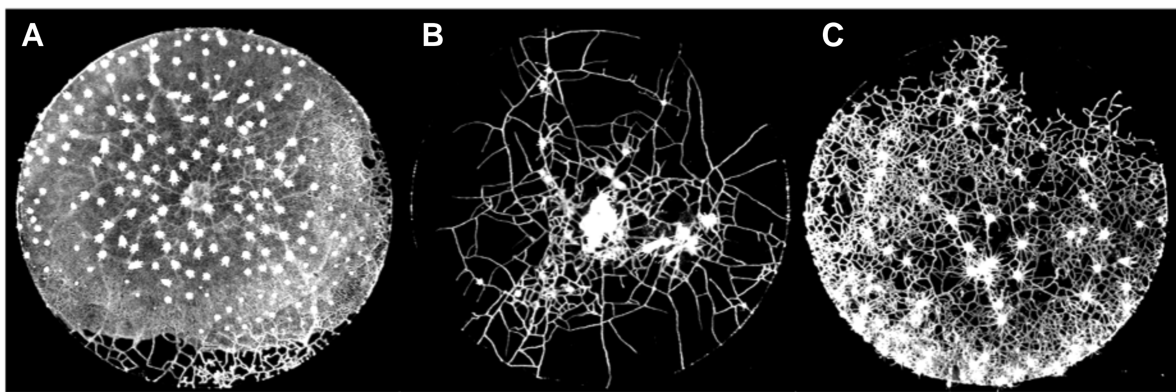

Fig. 6. Images of *Hydractinia symbiolongicarpus* colonies growing on 15 mm diameter glass coverslips at the time of covering the surface. (A) Control colony grown from an explant; (B) colony grown from an explant genetically identical to that in A and treated with 800 μmol l^{-1} azide for 12 h per day; (C) colony grown from a primary polyp offspring of the colony in A and treated with 800 μmol l^{-1} azide for 12 h per day.

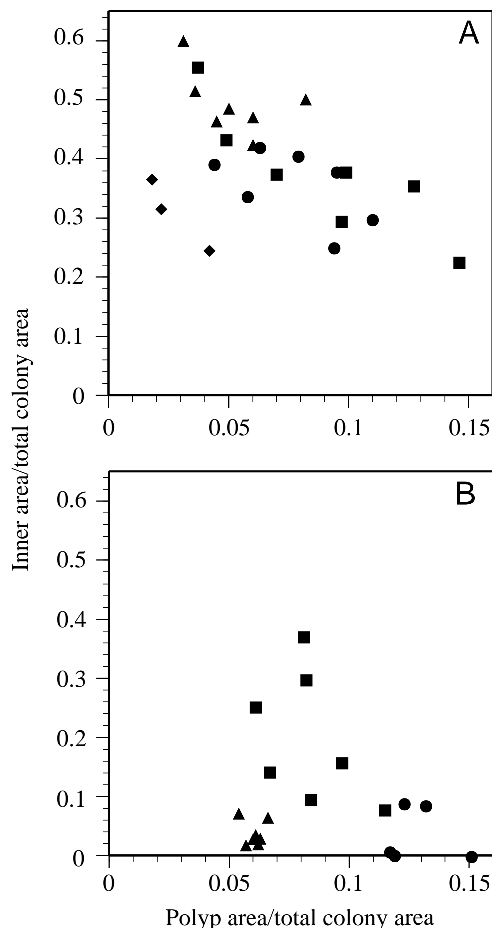

Fig. 7. Bivariate scatterplots of the amount of stolon development (inversely correlated to inner area/total colony area) and the amount of polyp development (polyp area/total colony area) for *Hydractinia symbiolongicarpus* colonies grown from explants and measured every 10 days. (A) Treated with 800 µmol l^{-1} azide for 12 h per day; (B) controls; squares, 10 days; circles, 20 days; triangles, 30 days. To facilitate between-treatment comparisons, results from two 20 day colonies with off-scale polyp area/total colony area measures (0.195 and 0.232) have been omitted from B. These two colonies had measures of inner area/total colony area similar to those of the other controls. Diamonds in A represent the three colonies grown from primary polyps and measured at 30 days.

