RESEARCH ARTICLE

Causes and consequences of stolon regression in a colonial hydroid

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SUMMARY

A cnidarian colony can be idealized as a group of feeding polyps connected by tube-like stolons. Morphological variation ranges from runner-like forms with sparse polyp and stolon development to sheet-like forms with dense polyp and stolon development. These forms have typically been considered in a foraging context, consistent with a focus on rates of polyp development relative to stolon elongation. At the same time, rates of stolon regression can affect this morphological variation; several aspects of regression were investigated in this context. More sheet-like forms were produced by periodic peroxide treatment, which induced high rates of stolon regression. Caspase inhibitors altered the effects of regression induced by peroxide or vitamin C. These inhibitors generally diminished physical regression and the abundance of associated reactive oxygen species. Caspase inhibitors also altered cellular ultrastructure, resulting in features suggestive of necrosis rather than apoptosis. At the same time, caspase inhibitors had little effect on reactive nitrogen species that are also associated with regression. Although regression is most easily triggered by pharmacological perturbations related to reactive oxygen species (e.g. peroxide or vitamin C), a variety of environmental effects, particularly restricted environments and an interaction between feeding and temperature, can also induce regression. Stolon regression may thus be a factor contributing to natural variation between runners and sheets.

Key words: cell death, clonal organism, cnidarian stress response, evolutionary morphology, hydroid, *Podocoryna*, *Podocoryne*, reactive oxygen species, reactive nitrogen species.

INTRODUCTION

Modular colonial sessile organisms, whether plant or animal, have common features that relate to their ability to respond to environmental challenges (Buss and Blackstone, 1991). Typically, a colony exhibits ramets, vegetative modules with the capacity for an independent existence, interconnected by a branching vascular system. Changing the timing and spacing of ramet and branch formation allows the sessile organism to adjust its growth morphology and respond appropriately to different environmental challenges. This morphological plasticity may allow the same clone to be successful in two very different environments or, alternatively, allow a single colony to be successful in a changing environment. Growth morphologies include runner-like forms at one extreme and sheet-like forms at the other. Runner-like forms grow quickly with long vascular connections and widely spaced ramets (Buss, 1979; Jackson, 1979). Sheet-like forms grow more slowly with short stolonal connections and closely spaced polyps. Often the terms 'guerilla' and 'phalanx' are applied to these growth forms, particularly in plants (Lovett Doust, 1981). Guerilla growth forms sample the environment to locate suitable conditions whereas phalanx forms commit to a location, securing a place in the current environment.

Colonial cnidarians have been central to the study of runner-like and sheet-like forms (Blackstone, 2009; Buss, 2001). Studies of these organisms have explored the physiological effects of gastrovascular flow (Dudgeon and Buss, 1996; Hale, 1964; Schierwater et al., 1992; Wyttenbach, 1968), the role of gene activity in colony development (Cartwright and Buss, 1999; Cartwright et al., 1999; Cartwright et al., 2006; Lange and Müller, 1991; Plickert et al., 1987; Schaller et al., 1989; Takahashi et al., 1997) and other mechanisms (Beloussov,

1973; Beloussov et al., 1989; Kossevitch et al., 2001). These investigations have elucidated processes involved in cnidarian colony development, particularly with regard to the outward growth of stolons and the initiation of polyp formation. Tissue regression has received less attention even though regressive processes are increasingly being recognized for their roles in organismal development. Tissue regression contributes to pattern formation in a variety of multicellular animals (Hori et al., 2000; Niswander, 2003; Reddien et al., 2005; Tata, 2006; Technau et al., 2003). Examples of tissue regression have been suggested in colonial hydroids (e.g. Crowell, 1991; Forteath et al., 1982; Hall-Spencer et al., 2007; Pfeifer and Berking, 1995; Ponczek and Blackstone, 2001). However, the significance of stolon regression has yet to be examined in the context of runner-like and sheet-like growth forms. If, for example, the growth of outward-reaching stolons is retarded or the stolons regress while polyp production remains constant, a sheet-like pattern can emerge. The regression of stolon tissue may thus contribute to pattern formation in colonial hydroids.

Previously, we introduced and characterized aspects of stolon regression in a colonial hydroid (Cherry Vogt et al., 2008). Detailed studies of this process were carried out using pharmacological manipulations to induce the simultaneous regression of a number of stolons. Accumulation of endogenous reactive oxygen species (ROS) and reactive nitrogen species (RNS) was found in the regressing tissue. Morphological features and DNA fragmentation indicated that cell death was occurring in the regressing stolons. A number of questions nevertheless remain unanswered, including: (1) what are the effects of high rates of stolon regression on colony morphology, (2) how do caspase inhibitors affect this process and (3) are there non-pharmacological, environmental factors that can

induce stolon regression? These questions are addressed by the current investigations, and the results are discussed in the general context of the cnidarian stress response (Weis, 2008).

MATERIALS AND METHODS
Study species and culture conditions

For all experiments, colonies of a single clone, P3, of *Podocoryna* (=*Podocoryne*) *carnea* Sars 1846 were cultured using standard methods at 20.5°C (e.g. Blackstone, 1999). In each series of experiments, clonal replicates were grown from single polyp explants of a source colony. Replicate colonies were grown on 15 or 18 mm diameter microscope cover glass. For transmission electron microscopy (TEM), colonies were grown on thin polyethylene tied to a glass slide for support.

Comparisons of colony morphology

Treatment with exogenous peroxide at low concentrations (20–50 μmol l^{-1}) has no effect on colony morphology (Blackstone et al., 2005). At higher concentrations (5 mmol l^{-1}), treatment with peroxide has been shown to trigger high rates of stolon regression (Cherry Vogt et al., 2008). Three treatments were begun on small colonies 18 days after explanting. In addition to a control treatment, peroxide was used at two concentrations (0.1 and 1 mmol l^{-1}). Brief intermittent treatments (1 h per day, three times per week on non-feeding days) were observed to trigger high rates of stolon regression. The justification for this treatment regime is twofold. First, continuous treatment seems to be more likely to alter rates of polyp and stolon initiation during growth. Second, inhabitants of shallow-water marine environments might be exposed to various environmental stresses on an intermittent basis, e.g. corresponding to the tidal cycle. Images were taken when a colony was covering the surface (defined as when the largest gap between any two stolons reaching the edge was less than one-fourth of the circumference of the cover glass). At the time of imaging, the ages of the colonies thus varied (treated colonies grew more slowly), but their sizes were comparable. After 3 months, some of the colonies treated with the high concentration of peroxide still failed to reach the edge of the cover glass. Because analyses were adjusted for the size of the colony, these 3 month images were nevertheless included.

