Increased number of mucocytes in *Aiptasia pallida* following bleaching

David Fransolet[1], Anne-Catherine Herman[1], Stephane Roberty[2], Jean-Christophe Plumier[1]

[1]Laboratoire d'écophysiologie et physiologie animale, Université de Liège, Liège Belgium
[2]Laboratoire d'écologie animale et d'écotoxicologie, Université de Liège, Liège Belgium
Corresponding author: d.fransolet@ulg.ac.be

Abstract. While many studies have investigated histological changes occurring in cnidarians during bleaching, only a few have focused on continuing changes in tissues during the recovery period. Here, we examine the response of the sea anemone *Aiptasia pallida* to a transient elevation of water temperature combined with high illumination. Following 30h of exposure to stress conditions (33 °C and 1900 µE/m^2.s), anemones show a significant reduction in their *Symbiodinium* concentration followed by a progressive recovery over 8 weeks. Histological analyses show an increase in cell proliferation in both ectoderm and gastroderm tissues one day following the stress. This increased proliferation seems to be sustained after 3 weeks before returning to normal after 8 weeks. Moreover, our results show a progressive increase in the number of ectodermal mucocytes over 3 weeks before returning to a normal level after 8 weeks. While the new cells formed in the gastroderm would most likely host new *Symbiodinium*, the fate of new cells in the ectoderm is still not completely understood. These new cells may contribute to the increased number of mucocytes which could eventually help shift the feeding mode temporarily to a heterotrophic state until restoration of the symbiosis.

Key words: Bleaching, Mucocyte, Cell proliferation, Histology, 5-ethynyl-2'-deoxyuridine.

Introduction

High water temperature and solar irradiance are known to disrupt the symbiosis established between scleractinian corals and algae of the genus *Symbiodinium*. Studies have shown that these environmental factors can act both separately (Brown and Suharsono 1990; Gates 1990; Jokiel and Coles 1990; Goreau and Hayes 1994; Brown et al. 1996; LeTissier and Brown 1996) and in combination (Lesser et al. 1990; Glynn 1993; Lesser and Farrell 2004; Hoegh-Guldberg et al. 2007; Weis 2008). This process, known as coral bleaching typically involves loss of algal pigmentation and can deprive the host from its main energy source. During the following weeks the nutritional state of the coral is therefore compromised. Depending on the symbiont/host association and the intensity of the stress, it could either die or recover with the same or altered algal communities (McClanahan 2004; McClanahan et al. 2004; Tchernov et al. 2011).

Although bleaching could sometimes be limited to a decrease in chlorophyll concentration, many studies have focused on the mechanisms involved in *Symbiodinium* loss. While many scenarios like symbiont digestion via autophagy (Dunn et al. 2007; Downs et al. 2009), symbiont expulsion (Steen and Muscatine 1987; Sandeman 2006) and host cell detachment (Gates et al. 1992; Brown et al. 1995; Sawyer and Muscatine 2001) have been considered (Weis 2008), most of the recent findings argue for a mechanism implying the death of the host cell either by necrosis or through the apoptotic pathway (Dunn et al. 2000; Dunn et al. 2002; Dunn et al. 2004; Kvitt et al. 2011; Pernice et al. 2011; Tchernov et al. 2011).

Although several studies have documented the recovery of coral from bleaching (Brown and Suharsono 1990; Hayes and Bush 1990; Fitt et al. 1993; Baker et al. 2004), little information has been gathered concerning the histological modifications occurring during this recovery (Hayes and Bush 1990). We hypothesize that host cells lost during bleaching should be replaced by new ones in order to regenerate the damaged gastroderm and so that it can host new algae. To address our hypothesis we investigated the cellular proliferation following a heat-induced bleaching in the zooxanthelate sea anemone *Aiptasia pallida,* often used as a model cnidarian (Lesser 1989; Muscatine et al. 1991; Cook and Davy 2001).

Material and Methods

Sea Anemones
All *Aiptasia pallida* specimens came from a few populations originating from the aquarium of the University of Liège. Those specimens were multiplied in an aquarium in our laboratory using artificial seawater held at 26°C and were fed on a weekly basis with frozen *Artemia* shrimp. For our

experiments we selected specimens presenting a column height of approximately 1cm.

