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# Variable Effects of Mitochondrial Inhibitors and Uncouplers Related to Feeding Times in Colonial Hydroids

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## Abstract

The mitochondria of a cell are the centers of oxidative phosphorylation in aerobic organisms. Certain compounds have been found to perturb the proton gradient created during energy metabolism causing an increase or decrease in the consumption of oxygen by a colonial hydroid. Azide, an inhibitor has been shown to decrease the consumption of oxygen while dinitrophenol, an uncoupler, has been shown to increase oxygen consumption. In colonial hydroids on non-feeding days, the gastrovascular flow of the colonies is inactive and the mitochondria are less responsive to environmental cues such as the presence of these compounds. Therefore, larger concentrations of the compounds on non-feeding days were required to induce an evident physiological response by the colonies.


## Introduction

Colonial hydroids are animals that grow most frequently in the surface areas of marine environments (Frank et al., 2001). They are members of the phylum Cnidaria and as such, share similar features with other species including jellyfish, sea anemones, and corals. However, hydroids offer more advantages for maintenance and experimental studies in the laboratory as compared to other classes. They are easily cultured in the laboratory, are fast growing and able to complete their life cycle within a reasonable time period, facilitate genetic studies and the establishment of inbred lines, are abundant and accessible in nature, are able to provide good perspectives for the application of modern research technologies such as gene expression manipulation or the creation of transgenic lines, and they are suitable for the study of a broad variety of scientific issues such as

allorecognition and the evolution of the vertebrate immune system. Hydroids are small, translucent, and make whole mount in situ hybridization analyses feasible in all life stages. Furthermore, they possess a large array of differentiated cells including striated muscle cells (in medusae) as well as morphologically identifiable pluripotent stem cells called interstitial cells (I-cells), found only in the stolonal network (Frank et al., 2001). Having a population of multi-potent stem cells throughout their lives grants colonial invertebrates a remarkable regenerative capacity. They consist of two layers of living tissue: the epidermis and the gastrodermis with a sometimes thick, nonliving gelatinous mesoglea in between. Hydroids possess cnidae (stinging cells) which are especially abundant on the feeding tentacles located at the mouth of the hydroid. The sensory organs of the hydroid are also present on these tentacles. Like all cnidarians, hydroids lack an anus thereby requiring all undigested food to pass back through the mouth before the next feeding. In addition to an anus, cnidarians lack a true central nervous system but instead possess a network of nerve cells and neurites. Hydroids are diploblastic animals meaning that their musculature does not originate in embryonic mesoderm. The muscle layers of a hydroid, composed of numerous ectodermal and endodermal cells, collectively known as epitheliomuscular cells, function in rhythmic contractile patterns within the feeding polyp in order to transport nutrients throughout the hydroid stolons as well as to capture small food particles and digest them intracellularly. Transport of gases in the hydroid occurs by diffusion across exposed epidermal and gastrodermal surfaces. As members of class Hydrozoa, hydroids are characterized by having greater representation of the polyp morph throughout their life cycle (Brusca and Brusca, 2003). The life cycle of *Podocoryna carnea* is metagenetic (asexual and sexual phases) with two phenotypes,

the sedentary, asexual colonial polyploid stage and the free, single living sexual medusae (Frank et al., 2001).

Hydroids are relatively persistent animals and occupy a given habitat for long periods of time (Blackstone, 2003). Due to their unusually long life spans, these animals encounter challenges not faced in other, shorter-lived animals. Over time, hydroids may face the dilemma of an ever changing food supply. In order to adapt to naturally changing conditions in the environment and the available food supply, a hydroid colony may alter its growth by modifying the production of stolons (vascular connections) and polyps (feeding structures; Blackstone, 1998). In these organisms, it is possible that redox signals ultimately control the development of the feeding structures and gastrovascular connections (Blackstone, 2003). This is considered by many as being an adaptation in adjusting the timing of development and spacing of structures in response to a variable food supply as well as to other environmental factors such as the presence of bacteria. Closely spaced polyps with short branching stolons, also known as sheet-like growth, adapts a colony to an area containing a high volume of food (Blackstone, 1999). This allows for the maximum amount of food to be acquired by the colony. In areas where food may be scarce but could increase in the near future, a runner-like growth may be adopted, consisting of widely spaced polyps with long reaching stolons connecting the polyps which would be energetically favorable for the colony (Blackstone, 1999). In addition, colonies displaying runner-like morphology also exhibit larger medusae and a greater amount of gastrovascular flow to the peripheral stolons than do sheet-like colonies of *Podocoryna carnea* (Blackstone, 1998). A significant characteristic of colonial hydroids is that substrates are shared amongst polyps. Contractions of polyp