Images were processed using Corel Photo Paint software (Corel, Ottawa, Canada) in order to enhance contrast and permit automatic measurement in Image Pro Plus software (Cherry Vogt et al., 2008). Processed images were checked against the original images for accuracy. Images were measured for total area of the colony, area of polyps and areas within the colony not occupied by the tissue of the stolons or polyps (i.e. empty areas). These measures allow us to distinguish runner-like and sheet-like growth (Blackstone, 1998). Data were analyzed with ANOVA and multivariate ANOVA (MANOVA). To meet the assumptions of parametric statistics, the data were natural-log transformed.

Effects of caspase inhibitors

Caspases are central to some cell death pathways in animals (Kroemer et al., 2009). Caspase homologues seem to participate in cell death in cnidarians (David et al., 2005; Dunn et al., 2006; Wittig et al., 2011). Fifteen putative caspases were identified from the whole-genome assembly of *Hydra magnipapillata* (Lasi et al., 2010). Although the specific homologies of these caspases are not yet clear, existence of a caspase-3 homologue is well established (Seipp et al., 2006; Wittig et al., 2011). Previous work indicated that cell death may be involved in the process of stolon regression (Cherry Vogt et al., 2008). Generally, the observed features were more consistent

with an apoptotic rather than a necrotic form of cell death, although the division between the two is becoming increasingly blurred (Kroemer et al., 2009). At the level of cellular ultrastructure, apoptosis is typically characterized by rounding up of the cell, reduction of cellular and nuclear volume, chromatin condensation and plasma membrane blebbing, whereas necrosis exhibits rupture of the plasma membrane, swelling of cytoplasmic organelles and more moderate chromatin condensation. Characterizing the particular form of cell death in the hydroid stolon system was complicated by inconsistent results from standard *in vivo* diagnostic tests (Cherry Vogt et al., 2008). Examination of caspase activity with the FAM Caspase Activity Kit (Imgenex Corp., San Diego, CA, USA) also provided inconsistent results (data not shown).

In this context, a logical next step is to investigate the effects of caspase inhibitors. When stolon regression was induced using vitamin C (Blackstone et al., 2005), the poly-caspase inhibitor z-DEVD-fmk [benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone; BioVision, Inc., Mountain View, CA, USA] was used. This inhibitor is thought to affect caspases 3 and 7 and possibly caspases 6, 8 and 9 as well (Duval et al., 2002; Fahy et al., 1999). Colonies were treated with a 2 μmol l^{-1} solution of z-DEVD-fmk and seawater. Vitamin C in seawater adjusted to pH 8 was then added to a final concentration of 100 μmol l^{-1} and the colonies were incubated for 1 h (Blackstone et al., 2005). Following the addition of a fluorescent probe for detecting peroxide [2′,7′-dichlorodihydrofluorescein diacetate (H$_2$DCFDA); Molecular Probes, Eugene, OR, USA], colonies were incubated for an additional hour before imaging three stolon tips per colony. Use and visualization of H$_2$DCFDA followed standard protocols (Cherry Vogt et al., 2008). To standardize the relative luminance, the foreground luminance of the tip was adjusted for the background luminance by subtraction.

Additional investigations used H$_2$O$_2$ because it was more effective at triggering a large number of stolons to regress within a single colony. These experiments used the caspase inhibitor Q-VD-OPh [quinolyl-valyl-O-methylaspartyl-(–2,6-difluorophenoxy)-methyl ketone; Imgenex Corp.]. The switch from z-DEVD-fmk to Q-VD-OPh was made for several reasons. Relative to z-DEVD-fmk, Q-VD-OPh inhibits more mammalian caspases, is less cytotoxic, exhibits increased uptake by the cell and is less likely to cross-react with other cysteine proteases (Chauvier et al., 2007; Knoblach et.al., 2004; Rozman-Pungercar et al., 2003; Schotte et al., 1999).

At least 1 day after feeding, control colonies, H$_2$O$_2$-treated (5 mmol l^{-1}) colonies and caspase-inhibited H$_2$O$_2$-treated (5 mmol l^{-1}) colonies were incubated at 20.5°C for 1 h. A few minutes prior to adding H$_2$O$_2$, the caspase-inhibited colonies were treated with a 0.1 mmol l^{-1} solution of Q-VD-OPh, which was solubilized in dimethyl sulfoxide and seawater. After 1 h, the treatment solutions were replaced with solutions of fluorescent probes for peroxide (H$_2$DCFDA) or nitric oxide [4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) diacetate; Molecular Probes] and incubated for an additional 1 h prior to imaging. Note that H$_2$DCFDA can only detect peroxide once it is inside a cell, so it will not interact with any remaining exogenous peroxide. The relative luminance of three stolon tips per colony was measured using standard methods (Cherry Vogt et al., 2008), and log-transformed data were analyzed with a nested ANOVA. Counts of regressed and unregressed stolon tips were also taken from colonies in the different treatments.

Environmental effects

Observation suggests that stolon regression can be triggered by a restricted environment and associated poor water quality. Instead
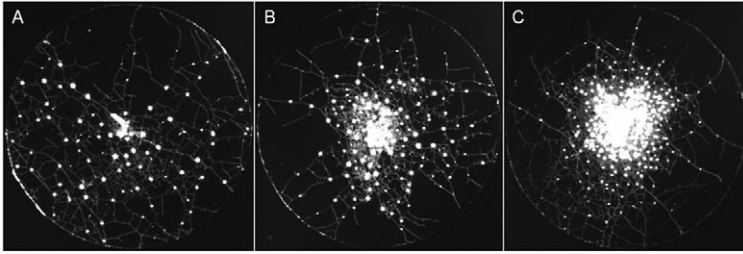
Fig. 1. Processed images of colonies of *Podocoryna carnea* growing on 18 mm diameter cover glass. Processed images were checked against originals to insure accuracy. Colonies treated with higher concentrations of peroxide exhibited more polyps and shorter stolonal connections: (A) control colony, (B) colony treated with 0.1 mmol l^{-1} peroxide, (C) colony treated with 1 mmol l^{-1} peroxide.

of the usual finger bowls (300 ml), colonies were thus confined to Petri dishes (50 ml). After several days without food, colonies in one group were fed before transfer to Petri dishes whereas colonies in the other group remained starved. After 10 h in Petri dishes, counts of regressed and unregressed stolon tips were taken from colonies in the different treatments.