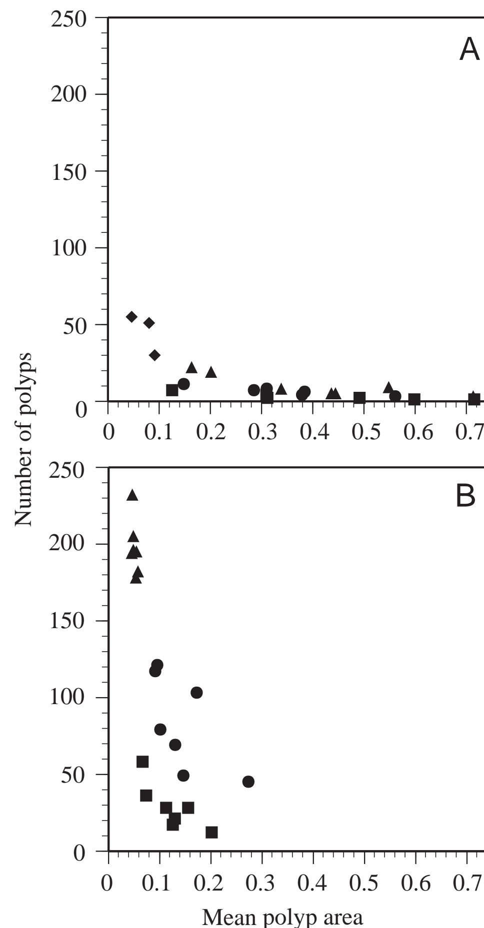

Fig. 8. Bivariate scatterplots showing the trade-offs between the number of polyps and mean polyp area (mm^2) for *Hydractinia symbiolongicarpus* colonies grown from explants and measured every 10 days. (A) Treated with 800 µmol l^{-1} azide for 12 h per day; (B) controls; squares, 10 days; circles, 20 days; triangles, 30 days. To facilitate between-treatment comparisons, results from a 10 day and a 20 day colony with single very large polyps (1.7 and 2.4 mean polyp areas) have been omitted from A. Diamonds in A represent the three colonies grown from primary polyps and measured at 30 days.

The *H. symbiolongicarpus* colonies which were bred from the colony used in the above studies of explants and then grown in azide from primary polyps (diamonds in Figs 7A, 8A) were more similar to the azide-treated explants than to the controls. These colonies grown from primary polyps, which never developed a stolonal mat, seemed to be able to initiate more polyps and more stolon branches than explants when grown in azide (Figs 6, 7A, 8A), although blastostyles were never produced. It may be that primary polyp colonies are competent to develop without a stolonal mat while explants of colonies already exhibiting a stolonal mat are less competent to do so, although further work is needed to support this observation. Finally, after being returned to normal culture conditions, all the azide-treated *H. symbiolongicarpus* colonies, whether from explants or primary polyps, quickly developed both a stolonal mat and blastostyles and subsequently matured in the normal fashion.

Video microscopic measurements of peripheral gastrovascular flow

Gastrovascular flow has pronounced effects on colony morphology in these hydroids (Blackstone and Buss, 1992, 1993; Dudgeon and Buss, 1996; Blackstone, 1996, 1997b). The premise for perturbing redox state with azide and with dinitrophenol was that these treatments would have equal effects on gastrovascular flow, while differentially perturbing redox state. This hypothesis was tested with the *P. carnea* colonies growing on 15 mm coverslips (Fig. 9). There is