epitheliomuscular cells largely drive the gastrovascular flow and these contractions probably constitute a major metabolic cost to the colony (Blackstone, 2001). The colony is therefore able to sustain its existence in a particular area, based upon interpretations of environmental cues, without putting at risk energy demands that cannot be met. These adaptations in growth allow the colonies to successfully inhabit a larger variety of marine environments thus enhancing survival fitness in the species.

In many animals, environmental signals can be transduced into gene activity by redox signaling (Blackstone, 2003). Redox signaling, involving a transfer of electrons and hydrogen atoms in the electron transport chain of the mitochondria, typically occurs when the redox states of electron carriers of the mitochondria are perturbed by substrate. Such perturbations can alter the rate of formation of reactive oxygen species (ROS). ROS are frequently, but not always, a key intermediary in metabolic and redox signaling (Blackstone, 2003). Such a mechanism may function in colonial animals as well.

The mitochondria are a principal source of the metabolic signals in the colony (Blackstone et al., 2004). Both NAD(P)H and peroxides are metabolic signals that emanate from the mitochondria. Because peroxides and other reactive oxygen species are frequently intermediaries in metabolic signaling pathways, such signaling may occur at polyp-stolon junctions, affecting colony pattern formation (Blackstone, 2003). The major sites of ROS formation are found at NADH dehydrogenase of complex I and at the interface between coenzyme Q and complex III at the electron transport chain of the mitochondria in the stolon-polyp junction of the hydroid (Blackstone, 2003). Inhibitors of complexes III and IV in the electron transport chain should therefore 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 down-regulate ROS from both sites. 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 (Blackstone, 2003). Inhibitors of complex I should have intermediate phenotypic effects (Blackstone, 2003).

Redox chemistry is central to energy conversion in cell respiration (Blackstone, 2000). Redox chemistry can be influenced in many ways, including by the state of mitochondrial activation, levels of reduced glutathione and other thiols, levels of NADH and NADPH, thioredoxin levels, and other factors (Smith et al., 2000). It was apparent, in an experiment previously conducted by Dr. Neil Blackstone, that upon manipulation of redox reactions in the electron transport chain, hydroids appeared to undergo morphological as well as polyp-driven gastrovascular changes when the colonial hydroid specie *Podocoryna carnea* was treated with both dinitrophenol, an uncoupler, as well as with azide, a reducing agent (Blackstone, 1999). In this experiment, several specific tests for the presence of reactive oxygen species, released from the mitochondrian-rich stolon-polyp junctions, were conducted (Blackstone, 1999). These same tests were conducted more recently over the past year, however with slight alterations in the procedure. The former experiment, using lower concentrations of azide and dinitrophenol ($800\mu mol l^{-1}$ and $60\mu mol l^{-1}$ respectively), confirmed that azide is an inhibitor of oxygen uptake in colonial hydroids and caused oxygen concentrations to fall more slowly in treated colonies than oxygen concentrations in control colonies (Blackstone, 1999).

Dinitrophenol, an uncoupler, increased the oxygen uptake, even more so than the control colonies, giving an opposite effect to that of azide.

The morphology of the hydroids, when grown in these compounds, displayed variations from normal colonial growth (Blackstone, 1999). When treated with azide, the colonies revealed a more runner-like growth consisting of less stolon branching with few large polyps. Dinitrophenol again differed from azide in that the morphology of the hydroid became even more sheet-like than the control colony and resulted in more stolon branching and many small polyps (Blackstone, 1999).