The effects of temperature and feeding were also investigated. As shown by the caspase-inhibitor experiments (see Results), the effects of regression are transitory and a limited number of replicates could be included in each experiment. These limitations on the size of the experiment ruled out a full factorial design. Instead, temperature-perturbed colonies at 27°C, both fed and unfed, were compared with unfed colonies at 20.5°C. Subsequent experiments compared fed colonies at 27 and 20.5°C as well as fed and unfed colonies at 20.5°C. All colonies in these comparisons were starved for several days prior to treatment. Colonies were treated with fluorescent probes for H_2O_2 and three tips per colony were imaged. Data were analyzed as described above. Counts of regressed stolon tips were also taken. Rates of gastrovascular flow in stolon tips were also measured using standard methods (Cherry Vogt et al., 2008).

Ultrastructure of regressing stolons

Ultrastructure in normal and regressing stolon tips was examined using TEM. Colonies were fixed in a solution of 2.5% glutaraldehyde and seawater for 3 h at 4°C. Fixation was followed by three 10 min rinses using Millonig's phosphate buffer. Colonies were postfixed in 1% osmium tetroxide for 2 h at room temperature. Again, the specimens were rinsed in Millonig's phosphate buffer three times for 10 min each rinse. Specimens were dehydrated in an ethanol series and cleared in acetone. Subsequently, colonies were infiltrated and embedded in EMbed 812 resin (Electron Microscopy Sciences, Hatfield, PA, USA). Sections, ~90 nm thick, were cut using a Reichert OmU2 ultramicrotome with a diatome diamond knife and collected on Formvar-coated slot grids or 75-mesh copper grids. Sections were stained with uranyl acetate for 20 min and then with lead citrate for 40 min. Following staining, sections were examined using a Hitachi H-600 transmission electron microscope. Micrographs of specimens were obtained using Kodak 4489 Electron Microscope Film and negatives were then scanned. Cellular structures were examined and differences noted.

RESULTS
Comparisons of colony morphology

In colonies of *P. carnea* treated briefly and intermittently with peroxide for up to 3 months, the total colony area did not differ significantly between treatments ($F_{2,18}=2.1$, $P>0.15$). However, the size of the areas within colonies not covered by stolons or polyps (i.e. the empty areas) differed dramatically (mean ± s.e.m.=0.28±0.045, 0.14±0.011 and 0.09±0.013 for controls, 0.1 peroxide and 1 mmol l^{-1} peroxide, respectively). Using log-transformed data, these differences are highly significant ($F_{2,18}=16.9$, $P<<0.001$). Given that sheet-like colonies cover the surface with

closely spaced polyps and stolons, these data suggest that higher concentrations of peroxide lead to more sheet-like morphologies. Similar conclusions are apparent from inspecting these colonies (Fig. 1) and from comparing the total amount of empty area within a colony with the total amount of polyp area (Fig. 2). The relationship between these variables differs significantly depending on the treatment (MANOVA, $F_{4,34}=12.2$, $P<<0.001$). Higher concentrations of peroxide result in greater areas of polyps and lesser areas not covered by tissue.

Effects of caspase inhibitors

Colonies treated with exogenous vitamin C exhibit stolon regression as well as elevated levels of ROS and RNS in stolon tips (Blackstone et al., 2004; Blackstone et al., 2005; Cherry Vogt et al., 2008). However, in colonies treated with the caspase inhibitor, z-DEVD-fmk, ROS levels are suppressed (Fig. 3A–F). In the first experiment with the inhibitor (four replicates in each treatment), there was a significant difference between treatment groups in the relative luminance of stolon tips ($F_{2,9}=60$, $P<<0.001$; Fig. 4A). An orthogonal contrast showed that relative luminance of the control group was not significantly different from that of the vitamin-treated, inhibited group ($F_{1,9}=0.5$, $P>0.45$). In contrast, the relative luminance of the vitamin-treated, inhibited group was significantly different from that of the vitamin-treated group ($F_{1,9}=96$, $P<<0.001$). Two subsequent experiments used similar protocols, but with seven replicates in each treatment (data not shown). Overall, these
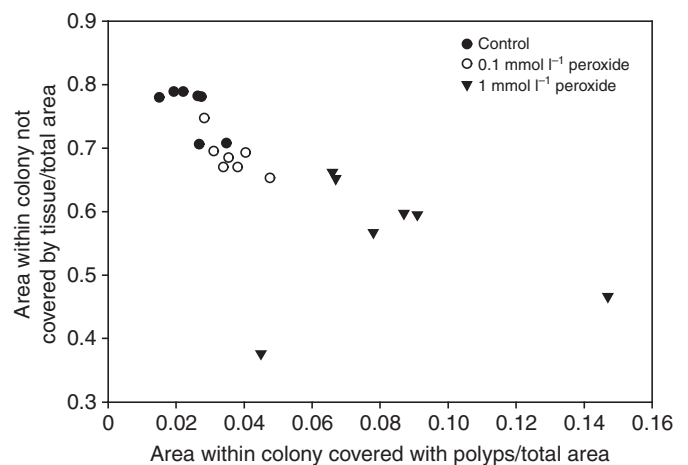

