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**HISTOLOGICAL RESPONSES OF THE SEA ANEMONE
A. PALLIDA TO BLEACHING INDUCING STRESSES**

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Abstract

Tropical Coral reefs are among the richest and most important ecosystem on Earth. This success would not be possible without the symbiosis established between corals and unicellular algae of the genus *Symbiodinium* that provide them with photosynthesis-derived carbon. Unfortunately, with the climatic upheaval that we witness today, the long-term survival of coral reefs could be in jeopardy. Massive loss of symbiotic algae, a phenomenon known as coral bleaching, becomes indeed more and more frequent throughout the globe and already urged scientists to study its mechanisms for more than a decade. Their research highlighted the central role of reactive oxygen species in the collapse of symbiosis. They also established that the expulsion of *Symbiodinium* from its host is mainly operated through the death of the host cell. The ensuing events, although determining the eventual survival of the energetically compromised coral, are however much less detailed. In this work, we decided to investigate these “post-bleaching” events and focused our efforts on the evaluation of cell proliferation and mucocyte number, for the role they may respectively play in regenerative processes and heterotrophic feeding. For this purpose, we worked with the sea anemone model *A. pallida* in which we analyzed the incorporation of a thymidine analogue (EdU). After preliminary experiments assessing the general repartition and the circadian variations of cellular proliferation in healthy specimens, we conducted a series of bleaching experiments using a variety of stresses. Every treatment, namely cold and darkness, heat and light or exposition to a photosynthesis inhibitor, drastically reduced the *Symbiodinium* density. This reduction was always accompanied by important histological modifications. In every case, we highlighted an increase in cellular proliferation in both the ectodermis and the gastrodermis as well as an increase in

ectodermal mucocyte density. These values returned then to normal as algae that survived the stress progressively repopulated anemones. Further experiments showed that, following bleaching, a small fraction of the newly produced ectodermal cells migrate to the gastrodermis. Along with new gastrodermal cells, they most probably operate a regeneration of the wounded tissue, differentiating into host cells in order to harbor new algae. Another experiment also indicated that a small but significant part of ectodermal newly produced cells might differentiate into mucocytes, therefore explaining their increased density in bleached individuals. We hypothesize that the higher amount of mucus produced, in addition to providing protection against various aggravating stresses, would be a way to efficiently increase the feeding capacity of the bleached cnidarians. This heterotrophic shift would therefore allow a sufficient energy income until full restoration of the symbiosis. This work emphasizes the need to focus more attention on the post-bleaching period, a critical time in which some modifications might be decisive for coral and coral reef survival.

Résumé

Les récifs coralliens tropicaux font partie des plus riches et plus importants écosystèmes sur terre. Ce succès ne serait pas possible sans la symbiose établie entre les coraux et les algues unicellulaires du genre *Symbiodinium* qui fournissent ces derniers en carbone d'origine photosynthétique. Malheureusement, avec le bouleversement climatique que nous observons aujourd'hui, la survie à long terme des récifs coralliens pourrait bien être en péril. La perte massive d'algues symbiotiques, un phénomène connu sous le nom de blanchissement corallien, devient en effet de plus en plus fréquente à travers le monde et a déjà poussé les scientifiques à en étudier les mécanismes depuis plus d'une décennie. Leurs recherches ont mis en évidence le rôle central joué par les espèces réactives de l'oxygène dans l'effondrement de la symbiose. Elles ont aussi établi que l'expulsion de *Symbiodinium* s'opère principalement par la mort de la cellule hôte. Les événements qui s'en suivent, bien que déterminant dans l'éventuelle survie du corail énergétiquement compromis, sont cependant beaucoup moins détaillés. Dans ce travail, nous avons décidé d'investiguer ces événements "post-blanchissement" et avons alors focalisé nos efforts sur l'évaluation de la prolifération cellulaire et du nombre de mucocytes, pour les rôles qu'ils pourraient respectivement jouer dans les processus de régénération et l'alimentation hétérotrophe. Pour ce faire, nous avons travaillé avec l'anémone modèle *A. pallida* chez laquelle nous avons analysé l'incorporation d'un analogue de la thymidine (EdU). Après quelques expériences préliminaires évaluant la répartition générale et les variations circadiennes de la prolifération cellulaire chez des spécimens sains, nous avons conduit une série d'expériences de blanchissement en utilisant une variété de stress. Chaque traitement, à savoir le froid et l'obscurité, le chaud et la lumière ou l'exposition à un inhibiteur de la photosynthèse, a réduit de manière drastique la