In addition to oxygen uptake experiments, redox state measurements were taken using fluorescent microscopic measures of NAD(P)H (Blackstone, 1999). It was observed that dinitrophenol shifted the redox state in the direction of oxidation while azide shifted the redox state in the direction of reduction. In the series of tests conducted during this time period, all colonies were utilized for testing 3-5 hours after feeding. Polyps 3-5h after feeding contract maximally. During this time, surges in metabolic demand shift the redox state in the direction of oxidation while it diminishes levels of reactive oxygen species (Blackstone, 2001). In this state of enhanced activity, the mitochondria are phosphorylating maximally and the electron carriers are oxidized (Blackstone, 2001). The polyps of colonial hydroids contract regularly after feeding and disperse food throughout the colony via the gastrovascular fluid (Blackstone et al., 2004). Such contractions may trigger signaling pathways that allow colonies to grow in an adaptive manner, i.e., to initiate development of more polyps in food-rich areas and to suppress polyp development in food-poor areas.

The experiments conducted more recently, however, have been altered slightly in order to further understand the effects of these compounds as well as the naturally occurring cell signaling used by hydroids in order to better adapt their growth and development to aspects of their environment. In order to accomplish this, the time period of treatment with the compounds was changed. The hydroids were utilized for experimentation >24 hours after feeding. In contrast to hydroid activity only 3-5h after feeding, 24h after feeding the polyps are relatively inactive, whereupon the mitochondria enter into a resting state. Thus, with lowered metabolic demands the redox state is shifted into a state of reduction and increased levels of reactive oxygen species are present (Blackstone, 2001). In addition to time modifications, higher concentrations of azide and dinitrophenol were required in order to incite an observable physiological effect. Although these adjustments in the procedure are significant, the results were expected to be similar to those of the previous experiment.

## Materials and Methods

### *Colony Preparation*

Colonies of *P. carnea* were prepared several weeks prior to the experiments. From a larger, mature colony of *P. carnea*, explants were scraped using a scalpel blade and placed onto glass coverslips 12mm in diameter under a plastic "thread" for each individual trial. All of the explants were genetically identical. The colonies were fed brine shrimp three days a week on Mondays, Wednesdays, and Fridays. In response to feeding, oxygen uptake in the colony increases and the redox state of the epitheliomuscular cells shifts in the direction of oxidation (Blackstone, 1997b, 1998a).

Following this period of oxidation, the colony enters into a redox state of reduction until the next feeding.

*Measures of oxygen uptake*

The oxygen uptake experiments were, as stated above, conducted on non-feeding days >24 hours after feeding. The colonies, which were grown on 12mm coverslips, were attached to a coverslip, itself cemented to a magnet, using silicone grease. In order to test for the levels of oxygen concentration, a sealed glass chamber (Strathkelvin RC300) 13mm in diameter was employed. Inside of the chamber, 0.7ml of sea water (filtered to .2mM) was placed. The temperature was held constant at 20.5±0.02°C using an external circulation water bath (Neslab RTE-100D). Two colonies, consisting of a control and an experimental colony of similar size (same approximate number of polyps and stolon connections) were tested. For an initial 30 minute period, each colony was placed in the chamber containing only sea water. The colonies were magnetically spun every 1.5 minutes with the concentration of oxygen recorded every 3 minutes. After the initial 30 minute period the chamber was opened. For both the control and the experimental colony the water was re-aerated. A small amount of sea water (varying depending on the treatment) was removed for the experimental colony with an equivalent volume of sodium azide added to a final concentration of 1mM, 2mM, 3mM, 5mM on non-feeding days and a final concentration of 1mM on a feeding day or dinitrophenol added to a final concentration of 100$\mu$M on a feeding day and 20$\mu$M on a non-feeding day. Again, each colony was tested for 30 minutes. Five pairs of colonies for azide were tested over a period of five weeks. After the azide experiments were conducted, the two tests of dinitrophenol were conducted. The initial concentration of azide that was tested

was greater than that of the 1999 experiment at 1mM of azide for testing oxygen uptake in the colonies. Five graphs were constructed to display the concentrations required to alter oxygen uptake in treated colonies.