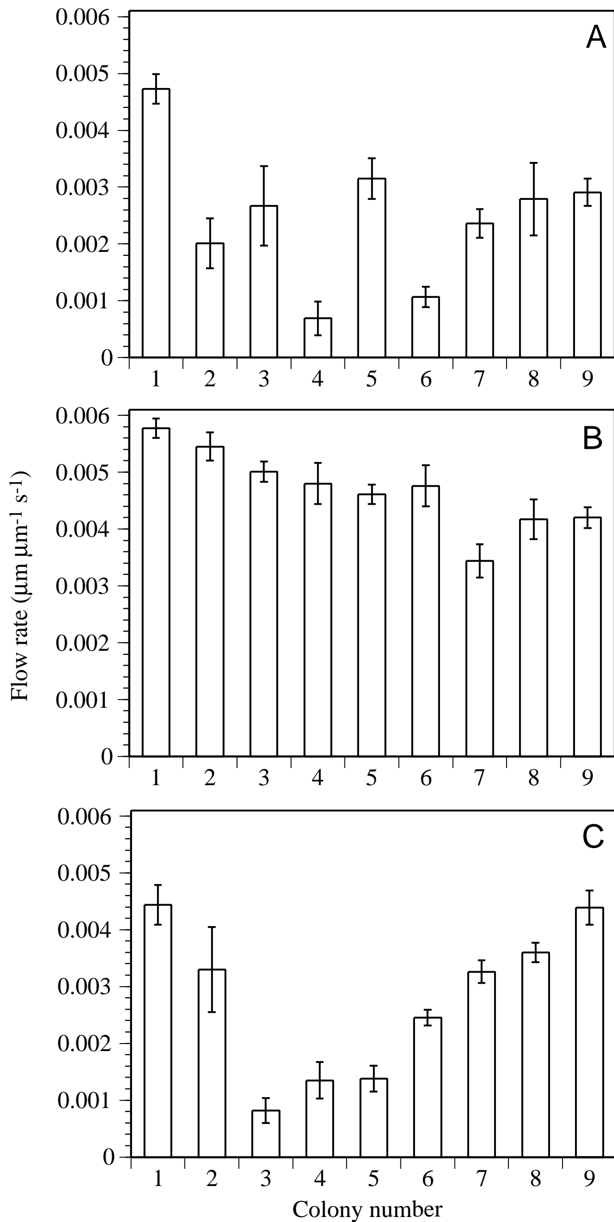

Fig. 9. Means and standard errors of flow rates (µm of lumen expansion and contraction per total µm of stolon width per second) for two stolons per replicate and nine replicates per treatment. (A) Treated with 800 µmol l^{-1} azide for 12 h per day; (B) control; (C) treated with 60 µmol l^{-1} dinitrophenol for 12 h per day. Standard errors provide a measure of between-stolon, within-replicate variation.