Fig. 2. Proportion of within-colony area not covered by polyps or stolons (i.e. empty area relative to total area) plotted against the proportion of within-colony area of polyps for *P. carnea* colonies treated with peroxide. Higher concentrations of peroxide generally resulted in lesser amounts of empty area and greater amounts of polyps, which are both characteristics of sheet-like growth. The outliers in the 1 mmol l^{-1} treatment were both colonies that barely grew, e.g. the colony in the lower-left quadrant of the graph was only 8.8 mm^2 after 3 months growth (cover glass area=254 mm^2).
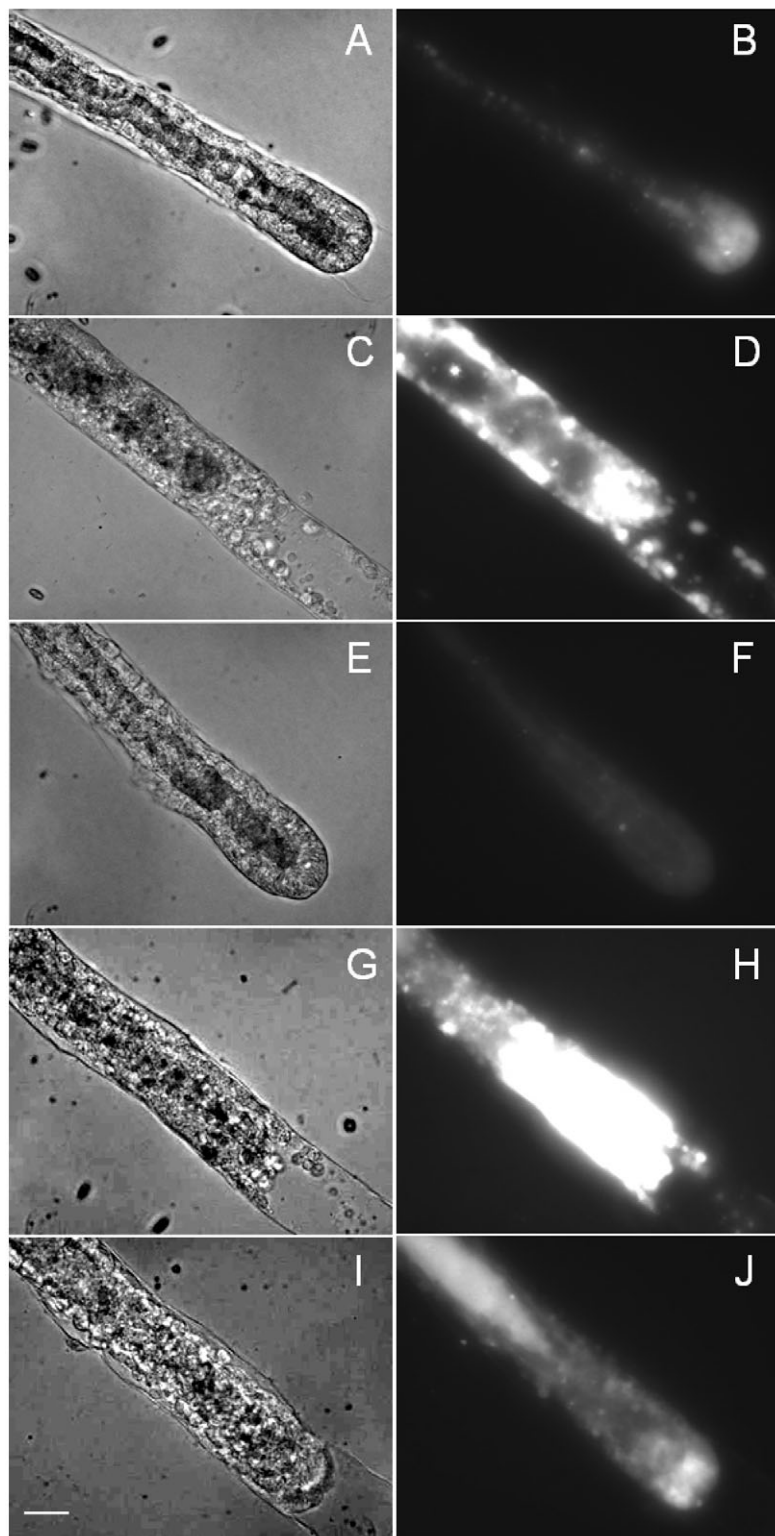
Fig. 3. Bright-field (A,C,E,G,I) and fluorescent (B,D,F,H,J) micrographs of stolon tips from colonies of *P. carnea* visualized with H_2DCFDA. (A) A healthy stolon tip from a control colony exhibited no regression and (B) relatively little ROS-related fluorescence. (C) After 2 h treatment with vitamin C, a stolon tip exhibited pronounced regression and (D) high levels of ROS-related fluorescence. (E) After 2 h treatment with a caspase inhibitor and vitamin C, a stolon tip exhibited little regression and (F) relatively little ROS-related fluorescence. (G) A stolon tip after 1 h treatment with exogenous H_2O_2 exhibited pronounced regression and (H) high levels of ROS-related fluorescence. (I) A stolon tip after 1 h treatment with a caspase inhibitor and exogenous H_2O_2, exhibited moderate regression and (J) intermediate ROS-related fluorescence. Scale bar (applies to all panels), 25 µm.

experiments showed a similar pattern, but sometimes with lower statistical significance. Examination of the time course of regression shows that the effects of vitamin C on ROS are transitory. The greater number of replicates necessarily required a greater total measurement time so the effects of vitamin C (and of the inhibitor) may have been attenuated for some replicates. This greater within-treatment variation may have produced the observed smaller between-treatment effects. These results influenced the design of

the 'environmental effects' experiments (see below). Nevertheless, the effects of both vitamin C and caspase inhibitors appear robust and repeatable.