Bleaching procedure
A first group of 6 anemones was subjected to our bleaching procedure in order to confirm its effectiveness. They were placed in Petri dishes in which the water was constantly renewed by a flow-through mechanism using a peristaltic pump. They were then exposed for 30 hours to a water temperature of 33°C and an illumination of approximately 2000 µmol of photons/m^2 (measured in the water of the dishes) produced by led bulbs (12W, 6000K, Elix Belgium). They were then allowed to recover in an isolated area of the stock aquarium (26°C, approx 30 µmol of photons/m^2) for 24h and 8 weeks. A second group of 15 anemones was subjected to the same treatment. They were also allowed to recover in an isolated area of the stock aquarium. Seven anemones were collected after 24h, another four after 3 weeks and a further four after 8 weeks for histological analysis. An equal number of healthy anemones were collected in the stock aquarium at each time point which served as controls.

Symbiodinium density
Symbiodinium density was evaluated in healthy anemones from the stock aquarium and bleached anemones having recovered for 24h and 8 weeks. Three anemones of each group were fixed using a 30% formaldehyde solution before being slightly dried in absorbent paper and weighed using an analytical scale. They were subsequently crushed in a glass potter with a precise quantity of filtered seawater (Muscatine et al. 1991; Perez et al. 2001). The number of *Symbiodinium* cells was estimated under the microscope using a haematocytometer. Six counts were averaged for each anemone. Using the weight of the anemones and the quantity of water used, we calculated the density of *Symbiodinium* per mg of fresh tissue (wet weight).

Cell proliferation
Cell proliferation assays consisted of counting nuclei which incorporated thymidine analogue during DNA synthesis. They were conducted on control and bleached anemones isolated 24h, 3 weeks and 8 weeks after bleaching. To do so, each anemone of these treatments was incubated for 24h in a solution of 1 µM EdU (5-ethynyl-2'-deoxyuridine, thymidine analogue, Invitrogen, Eugene-Oregon-USA) in seawater. Anemones were then anesthetized for 20 minutes in a 1:1 solution of seawater and 0.37M MgCl$_2$ before fixation in a solution of 4% paraformaldehyde in seawater. Fixed specimens were subsequently dehydrated, embedded in paraffin (paraplast Xtra, Sigma), cut into 5µm thick slices and finally placed on silane-coated slides. After dissolution of the paraffin, re-hydration and PBS washes, the slides were incubated for 10 minutes in a blocking solution of 3% BSA in PBS in order to prevent non-specific interactions. This was followed by a permeabilization procedure of 20 minutes in a solution of 0.5% Triton x-100 in PBS prior to three PBS washes and incubation for 30 minutes in the reaction mix made from the Click-iT EdU kit (Click-it EdU Alexa Fluor 488 Imaging Kit, Invitrogen, Eugene-Oregon-USA). The fluorescent dye provided in this kit binds to EdU using a simple chemical reaction (azide/alkyne) and therefore doesn't require DNA denaturation as needed when using antibodies. Finally, the slides were washed three more times in PBS, dried and mounted for epifluorescence microscopy (Vectashield + DAPI, Vektor labs).

Mucus staining
Mucus staining was performed on slides obtained from the same specimens as for cell proliferation assays. After deparaffinisation, the slides were incubated for 2 minutes in alcian blue (Merk). They were then washed several times in distilled water, dehydrated and mounted for light microscopy.

Analysis and Statistics
EdU-positive nuclei and mucocyte mean densities were calculated from five counts made in tentacle sections using Nikon NIS software v3.1. Densities were estimated from the surface of the ectoderm and expressed per square millimeter. Ectodermal surface densities were used because the gastroderm surface is affected by the bleaching and the tentacle circumference doesn't correlate to the amount of cells present in the slice because of the eventual contraction of the tentacle. Statistical analyses revealed that there was no significant difference between controls obtained from 24h, 3 weeks and 8 weeks. They were therefore pooled together as a single control group for subsequent analyses. Statistical analyses (non-parametric Kruskal-Wallis test or ANOVA) were performed using Statistica v8.0.

Results
Bleaching
The light and temperature treatment successfully bleached anemones, reducing their *Symbiodinium* density by almost a factor 10 (Fig. 1). Anemones of the control group showed a density of $122 \pm 6.3 \times 10^3$ (mean ± SE) algae per mg of fresh tissue while this value dropped to $15.3 \pm 2.1 \times 10^3$ 24 hours after the end of the treatment ($p < 0.05$).