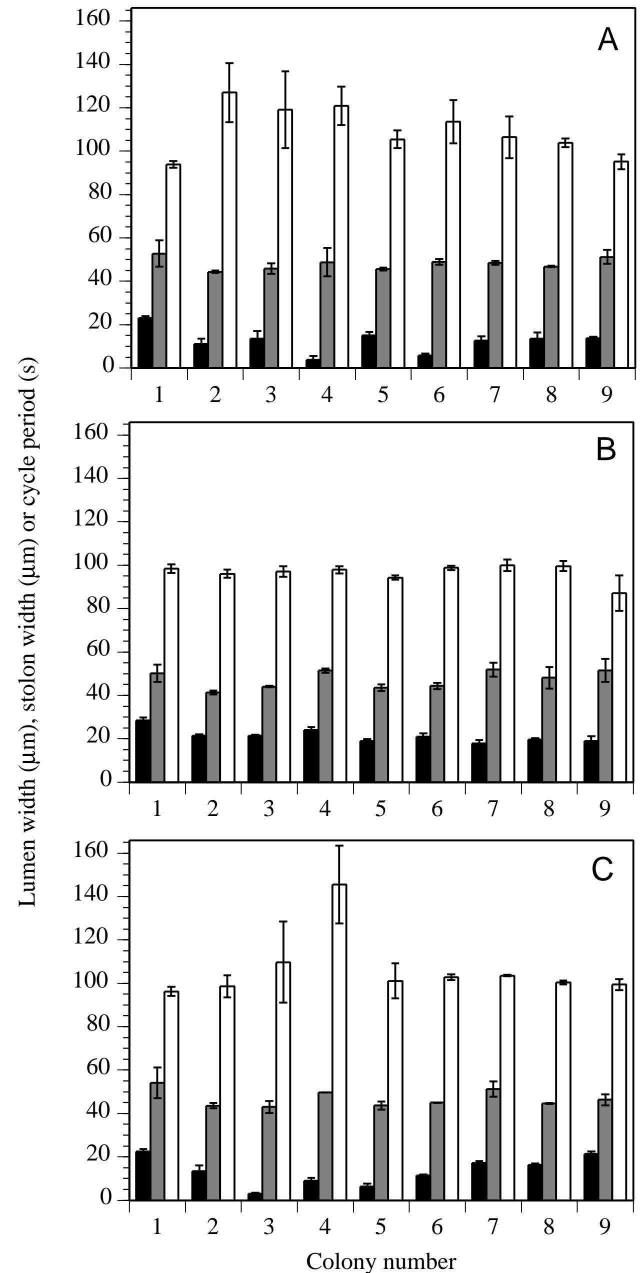

Fig. 10. Means and standard errors for the three flow variables that provide the rate measures in Fig. 9 (filled columns, change in lumen width in µm; shaded columns, stolon width in µm; open columns, cycle period in s) for two stolons per replicate and nine replicates per treatment. (A) Treated with 800 µmol l^{-1} azide for 12 h per day; (B) control; (C) treated with 60 µmol l^{-1} dinitrophenol for 12 h per day. Standard errors provide a measure of between-stolon, within-replicate variation.

significant between-replicate, within-treatment variation in flow rate (using the stolons-within-replicates effect as the error term, F=2.44, d.f.=24,27, P<0.05). In previous analyses using measures of three stolons per replicate colony (Blackstone, 1997b, 1998a,b), this effect is usually not significant. It may be that measuring two stolons per replicate insufficiently samples within-replicate, between-stolon variation, and this variation inflates the between-replicate variation. Nevertheless, despite the significant between-replicate, within-treatment variation, the between-treatment effect is still highly significant (using the replicates-within-treatments effect as the error term, F=11.2, d.f.=2,24, P<0.001). Replicate colonies treated with azide and dinitrophenol show diminished flow relative to the controls (Fig. 9). Since flow rate is a composite of three measured flow variables (net change in lumen width per cycle,

contraction cycle period and stolon width), it is useful to examine the between-treatment difference in these variables individually (Fig. 10). Stolon width shows no between-treatment effect (using the replicates-within-treatments effect as the error term, F=0.2, d.f.=2,24, P>0.8), while contraction cycle period shows a weak between-treatment effect (using the replicates-within-treatments effect as the error term, F=4.0, d.f.=2,24, P<0.05). The net change in lumen width during each cycle, however, shows a large between-treatment effect (using the replicates-within-treatments effect as the error term, F=7.9, d.f.=2,24, P<0.01). Thus, the combination of slightly shorter cycle periods and somewhat larger openings and closings of the lumen with each contraction cycle produces the greater flow rates seen in control replicate colonies compared with those treated with either azide or dinitrophenol. Since flow is generated by contractions of the polyp epitheliomuscular cells, this pattern is consistent with the predicted effects of diminished ATP formation, caused by uncoupling or blocking the electron transport chain, on such an ATP-requiring physiological process.

Assays of cellular redox state using fluorescent microscopic measures of NAD(P)H

In these hydroids, treatment with dinitrophenol has been shown to shift measures of NAD(P)H in the direction of oxidation (Blackstone, 1997b, 1998a,b), probably because the [ATP]/[ADP] ratio is diminished and oxidation of substrate and electron transport are thus activated (Heytler, 1981). Similarly, 3–5 h after feeding, polyps are contracting maximally (Dudgeon et al., 1999), and this metabolic demand probably diminishes the [ATP]/[ADP] ratio, activates oxidative phosphorylation and shifts the redox state in the direction of oxidation. In contrast, even in colonies 3–5 h after feeding, treatment with azide shifts the redox state in the direction of reduction (Fig. 11). Compared with controls and with replicates treated with dinitrophenol, azide-treated replicates exhibit a greater total cross-sectional area of visible epitheliomuscular cell fibers (Fig. 11A; using the replicates-within-treatments effect as the error term, F=14.2, d.f.=2,12, P<0.001), a larger number of visible fibers (Fig. 11B, using the replicates-within-treatments effect as the error term, F=7.9, d.f.=2,12, P<0.01) and greater relative luminance of the fibers (Fig. 11C; using the replicates-within-treatments effect as the error term, F=49.6, d.f.=2,12, $P \ll 0.001$). While the control and the dinitrophenol-treated colonies are indistinguishable at this time (see standard errors in Fig. 11), when polyps become quiescent 12–24 h after feeding, the redox states of control colonies, but not of the dinitrophenol-treated ones, shift in the direction of reduction (Blackstone, 1997b, 1998a,b).