Experiments using exogenous H_2O_2 yielded similar results (Fig. 3G–J). With seven replicates in each treatment, proportions of regressing stolon tips in each colony were highest in the peroxide treatment (mean ± s.e.m.=0.134±0.03) and similar in controls (0.008±0.005) and peroxide-treated, caspase-inhibited
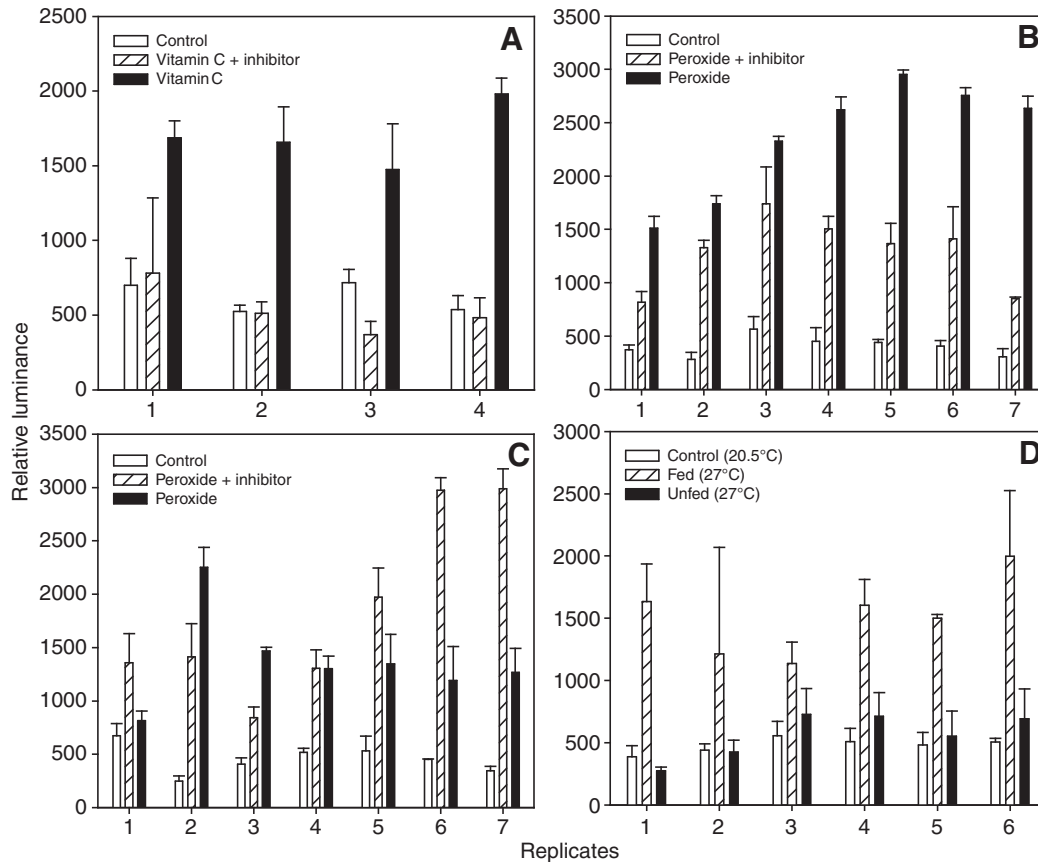
Fig. 4. Mean ± s.e.m. relative luminance for three stolon tips from the indicated number of replicate *P. carnea* colonies. (A) Four replicate colonies visualized with H_2DCFDA. Luminance indicates accumulation of ROS and is associated with stolon regression (Fig. 3C,D). Vitamin C treatment triggered high levels of regression and fluorescence whereas caspase inhibitors diminished this effect. (B) Seven replicate colonies visualized with H_2DCFDA. Luminance indicates accumulation of ROS and is associated with stolon regression (Fig. 3G,H). Peroxide treatment triggered high levels of regression and fluorescence whereas caspase inhibitors diminished this effect. Replicates were measured sequentially in time; note that the treatment effect builds to a peak and then declines. (C) Seven replicate colonies visualized with DAF-FM diacetate. Luminance indicates accumulation of RNS and is associated with stolon regression (Fig. 5). Peroxide treatment triggered high levels of regression and considerable fluorescence whereas caspase inhibitors did not consistently diminish this fluorescence. (D) Six replicate colonies visualized with H_2DCFDA. Note that control colonies are unfed. Luminance indicates accumulation of ROS and is associated with stolon regression. Temperature treatment triggered high levels of regression and fluorescence in fed colonies, but less so in unfed colonies.

colonies (0.014±0.003). There was also a significant difference between treatment groups in the relative luminance of stolon tips ($F_{2,18}$=48.5, P<<0.001; Fig. 4B). An orthogonal contrast showed that relative luminance of the control group was significantly different from that of the peroxide-treated, inhibited group ($F_{1,18}$=19.8, P<0.001). The relative luminance of the peroxide-treated, inhibited group was also significantly different from the peroxide-treated group ($F_{1,18}$=29, P<<0.001). Examination of the data suggests that the inhibitor diminished endogenous ROS considerably (Fig. 4B).

In contrast, the caspase inhibitor does not affect RNS in the same manner that it affects ROS (Fig. 5). With seven replicates in each treatment, there was a significant difference between treatment groups in the relative luminance of stolon tips ($F_{2,18}$=11.6, P<0.001; Fig. 4C). An orthogonal contrast showed that relative luminance of the control group was significantly different from that of the peroxide-treated, inhibited group ($F_{1,18}$=22.2, P>0.001). However, the relative luminance of the peroxide-treated, inhibited group was not significantly different from that of the peroxide-treated group ($F_{1,18}$=2.5, P>0.1). Thus no effect of the inhibitor on endogenous RNS was apparent.

Environmental effects

Unfed colonies confined to Petri dishes for 10 h showed high proportions of regressing stolon tips (N=7, mean ± s.e.m.=0.377±0.064) whereas feeding had a protective effect (N=7, 0.033±0.011). Temperature stress and feeding showed a more complex relationship. In an initial experiment, feeding resulted in lower proportions of regressing stolon tips in each colony (20.5°C, unfed, N=7, 0.097±0.013; 27°C, unfed, N=7, 0.442±0.025; 27°C, fed, N=7, 0.208±0.023). Examination of ROS detected with H_2DCFDA in stolon tips using a similar experimental design nevertheless suggested that, in some cases, starvation may protect against the effects of temperature. Unfed colonies at 20.5°C showed the least fluorescence (N=5, mean luminance ± s.e.m.=505±73) whereas fed colonies at 27°C showed the most (N=5, 1100±71) and unfed colonies at 27°C were intermediate (N=5, 766±91). The difference between the treatment groups in the relative luminance of stolon tips was statistically significant ($F_{2,12}$=14.5, P<0.001). An orthogonal contrast showed that relative luminance of the unfed 20.5°C group was significantly different from that of the unfed 27°C group ($F_{1,12}$=5.3, P<0.05). The relative luminance of the unfed 27°C group was also significantly different
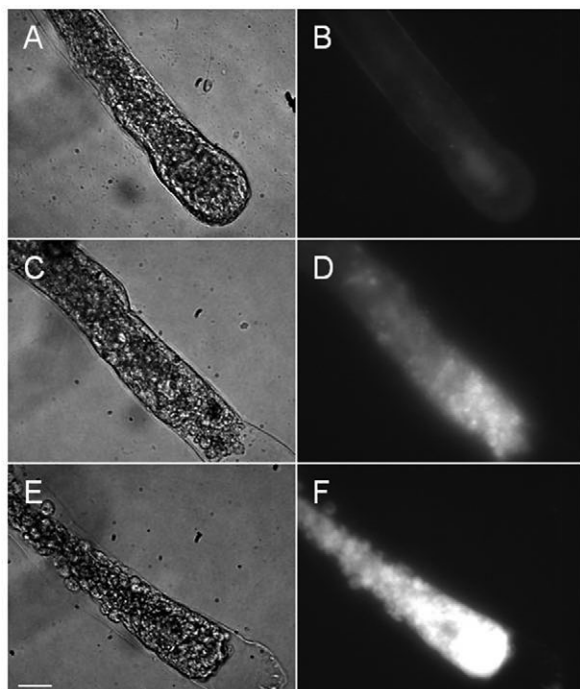
Fig. 5. Bright-field (A,C,E) and fluorescent (B,D,F) micrographs of stolon tips from colonies of *P. carnea* visualized with DAF-FM diacetate. (A) A healthy stolon tip from a control colony exhibited no regression and (B) relatively little NO-related fluorescence. (C) After 1 h treatment with H_2O_2, a stolon tip exhibited pronounced regression and (D) an intermediate level of NO-related fluorescence. (E) After 1 h treatment with a caspase inhibitor and H_2O_2, a stolon tip exhibited regression and (F) a high level of NO-related fluorescence. Scale bar (applies to all panels), 25 μm.