densité en *Symbiodinium*. Cette réduction a alors toujours été accompagnée par des modifications histologiques importantes. Dans chaque cas, nous avons mis en évidence une augmentation de la prolifération cellulaire tant au sein de l'ectoderme que de l'endoderme ainsi qu'une augmentation de la densité en mucocytes ectodermiques. Ces valeurs retournèrent ensuite à la normale alors que les algues ayant survécu au stress recolonisaient progressivement l'anémone. Des expériences supplémentaires ont montré que, suite au blanchissement, une faible fraction des nouvelles cellules ectodermiques migrent vers le gastroderme. Accompagnées des nouvelles cellules d'origine endodermique, ces dernières opèrent probablement une régénération du tissu blessé, se différenciant en cellules hôtes de manière à abriter de nouvelles algues. Une autre expérience a également indiqué qu'une faible mais significative partie des nouvelles cellules ectodermiques se différencieraient en mucocytes, expliquant dès lors leur densité accrue chez les individus blanchis. Nous faisons l'hypothèse que la quantité supérieure de mucus produite, en plus de fournir une protection contre divers stress aggravants, pourrait être un moyen d'accroître efficacement la capacité à se nourrir des cnidaires blanchis. Ce shift hétérotrophique pourrait dès lors permettre un apport énergétique suffisant jusqu'à la restauration complète de la symbiose. Ce travail souligne la nécessité de se concentrer d'avantage sur la période post-blanchissement, un moment critique durant lequel certaines modifications pourraient être décisives pour la survie du corail et des récifs coralliens.

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List of figures

Figure 1 Coral reef distribution and number of species.	4
Figure 2 Classification of cnidarians.	7
Figure 3 Anatomy of the coral polyp and its skeleton.	11
Figure 4 Structure and functioning of the cnidocyte.	13
Figure 5 Structure and components of the mucine monomer.	16
Figure 6 <i>Symbiodinium</i> life cycle.	26
Figure 7 Schematic illustration of <i>Symbiodinium</i> infection of a cnidarian host cell.	34
Figure 8 Illustration of a bleached <i>Acropora sp.</i>	45
Figure 9 Oxidative stress mechanisms responsible for bleaching. ...	47
Figure 10 Host-controlled cellular processes involved in <i>Symbiodinium</i> loss.	52
Figure 11 <i>In toto</i> illustrations of the model anemone <i>Aiptasia pallida</i>	64
Figure 12 Histological illustration of the model anemone <i>Aiptasia pallida</i> (H&E staining).	65
Figure 13 Advantages of <i>Aiptasia</i> over corals as a model organism.	66
Figure 14 Illustration of the "Click" reaction between the Alexa fluor 488 azide and the ethynyl group of the EdU molecule incorporated in the DNA.	67
Figure 15 Summary of the cold-induced bleaching experiment.	69

Figure 16 Diel variations of EdU incorporation.	72
Figure 17 Loss of <i>Symbiodinium</i> following cold-shock stress.	73
Figure 18 Increase of cell proliferation after cold-induced bleaching.	74
Figure 19 Increase of mucocyte density after cold-induced bleaching.	75
Figure 20 Transient reduction of <i>Symbiodinium</i> density following photic/thermic stress.....	88
Figure 21 Loss of <i>Symbiodinium</i> following photic/thermic stress. ...	89
Figure 22 EdU and WGA labeling.....	90
Figure 23 Increase of cell proliferation after heat/light-induced bleaching.....	91
Figure 24 Increase of mucocyte density after heat/light-induced bleaching.....	93
Figure 25 Experimental treatments and sampling strategy.	103
Figure 26 The maximal photochemical quantum yield in <i>Symbiodinium</i> of the sea anemone <i>Aiptasia pallida</i> , before, during and after treatment with 20 mM DCMU.....	107
Figure 27 <i>Symbiodinium</i> density in control and DCMU-treated sea anemones <i>Aiptasia pallida</i>	108
Figure 28 Apoptotic cell death and <i>Symbiodinium</i> density in tentacles of <i>Aiptasia pallida</i> , before the treatment with 20 mM DCMU (A), after 2 days (B) and 1 week (C).	109