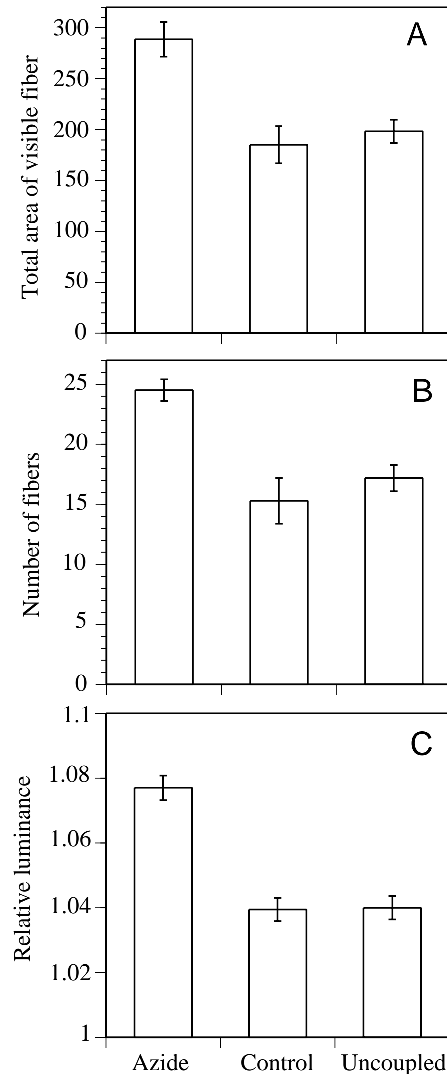

Fig. 11. Means and standard errors of measures of NAD(P)H fluorescence for epitheliomuscular cell fibers of two polyps of each of five colonies for each treatment. Azide, treated with 800 μmol l^{-1} azide for 12 h per day; control; uncoupled, treated with 60 μmol l^{-1} dinitrophenol for 12 h per day.

Discussion

In hydroid colonies, gastrovascular flow and cellular redox state are closely interrelated. Generating gastrovascular flow probably constitutes a major portion of the metabolic work performed by a polyp. The muscular contractions that drive flow may therefore comprise a large part of the polyp's metabolic demand and strongly influence its redox state. Gastrovascular flow also carries nutrients that influence cellular redox state both directly, *via* metabolic pathways, and indirectly, by stimulating muscular contractions and metabolic demand. The interaction between flow and redox state is therefore complex and multifaceted.

Given this complex relationship, it is understandably difficult to separate the effects of gastrovascular flow from redox state. In the current series of experiments, the *P. carnea* explants were similar developmentally at the time they were first treated with either azide, which blocks the electron transport chain, or dinitrophenol, which uncouples oxidative phosphorylation. Despite significant within-treatment variance, the overall treatment effect on flow was highly

significant, and both azide and dinitrophenol result in similarly diminished flow, probably resulting from a reduction in the rate of ATP formation. Redox states, in contrast, were differentially perturbed; dinitrophenol has been shown to shift the redox state in the direction of oxidation (Blackstone, 1997b, 1998a,b), whereas azide shifts the redox state in the direction of reduction. Since the azide- and dinitrophenol-treated colonies subsequently diverged strikingly in their development, an effect of redox state independent of that of gastrovascular flow may therefore be suggested.

Several factors complicate this interpretation. Flow rates and redox states were measured immediately after treatment was begun, while morphology was not measured for several weeks to allow the colonies to reach recognizable developmental landmarks. Between-treatment differences in colony morphology were nevertheless apparent almost immediately. However, the interaction between gastrovascular flow and colony morphology (Blackstone and Buss, 1993; Dudgeon and Buss, 1996) prevents the effects of flow from being entirely absent or controlled once the colony morphology has been perturbed. Thus, after several weeks, some portion of the observed changes in colony development are probably due to this morphology-by-flow interaction. It is also plausible that redox state can have a direct effect on gastrovascular flow. For instance, changes in redox state can affect the formation of reactive oxygen species (ROS; Poyton and McEwen, 1996; Scheffler, 1999), and ROS can in turn affect the stimulation of vascular smooth muscle cells, at least in vertebrates (Sundaresan et al., 1995).