## Results

As seen on Figure 1, for 1mM azide on a non-feeding day, there is very little difference between the slopes of the untreated and treated colony and thus, the compound had no apparent effect on the treated colonies uptake of oxygen (paired comparison t-test, t=-0.10, P>0.9, N=7). Next, the unfed experimental colonies were treated with a concentration of 2mM azide. This time there was a near-significant change in oxygen uptake between the treated and untreated colonies as shown by Figure 2 (t=2.1, P>0.05, N=10). The concentrations of the azide was further increased to 3mM (Fig. 3), and showed azide treated colonies with an unchanged rate of decline in oxygen concentration (t=0.7, P>0.5, N=6). Figure 4 shows the most significant decline in oxygen uptake for unfed colonies when treated with a large concentration (5mM) of azide (t=9.42, P<0.001, N=5). Figures 4 and 5 display similar changes in rates between treated and control colonies. Figure 5 displays the effect of 1mM azide on a colony only 3-5h after feeding (t=7.95, P=0.001, N=5). Thus, a five-fold increase in azide was required to elicit the same response in an unfed colony as a fed colony.

Dinitrophenol produced results opposite to those of azide. The oxygen uptake was increased by the colonies at a concentration of 20$\mu$M (treated colony: t=-8.37, P=0.001, N=5; control colony: t=0.23, P>0.8, N=5) on a feeding day and at a concentration of 100$\mu$M on a non-feeding day (treated colony: t=-7.00, P<0.01, N=5;

control colony: t=-2.62, P>0.05, N=5). Dinitrophenol, like azide, was required at a five-time higher concentration on non-feeding days to elicit the same physiological response in the colonies.

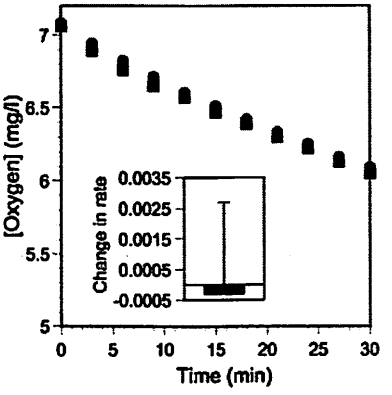


Fig.1. For colonies on non-feeding days, rate of decline in oxygen concentration for a *Podocoryna carnea* colony before (squares) and after (circles) treatment with 1mM. The inset plot shows the mean + S.E.M. of the before/after difference in the rate of decline in oxygen concentration for the treated colony, where this decline is measured by the least-squared slope of oxygen concentration *versus* time. The difference in rate was not significant at the concentration of 1mM.

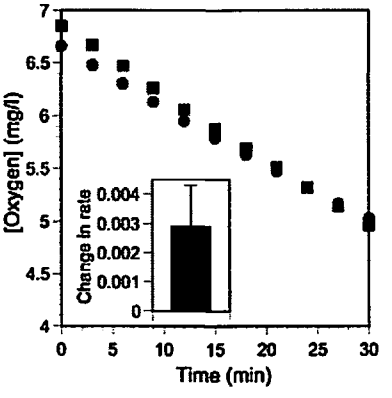


Fig. 2. For colonies on non-feeding days, rate of decline in oxygen concentration for a *Podocoryna carnea* colony before (squares) and after (circles) treatment with 2mM. The inset plot shows the mean + S.E.M. of the before/after difference in the rate of decline in oxygen concentration for the treated colony, where this decline is measured by the least-squared slope of oxygen concentration *versus* time. The difference in rate for azide at a concentration of 2mM was near significant.

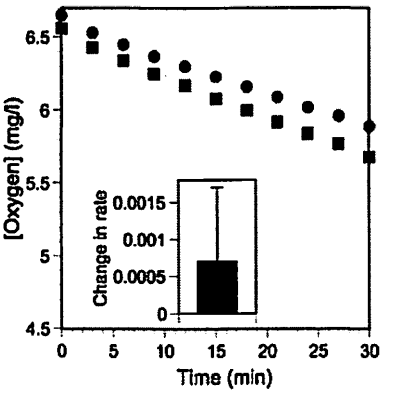


Fig. 3. For colonies on non-feeding days, rate of decline in oxygen concentration for a *Podocoryna carnea* colony before (squares) and after (circles) treatment with 3mM. The inset plot shows the mean + S.E.M. of the before/after difference in the rate of decline in oxygen concentration for the treated colony, where this decline is measured by the least-squared slope of oxygen concentration *versus* time. The difference in rate was not progressively more positive, as seen in the figure, as this concentration of azide at 3mM was not significant.