Anemones recovered from bleaching and *Symbiodinium* densities returned to pre-treatment values 8 weeks after the end of the treatment with a density of $95.4 \pm 11.4 \times 10^3$ ($p > 0.05$).
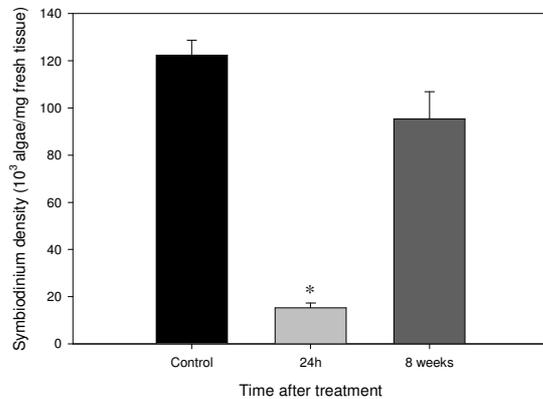

Figure 1: *Symbiodinium* mean densities in anemones of the control group and the bleaching treatment groups (24h and 8 weeks of recovery). Bars represent standard error. (* $p < 0.05$)

Cell proliferation
In the gastroderm of the bleached anemones, the number of EdU-positive nuclei exhibited a transient increase following the stress period (Fig. 2). The control group showed a density of positive nuclei of $36 \pm 6/mm^2$ while the 24h group underwent a significant increase in density to 323 ± 76 positive nuclei/mm^2 ($p < 0.001$). After 3 weeks, the number of positive nuclei showed an intermediate value (157 ± 30 positive nuclei/mm^2), which was not significantly different from either the control group or the 24h group. After 8 weeks, the density returned to control values (32 ± 8 positive nuclei/mm^2).
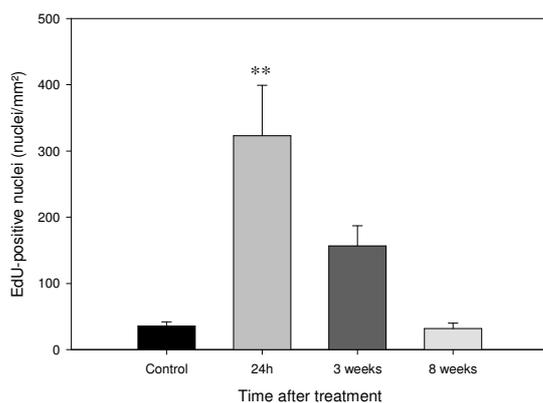

Figure 2: EdU-positive nuclei mean densities in the gastroderm of anemones of the control group and the bleaching treatment groups (24h, 3 weeks and 8 weeks of recovery). Bars represent standard error. (** $p < 0,001$)
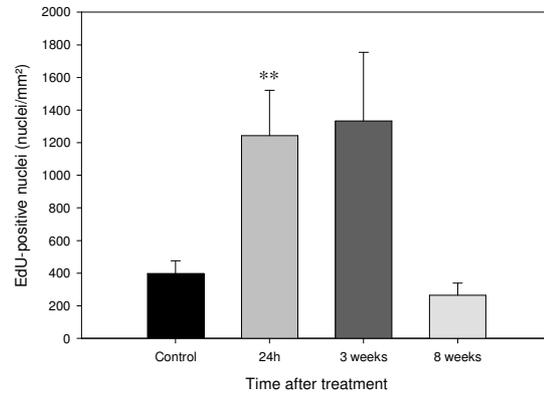

Figure 3: EdU-positive nuclei mean densities in the ectoderm of anemones of the control group and the bleaching treatment groups (24h, 3 weeks and 8 weeks of recovery). Bars represent standard error. (** $p < 0,001$)

In the ectoderm, we observed the same trend as in the gastroderm, that is, a transient increase in the number of EdU-positive nuclei (Fig. 3). The control group had 398 ± 78 positive nuclei/mm^2; increasing to 1244 ± 277 positive nuclei/mm^2 in the 24h group ($p < 0.001$). After 3 weeks the number of positive nuclei was still high (1333 ± 420 positive nuclei/mm^2) but not significantly different from either the control or the 24h group. Finally, 8 weeks following the stress period, the densities declined to 264 ± 75 positive nuclei/mm^2, similar to those of the control group.
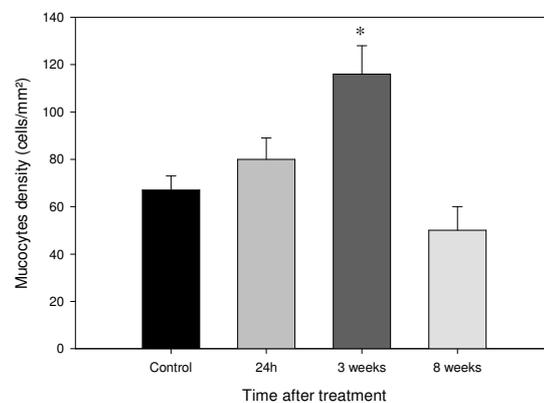