Ultrastructure of regressing stolons

Comparisons of stolon tip ultrastructure reveal several characteristics of dead and dying cells [following recommendations of Kroemer et al. (Kroemer et al., 2009)]. For comparison, a healthy control tip shows cells with irregular margins, nuclei that are approximately 4–8 μm in diameter and mitochondria that are roughly 0.5–0.75 μm in length (Fig. 6A–C). However, the cells of a spontaneously regressing tip have very few recognizable features (Fig. 6D,E). In part this may be because it was difficult to gauge the time of onset of regression that was not deliberately perturbed (i.e. this may be the late stages of regression). Nevertheless, membrane rupture is evident in these tips, and this is consistent with necrosis. The cells of stolon tips treated with peroxide exhibit characteristics found with previous work (Cherry Vogt et al., 2008), including membrane blebbing, rounding-up of the cell body, a reduction of nuclear size to approximately 3 μm in diameter and chromatin condensation (Fig. 7A–D; Table 1). Peroxide-treated, caspase-inhibited tips exhibit features more consistent with necrosis rather than apoptosis (Fig. 7E–G; Table 1).

DISCUSSION

Many colonial organisms exhibit plasticity of morphological phenotypes. Colonial cnidarians, in particular, exhibit wide variation in growth forms. At the extremes of this variation are sheet-like growth forms with dense polyp and stolon growth and runner-like growth forms with sparse polyp and stolon growth. Indeed, both types of growth can be seen within individual colonies exposed to environmental gradients (Blackstone, 2001; Bumann and Buss, 2008). This variation may develop in part through different rates of polyp initiation and stolon elongation relative to stolonal branching. The data presented here suggest that stolon regression may also be relevant to this variation. Although elongation, initiation and regression are likely all affected simultaneously by any perturbation, previous work (Blackstone et al., 2005; Cherry Vogt et al., 2008) and the results of the present study suggest that brief, intermittent treatment with exogenous peroxide primarily triggers stolon regression. Greater concentrations of peroxide and presumably greater rates of regression result in more sheet-like colony morphologies. On the one hand, this result is unsurprising because stolon regression is negative elongation and thus has the opposite effect of positive elongation on colony morphology. On the other hand, recognition of stolon regression as a mechanism that can generate morphological diversity in cnidarian colonies provides greater depth to the understanding of this diversity.

Although previous work (Cherry Vogt et al., 2008) employed pharmacological perturbations to induce stolon regression, the present study shows that potentially common environmental effects (poor water quality, temperature and feeding) can also serve as inducers of stolon regression. Stolon regression may thus be fairly common in the field. Nevertheless, the data suggest that the same stimulus may not always elicit the same effect. For instance, higher temperature produced an effect on ROS in some, but not all, experiments. In part, this reflects the tendency of some groups of control colonies to undergo high rates of spontaneous regression. These groups of control colonies are likely to have experienced an unplanned perturbation while under normal culture conditions (e.g. during a water change or while being examined in a small dish under a microscope). The role of feeding also seemed ambiguous. In some experiments, feeding seemed to have a protective effect against stolon regression, whereas in others it seemed to potentiate the effects of temperature. It may be that feeding has countervailing effects. At the level of the colony, feeding typically stimulates

from that of the fed 27°C group ($F_{1,12}$=9.3, $P<0.01$). This experiment was repeated with six colonies in each group, and results were similar (Fig. 4D). Overall, there was a significant difference between treatment groups in the relative luminance of stolon tips ($F_{2,15}$=43.9, $P<<0.001$). Orthogonal contrasts showed that relative luminance of the unfed 20.5°C group was not significantly different from that of the unfed 27°C group ($F_{1,15}$=0.5, $P>0.5$), but the relative luminance of the unfed 27°C group was significantly different from that of the fed 27°C group ($F_{1,15}$=60, $P<<0.001$). Nevertheless, a subsequent experiment with fed colonies at 20.5°C (N=6) and at 27°C (N=6) showed no difference in relative luminance ($F_{1,10}$=0.2, $P>0.6$). A follow-up experiment showed no difference between fed (N=6) and unfed colonies (N=6) at 20.5°C ($F_{1,10}$=0.1, $P>0.7$). In both of these follow-up experiments, high rates of spontaneous regression in the control colonies diminished the between-treatment effect.