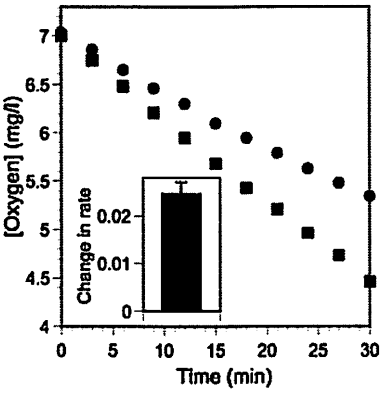


Fig. 4. For colonies on non-feeding days, rate of decline in oxygen concentration for a *Podocoryna carnea* colony before (squares) and after (circles) treatment with 5mM. The inset plot shows the mean + S.E.M. of the before/after difference in the rate of decline in oxygen concentration for the treated colony, where this decline is measured by the least-squared slope of oxygen concentration *versus* time. The uptake of oxygen by adding a concentration of 5mM azide was further inhibited and the result was significant.

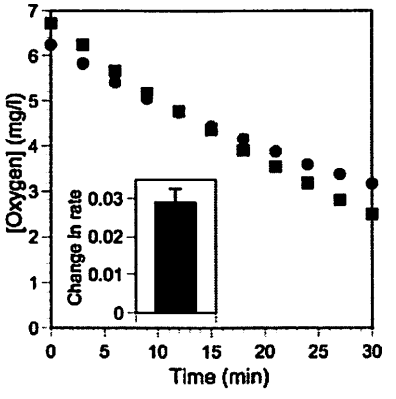


Fig. 5. For colonies on feeding days, rate of decline in oxygen concentration for a *Podocoryna carnea* colony before (squares) and after (circles) treatment with 1mM. The inset plot shows the mean + S.E.M. of the before/after difference in the rate of decline in oxygen concentration for the treated colony, where this decline is measured by the least-squared slope of oxygen concentration *versus* time. In a fed colony treated with a concentration of 1mM azide, the change in rate of decline in oxygen concentration is very similar to the change in rate for an unfed colony at a concentration five times higher.

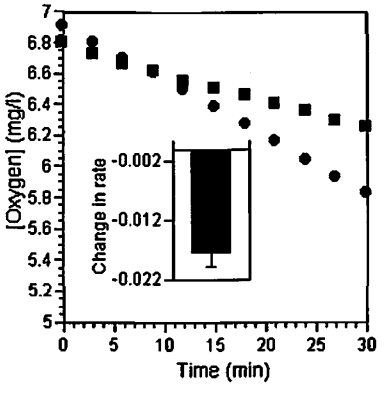


Fig. 6. For colonies on non-feeding days, rate of increase in oxygen concentration for a *Podocoryna carnea* colony before (squares) and after (circles) treatment with 100$\mu$M. The inset plot shows the mean + S.E.M. of the before/after difference in the rate of decline in oxygen concentration for the treated colony, where this decline is measured by the least-squared slope of oxygen concentration *versus* time. Dinitrophenol, at a concentration of 100$\mu$M, produces a significant increase in the uptake of oxygen.

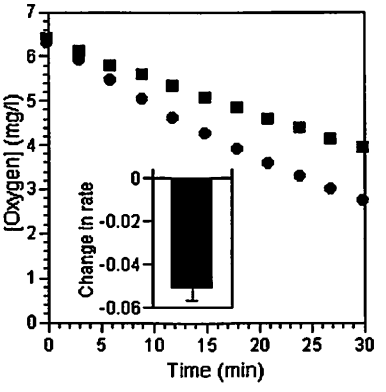


Fig. 7. For colonies on a feeding days, rate of decline in oxygen concentration for a *Podocoryna carnea* colony before (squares) and after (circles) treatment with 20$\mu$M dinitrophenol. The inset plot shows the mean + S.E.M. of the before/after difference in the rate of decline in oxygen concentration for the treated colony, where this decline is measured by the least-squared slope of oxygen concentration *versus* time. Dinitrophenol, at a concentration of 20$\mu$M, produced a similar effect on a feeding day as a concentration five-times higher on a non-feeding day.