Figure 3: Mucocyte mean densities in the ectoderm of anemones of the control group and the bleaching treatment groups (24h, 3 weeks and 8 weeks of recovery). Bars represent standard error. (* $p < 0,05$)

Mucocytes
Anemones of the control group had 67 ± 6 mucocytes per square millimeter of ectoderm. This value was similar in the 24h group with a density of 80 ± 9 mucocytes/mm^2. Mucocyte density increased significantly to $116 \pm 12/mm^2$ in the group sampled at 3 weeks ($p < 0.005$) before returning to levels similar

to that of the control group ($50 \pm 20/mm^2$) after 8 weeks of recovery.

Discussion

Recent studies have put emphasis on mechanisms involved in the loss of symbiotic algae during coral bleaching. Many highlighted that this event could occur through the loss of the gastrodermal host cells, leaving this tissue heavily damaged (Gates et al. 1992; Sawyer and Muscatine 2001). Here we showed an increase in the cellular proliferation following bleaching in the sea anemone *Aiptasia pallida*.

Our results show a rapid increase in the number of EdU-positive nuclei contained in the host gastroderm, reaching values almost ten times higher than the control group only 24 hours after the end of the bleaching procedure. This suggests that a massive cellular proliferation is initiated in the gastroderm of the already bleached anemones, most likely to regenerate the damaged tissue. The proliferation rate slows down during the weeks following the bleaching and finally returns to normal values after 8 weeks. By this time, anemones progressively regained their algal symbionts whose densities, initially reduced by 90%, were then similar to unbleached anemones. These new algae most likely come from the proliferation of those that remained in the bleached host as the seawater used in our experiments was artificial and thus lacked any live *Symbiodinium*. These observations firstly confirm previous studies reporting the loss of host cells during bleaching (Dunn et al. 2002; Dunn et al. 2004; Tchernov et al. 2011) and secondly suggest that gastrodermal regeneration could be an important step in the recovery of bleached cnidarians.

Our results also illustrate a more surprising increase in the amount of EdU positive nuclei in the ectoderm of bleached anemones. The ectoderm is reported in the literature to suffer only little or no damage following bleaching (Dunn et al. 2004); our observations are thus not likely to be related to some kind of regeneration process. Another plausible explanation would be an augmentation in the production and turnover of some cellular phenotypes that could improve the survival of the bleached host. We chose here to focus on mucocytes, a cellular type that has often been reported to be crucial for the holobiont (Bythell and Wild 2011) and whose response to bleaching is not yet completely understood (Lasker et al. 1984; Glynn et al. 1985; Niggl et al. 2008; Fitt et al. 2009; Piggot et al. 2009; Wooldridge 2009). Mucus has multiple functions in the holobiont such as UV protection, microbial defense, sediment cleansing, energy carrying and particle trapping (Lewis and Price 1975; Lewis 1977; Schlichter and Brendelberger 1998; Goldberg 2002; Brown and Bythell 2005; Niggl et al. 2008; Bythell

and Wild 2011). In the bleached anemone, the mucus ability to trap particles and carry them to the mouth of the host could be of great interest after bleaching. Heterotrophic feeding could indeed sustain the host energy incomes and compensate for the missing algal autotrophic contribution (Brown and Bythell 2005; Grottoli et al. 2006; Niggl et al. 2008). Augmentation of mucus production could also help to protect the bleached and thus more susceptible host against UV radiation or pathogen aggressions (Brown and Bythell 2005; Niggl et al. 2008). Here we observed a significant augmentation of mucocyte density in the ectoderm 3 weeks after bleaching. This density returned to values similar to those of the control group after 8 weeks recovery. Considering this, some of the newly produced EdU-positive nuclei in the ectodermal layer could differentiate into mucocytes and explain the higher number of mucocytes in the bleached anemones. The lag period observed between the augmentation of EdU-positive nuclei and the augmentation of mucocytes would then account for the time needed for their migration and differentiation.

However, it is still unclear whether it is the stress itself or the consequent reduction in algal number that induces the cellular proliferation we highlighted here. This remark especially concerns the ectoderm which is not directly affected by the effects of algal loss. Further investigations with shorter incubation periods conducted during and directly after the stress treatment will help clarify this matter.

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