Differences between fed and unfed colonies might be mediated by the effects of gastrovascular flow. Contraction cycles at three stolon tips for five colonies per treatment were used to assess flow with standard methods (Cherry Vogt et al., 2008). Even at high temperatures, fed colonies showed significantly greater relative amplitudes ($F_{1,8}$=27, $P<0.001$) and contraction cycle rates ($F_{1,8}$=32.8, $P<0.001$) than unfed colonies. In contrast, unfed colonies at different temperatures showed no differences in relative amplitude or contraction cycle rate ($F_{1,8}$=0.44, $P>0.5$ and $F_{1,8}$=0.42, $P>0.5$, respectively). Unfed colonies typically showed low and variable gastrovascular flow rates.
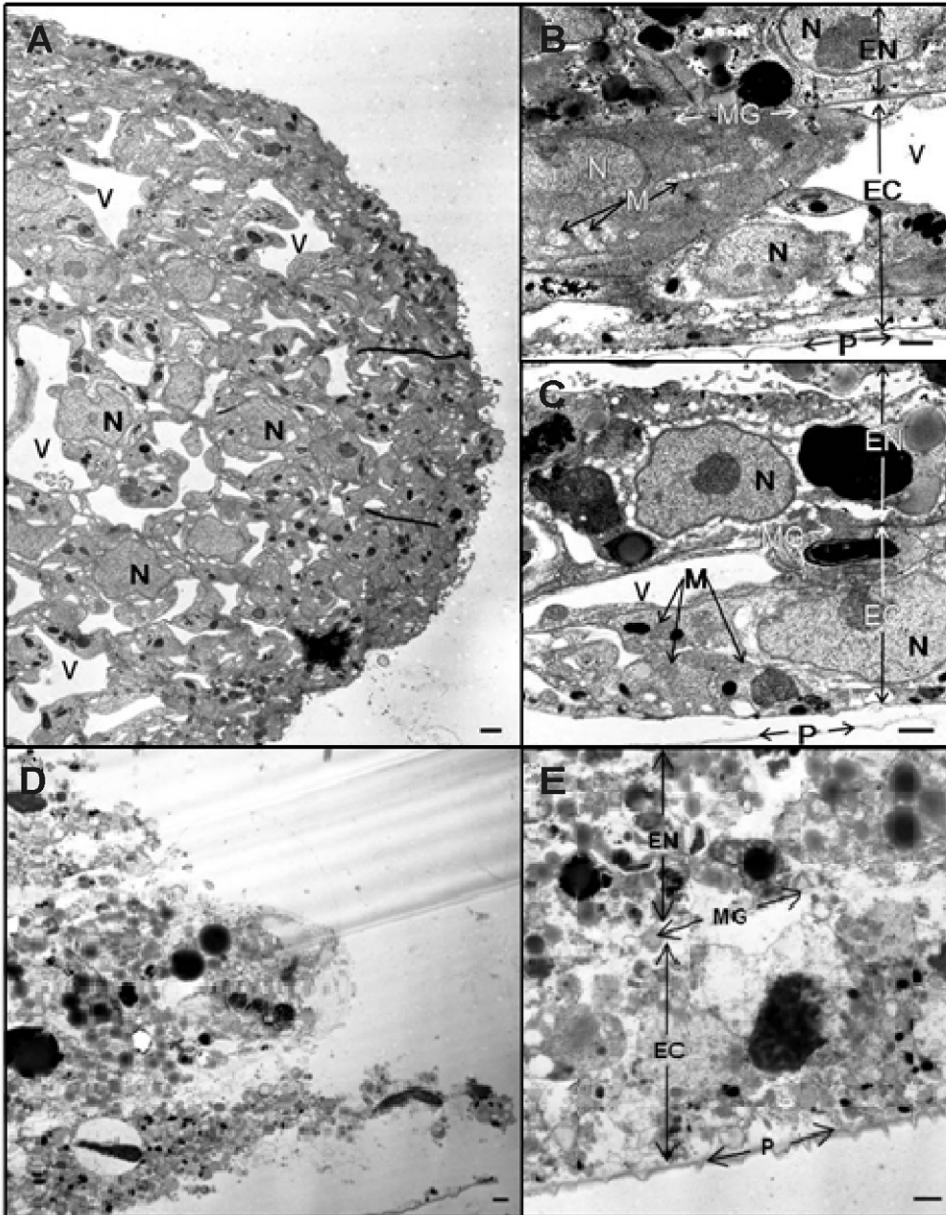
Fig. 6. Micrographs (TEM) of a healthy (A–C) and a naturally regressed (D,E) stolon. (A) A healthy, non-regressed stolon tip has a smooth and rounded appearance. Healthy stolon tip cells are typically large with irregular margins. (B) The ectoderm is relatively wide (as indicated by the space between the perisarc and the mesoglea), and nuclei of epitheliomuscular cells (EMCs) are often seen suspended near a large vacuole. The mitochondria of healthy EMCs are usually approximately 0.5 to 0.75 μm in length. (C) Healthy EMCs have nuclei approximately 4 to 8 μm diameter. (D) The tip of a regressed stolon in a control colony is irregular in appearance with few identifiable components. (E) Very few identifiable components can be found amongst the cellular debris along the edge of the stolon. The ectoderm is relatively wide (as indicated by the space between the perisarc and mesoglea); however, the structure of the mesoglea seems non-existent. EC, ectoderm; EN, endoderm; M, mitochondrion; MG, mesoglea; N, nucleus; P, perisarc; V, vacuole. Scale bars, 1 μm.

gastrovascular flow. Higher rates of flow may protect peripheral stolon tips from regression (Cherry Vogt et al., 2008). At the level of the cell, however, starvation may activate a survival stress response (Xu et al., 2007), and feeding may abrogate this response (Wellen and Thompson, 2010).

Given previous indications that cell death may be involved in the process of stolon regression (Cherry Vogt et al., 2008), a logical next step was to investigate the effects of caspase inhibitors on this process. Although caspase inhibitors diminish the physical manifestations of regression (i.e. fewer stolons actually regress), the processes of cell death are still very evident in the tissue of the regressing stolon. Nevertheless, caspase inhibitors seem to shift the features of cell death from more apoptosis-like to more necrosis-like (Table 1). Indeed, it has been generally noted that caspase inhibition may delay cell death, cause a change in the type of morphology the dying cells exhibit, or both (Kroemer et al., 2009; Zhang et al., 2009).

A striking feature of stolon regression is the accumulation of ROS and RNS in the tissue of the regressing stolon. This accumulation is found whether the regression occurs spontaneously, in response to environmental perturbations or as a result of pharmacological perturbations (Blackstone et al., 2004; Blackstone et al., 2005; Cherry Vogt et al., 2008). Here, caspase inhibitors apparently have a differential effect. The accumulation of ROS is blocked in whole or in part, but the accumulation of RNS is not. At this point, the significance of these results is not clear, although they may provide a guide to future investigations. Nevertheless, the clear effects of caspase inhibitors on the accumulation of ROS suggest that caspases have an active role in the process of stolon regression.