## Discussion

Both compounds, azide and dinitrophenol, act on the mitochondria of a cell but each elicits very different responses in the hydroid colonies. The compounds perturb the steps of oxidative phosphorylation whereby the degradation of carbohydrates, fats, and amino acids converge at a final stage of cellular respiration, driving the synthesis of ATP. This involves the reduction of $O_2$ to $H_2O$ with electrons donated by NADH and $FADH_2$. The mitochondrial respiratory chain consists of sequentially acting electron carriers, most of which are integral proteins with prosthetic groups capable of accepting and donating either one or two electrons (Nelson and Cox, 2005). Azide, an inhibitor, binds to cytochrome oxidase (complex IV and the final step of cellular respiration) of the electron transport chain and blocks the flow of the electrons preventing the reduction of oxygen into water. Dinitrophenol is a proton ionophore, creating a proton channel that allows the flow of electrons across the inner mitochondrial membrane into the mitochondrial matrix instead of going to ATP synthase. This flow of electrons through this newly created channel causes proton-motive force to be dissipated and the formation of ATP to be slowed considerably.

As predicted by the previous experiments conducted, azide, as an inhibitor, decreased the rate of oxygen uptake in the mitochondria of colonial hydroids. At a concentration of 1mM azide there was no effect on the colonies and it actually appears as though the effects were in contrast to those expected but these effects are considered to be negligible because of the very little change in slope. However, various concentrations gave effects that were slightly unexpected. The concentration of 2mM azide displayed a greater effect on the colonies than did 3mM. Despite the fact that 2mM azide gave a near

significant effect while 3mM azide did not, both concentrations suggest a weak and variable effect on the colonies and thus, are viewed as statistically insignificant and if conducted again, the results at these concentrations could be varied. 5mM azide, however, produced the greatest inhibition in the colonies uptake of oxygen and the result shows a strong effect at this concentration.

For dinitrophenol, the result was the same. The colonies were less responsive to dinitrophenol on non-feeding days and produced an almost identical slope in the increased uptake of oxygen by the hydroids when the concentration was magnified five-times. This data supports the belief that gastrovascular flow is closely related to the redox state of the electron transport chain (Blackstone, 1999). On feeding days, the gastrovascular activity is much greater and the redox state of the colony is oxidizing. In this redox state, as the results show, colonies on feeding days are more sensitive to the inhibitors and uncouplers than colonies on non-feeding days. The data suggests that there are differences in the mitochondria on these days.

Food acts as a substrate for the mitochondria in colonial hydroids. Therefore, when the mitochondria are attached to substrate, they appear to be more sensitive to these compounds. However, data from previous experiments suggest that at times 24hr after feeding, the hydroids are not out of substrate but are still in possession of some substrate. In other words, they are not yet in a state of starvation. The redox state is reducing but the gastrovascular pumping may be inactive. Studies have shown that most of the mitochondria in the hydroid lie in the polyp-stolon junction in muscle cells. It has been determined in rescent studies that contractions that drive flow are stimulated by feeding and begin 5-15 min after ingestion (Dudgeon et al., 1999). At 3-5hr the mitochondria are

contracting maximally and at 24hr after feeding, these regions would not be contracting. When the behavior of these regions is active they are more sensitive to environmental cues and are searching for input. The hydroids, at this time, are integrating environmental signals more strongly. The integration of information in the mitochondria has been shown to play a large role in early-evolving animals such as colonial hydroids (Blackstone et al., 2005). If input comes in the form of azide or dinitrophenol, these compounds appear to produce a greater physiological response in the mitochondria of the colonies. In addition, the metabolic demands are far greater after feeding than the demands 24hr after feeding and therefore, the uptake of oxygen is naturally greater. With slight inhibition from a small concentration, distinct visible changes to the uptake of oxygen take place. However, these compounds probably affect the uptake of oxygen in these same low concentrations on non-feeding days but the effects are not observable due to an overall lack of activity.

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