Additional complexities might be caused by unanticipated effects of azide or dinitrophenol on signaling pathways unrelated to redox state; however, this possibility is considered unlikely for several reasons. Agents that affect the electron transport chain have been subject to an enormous amount of study (Scheffler, 1999), so much so that their action is generally taken for granted (e.g. Springer et al., 1994; Vander Heiden et al., 1999). Also, these are usually relatively simple molecules (e.g. azide, N_3), and possible unanticipated effects are rather limited. Future work, however, can and should take advantage of the diversity of these agents. For instance, antimycin A, which binds to cytochrome bc_1, can be substituted for azide, while tributyltin can be substituted for dinitrophenol. Similar outcomes using chemically different agents would strongly support the hypothesis of redox-mediated effects.

These complexities notwithstanding, the effects of redox state on colony development may conform to expectations of the adaptive control of colony morphology by a food supply that may be temporally and spatially variable. A colony growing in an environment where some polyps are feeding more frequently and to a greater extent than others may experience metabolic gradients. Polyps that frequently consume large amounts of food would constantly be stimulated to contract and to pump food-containing gastrovascular fluid throughout the colony (Dudgeon et al., 1999). In such polyps, the large metabolic demands on the epitheliomuscular cells, which generate the gastrovascular flow, would stimulate maximal oxidative phosphorylation and shift the redox state in the direction of oxidation (Blackstone, 1998a). In contrast, polyps that feed infrequently would perhaps be less stimulated to contract, and the metabolic demands on the epitheliomuscular cells might be minimal. Nevertheless, these polyps would be well supplied with substrate (provided by the polyps of the colony that are feeding and contracting), and the mitochondria of the epitheliomuscular cells would enter the resting state, shifting the redox state in the direction of reduction (e.g. Chance and Baltscheffsky, 1958). This putative within-colony metabolic gradient could result in the well-fed polyps and stolons of the colony growing in a manner similar to colonies that are treated with an uncoupler or fed more frequently, i.e. initiating many polyps and stolon tips, while the poorly fed polyps and stolons would grow in a manner similar to those treated with azide, i.e. initiating few polyps and stolon tips. In both cases, the resulting growth form is adaptive. Sheet-like growth in the area of rich food supply would capture still greater amounts of resources for the colony. Runner-like growth in the area of poor food supply would minimize the resources invested in the food-poor area and result in the rapid growth of the colony out of this area and into potentially richer microhabitats. Putative within-colony metabolic gradients and the response of colony growth to such gradients can be elucidated by relatively simple differential feeding experiments and the use of NAD(P)H fluorometry.

Evolutionary morphology and heterochrony may be governed by redox control as well. Consider the evolution of a sheet-like morphology. Species that specialize in food-rich microhabitats would consistently develop with a colony-wide redox state shifted in the direction of oxidation. High rates of polyp and stolon tip initiation would result, and a sheet-like morphology would consistently develop. In such a species, this phenotypically controlled process might occur repeatedly for generation after generation. In time, genes might be recruited to direct the process of developing high rates of polyp and stolon tip initiation. Such ‘gene accumulation’ is thought to occur frequently under these circumstances in evolution (Bonner, 1996; Schlichting and Pigliucci, 1998). In such a species, features other than the timing of polyp and stolon tip initiation might also depend on the gene activity triggered by relative oxidation. Features such as the stolonal mat and the blastostyles of *H. symbiolongicarpus* should be considered in this context. These features characterize this sheet-like species and are never found in the runner-like *P. carnea*. While both structures develop in *H. symbiolongicarpus* colonies treated with dinitrophenol (Blackstone and Buss, 1993), neither develops in *H. symbiolongicarpus* colonies treated with azide. The gene activity associated with the formation of these features may be sensitive to redox state. Further, it may be that the stolonal mat and the blastostyles involve similar morphogenetic pathways, since the blastostyles always develop on the stolonal mat, and since the Hox gene *Cnox-2* may have a regulatory role in the differentiation of both of these structures (Cartwright et al., 1999; Cartwright and Buss,

1999). While there are as yet no data on the mechanism by which redox state triggers gene activity in these hydroids, ROS are likely candidates. ROS are widely used in redox signaling (Poyton and McEwen, 1996; Foyer and Noctor, 1999; Karpinski et al., 1999) and have been implicated in pattern formation in *Hydra* (Jantzen et al., 1998). Further investigations in this area will probably be informative.

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