Perhaps the most provocative implication of these results is the suggestion of a connection between stolon regression and the cnidarian stress response. The presence of ROS, RNS and dying cells as well as a role for caspases all suggest such a connection. This is in spite of the fact that the stress response is typically investigated in cnidarians that contain symbiotic dinoflagellates, which are photosynthetic (Venn et al., 2008; Weis, 2008). Indeed, current interpretations of the stress response focus nearly exclusively on both the symbiosis and photosynthesis (Venn et al., 2008; Weis, 2008),
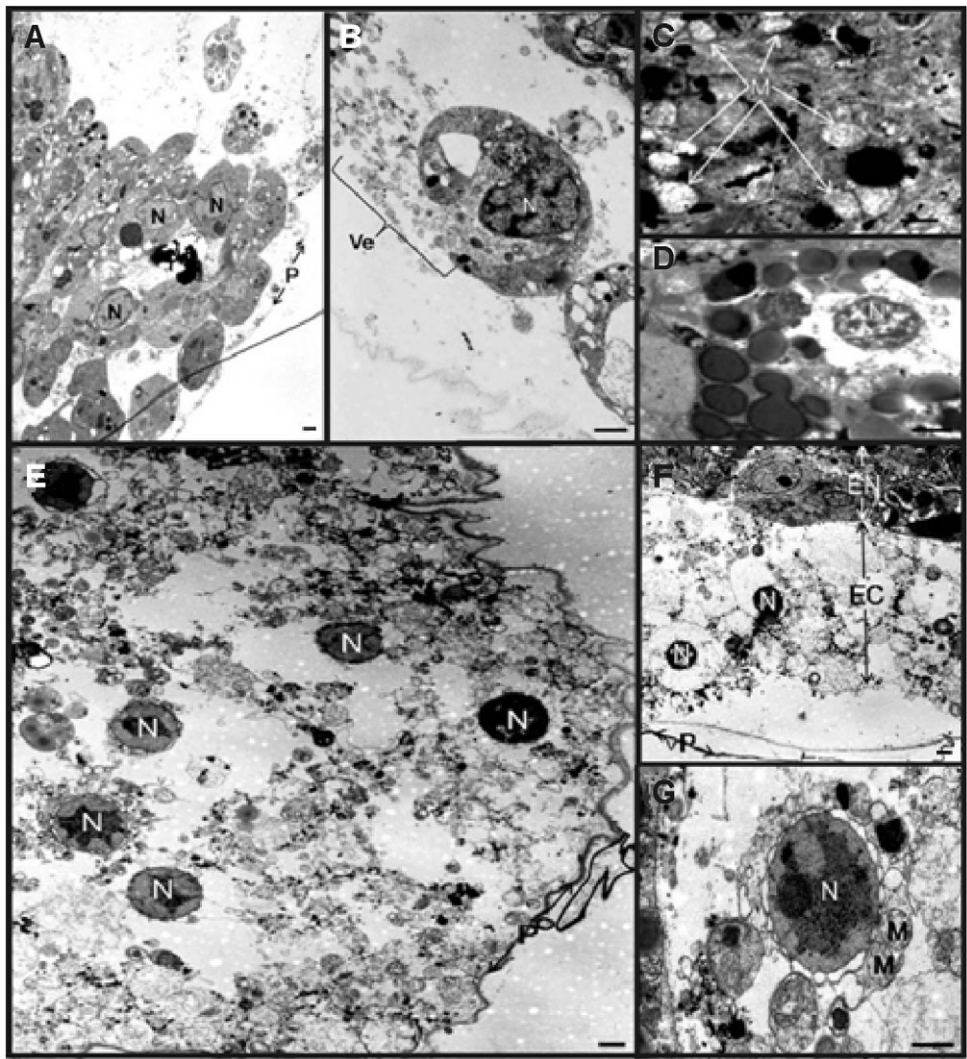
Fig. 7. Micrographs (TEM) of a regressed stolon treated with $5\,\mathrm{mmol\,l^{-1}}$ H_2O_2 (A–D) and a stolon treated with a $0.1\,\mathrm{mmol\,l^{-1}}$ solution of Q-VD-OPh prior to and during treatment with $5\,\mathrm{mmol\,l^{-1}}$ H_2O_2 (E–G). (A) Cells in the tip of a regressed stolon treated with $5\,\mathrm{mmol\,l^{-1}}$ H_2O_2 are shrunken and rounded compared with their control counterparts (e.g. Fig. 6A). (B) The epitheliomuscular cells exhibit numerous small vesicles, (C) enlarged mitochondria 0.75–$1.25\,\mu m$ in length and (A,B,D) a reduction in nuclear size to 2–$3\,\mu m$ in diameter, which is (B,D) often accompanied by condensation of chromatin. (E) Cellular features in the tip of a stolon treated with $0.1\,\mathrm{mmol\,l^{-1}}$ solution of Q-VD-OPh prior to and during treatment with $5\,\mathrm{mmol\,l^{-1}}$ H_2O_2 are virtually non-existent amongst the cellular debris with the exception of small nuclei (approximately $3\,\mu m$ in diameter) exhibiting condensed chromatin. (F) The epitheliomuscular cells along the edge of the stolon remain intact in the endoderm, but have lost identity in the ectoderm. Again, the only feature remaining amidst the cellular debris is small rounded nuclei with extensive condensation of chromatin. (G) The mitochondria in these cells are slightly enlarged at approximately 0.75–$1\,\mu m$ in length. EC, ectoderm; EN, endoderm; M, mitochondrion; N, nucleus; P, perisarc; Ve, vesicle. Scale bars, $1\,\mu m$.

yet *P. carnea* colonies lack prominent symbionts, photosynthetic or otherwise. Many if not all of the features of the stress response nevertheless are apparent: in response to an environmental perturbation, ROS and RNS accumulate in the tissue, and cell death occurs. It is tempting to view stolon regression as a related manifestation of a conserved cnidarian response. Indeed, laboratory experiments with a symbiont-containing stoloniferan octocoral (Parrin et al., 2010) have shown that temperature perturbation often leads to stolon regression as well as symbiont-related effects. Further work will better clarify the connections between stolon regression in hydroids and the possibly related processes that occur in symbiont-containing cnidarians and other organisms (Chen et al., 2010).

Table 1. Features of apoptotic and necrotic cells (from Kroemer et al., 2009), showing that caspase inhibitors shift peroxide-treated colonies from a more apoptosis-like morphology to a more necrosis-like one

	Control	Regressed control	Peroxide-treated	Peroxide-treated and caspase-inhibited
Apoptotic features				
Rounding up of the cell			×	
Retraction of pseudopodes				
Reduction of cellular and nuclear volume			×	×
Chromatin condensation			×	×
Nuclear fragmentation (karyorrhexis)				
Minor modification of cytoplasmic organelles				
Plasma membrane blebbing			×	
Engulfment by resident phagocytes *in vivo*				
Necrotic features				
Cytoplasmic swelling (oncosis)				
Rupture of plasma membrane		×		×
Swelling of cytoplasmic organelles			×	×
Moderate chromatin condensation			×	×

LIST OF ABBREVIATIONS

DAF-FM   4-amino-5-methylamino-2′,7′-difluorofluorescein
H_2DCFDA 2′,7′-dichlorodihydrofluorescein diacetate
Q-VD-Oph   quinolyl-valyl-O-methylaspartyl-(–2,6-difluorophenoxy)-methyl ketone
RNS   reactive nitrogen species
ROS   reactive oxygen species
TEM   transmission electron microscopy
z-DEVD-fmk benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone

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