Figure 29 Cell proliferation in the ectodermis (A) and the gastrodermis (B) of control and DCMU-treated sea anemones <i>Aiptasia pallida</i>	110
Figure 30 Mucocyte density in the ectodermis in control and DCMU-treated sea anemones <i>Aiptasia pallida</i>	111
Figure 31 <i>In toto</i> labeling of mucocytes and proliferating cells.	121
Figure 32 <i>In toto</i> EdU labeling.	125
Figure 33 Cell proliferation following photic/thermic stress and HU incubation in the ectodermis of <i>A. pallida</i>	126
Figure 34 Cell proliferation following photic/thermic stress and HU incubation in the gastrodermis of <i>A. pallida</i>	127
Figure 35 Confocal picture of a WGA+EdU+ cell in the ectodermis of <i>A. pallida</i> 7 days after bleaching treatment.	128
Figure 36 Ratio of EdU+ mucocytes 5 and 7 days following photic/thermic stress in <i>A. pallida</i>	129

Abbreviations

·OH :	hydroxyl radical
$^1\text{O}_2$:	singlet oxygen
ABC :	ATP-binding cassette
ABH :	adaptative bleaching hypothesis
APX :	ascorbate peroxidase
ATP :	adenosine-5'-triphosphate
BrdU :	5-bromo-2'-deoxyuridine
BSA :	bovine serum albumin
CA :	carbonic anhydrase
CHAR :	contribution of heterotrophically acquired carbon to daily animal respiration
Ctrl :	control
CZAR :	contribution of <i>Symbiodinium</i> -acquired carbon to animal respiration
DAB :	diaminobenzidine
DCMU :	3-(3,4-Dichlorophenyl)-1,1-dimethylurea
DOM :	dissolved organic matter
ECM :	extracellular calcifying medium

EdU :	5-ethynyl-2'-deoxyuridine
F ₀ :	initial fluorescence level
F _M :	maximum fluorescence level
FP's :	fluorescent pigments
F _V /F _M :	maximum potential quantum yield
GPCR :	G-protein-coupled receptors
GTP :	guanosine-5'-triphosphate
H ₂ O ₂ :	oxygen peroxide
H&E :	hematoxylin and eosin
HRFs :	host release factors
HSP's :	heat-shock proteins
HU :	hydroxyurea
MAA's :	mycosporine-like amino acids
NADP ⁺ :	oxidized NADPH
NADPH :	nicotinamide adenine dinucleotide phosphate
NO :	nitric oxide
O ₂ ⁻ :	superoxide anion
ONOO ⁻ :	peroxynitrite
PAH :	phagosome arrested hypothesis

PBS :	phosphate buffer saline
PCD :	programmed cell death
POM :	particulate organic matter
PRRs :	pattern recognition receptors
PSII :	photosystem II
ROS :	reactive oxygen species
S.E.M. :	standard error of the mean
SOD :	superoxide dismutase
SST :	sea surface temperature
TdT :	terminal deoxynucleotidyl transferase
TGFβ :	transforming growth factor beta
UV :	ultraviolet
WGA :	wheat germ agglutinin

Table of contents

Abstract.....	v
Résumé	vii
Remerciements.....	ix
List of figures	xi
Abbreviations	xiv
Table of contents	xvii
Preamble	xxi
Chapter 1.....	1
General Introduction.....	1
Coral Reefs	1
Origins.....	1
Repartition	3
Value	4
Cnidarians.....	7
Phylogeny	7
Ontogeny	8
Anatomy	9
Histology	11
Ectodermis	12
Mucus.....	15
Calicodermis.....	19
Mesoglea.....	21
Gastrodermis	22
<i>Symbiodinium</i>	24
Phylogeny	24
Habitats.....	24
Lifecycle	25
Photosynthesis.....	26
Clades.....	27

Symbiosis.....	31
Transmission	32
Acquisition	33
Establishment	39
Exchanges	41
Regulation.....	43
Bleaching.....	44
Causes	45
Mechanisms.....	47
Countermeasures	49
Algal loss	50
Host-cell persistence	51
Host-cell loss	52
Adaptative bleaching	54
Recovery	55
Tissue regeneration	55
Heterotrophy	56
Objectives.....	61
Chapter 2.....	63
Cold Shock Response and circadian rhythmicity of EdU	
incorporation in <i>A. pallida</i>.....	63
Introduction	63
Material and Methods	68
Biological material	68
Experimental treatment	68
Tissue histology.....	70
Countings and statistical analyses	71
Results	71
Diel variations of cell proliferation	71
Cold Stress and <i>Symbiodinium</i> density	72
Cell proliferation in bleached tissues.....	73
Mucocytes.....	75
Discussion.....	75

Chapter 3.....	81
Increased cell proliferation and mucocyte density in the sea anemone <i>Aiptasia pallida</i> recovering from bleaching.....	81
Introduction	81
Material and Methods	84
Biological material	84
Induction of bleaching by thermal/photic stress.....	84
<i>Symbiodinium</i> identification and population density	85
Tissue histology.....	86
Analyses and Statistics	87
Results	88
Population density of <i>Symbiodinium</i>	88
Proliferation of cells within ectoderm and gastrodermal tissues.	90
Mucocytes.....	92
Discussion.....	93
Chapter 4.....	99
Impairment of symbiont photosynthesis increases cell proliferation in the ectodermis of the sea anemone <i>Aiptasia pallida</i>.	99
Introduction	99
Material and Methods	101
Biological material	101
Experimental treatments.....	102
Chlorophyll fluorescence measurements	103
Tissue histology.....	103
Counting and statistical analysis	105
Results	106
Treatment with DCMU inhibited the photosynthetic electron transport through PSII.....	106
The inhibition of photosynthesis led to bleaching.....	107
Cells proliferated within ectodermal and gastrodermal tissues	109

Mucocyte density increased after DCMU treatment.....	111
Discussion.....	112
Chapter 5.....	117
Trans-tissular migration and mucocyte differentiation of	
bleaching-induced proliferating cells.	117
Introduction	117
Material and Methods	121
Biological material	121
<i>In toto</i> cell proliferation	122
Trans-tissular cell migration	122
WGA and EdU co-labeled cells.....	123
Counting and statistics.....	124
Results	125
<i>In toto</i> cell proliferation	125
Trans-tissular cell migration	125
EdU localization in WGA+ cells	127
Discussion.....	129
Chapter 6.....	133
General Discussion.....	133
Post-bleaching tissular modifications	134
Cell proliferation and tissue regeneration	136
Cell proliferation and mucocyte density.....	139
Perspectives	141
Conclusion.....	143
References.....	145

Preamble

In the beginning, there was light.

And with light came energy.

And thanks to this energy emerged life in all its exuberance.

From then on, the story is well-known, mostly hard working plants being eaten and so injecting carbon into the food chain.

Some organisms, though, opted for a more, let's say... fair trade policy. These organisms chose to fuse with the vegetable instead of eating it and yet managed to build some of the most precious ecosystems of earth: the coral reefs.

Unfortunately, nowadays, light and the precious energy it carries overwhelm this fragile association. These same sunrays that once gave birth to coral reefs now threaten them and may one day cause their demise.

For the sake of those magnificent structures, not only for their beauty and what they are in essence but also for what they represent for mankind, we have to try something. We have to grasp any pieces of knowledge leading to a better understanding of coral reef ecosystems for, even trivial, they can be paving stones on the road leading to their salvation.

Such is the purpose of this manuscript, trying humbly to unravel a few very specific, although important, details of coral histology. Helping to beat the odds and possibly allowing us to consider a future in which coral reefs will keep flourishing from the light.

Chapter 1

General Introduction

First of all, it is important to make clear that our work fits into the framework of a need, currently supported by many scientists, for using model organisms to progress in our understanding of the coral biology [396]. Except for its lack of skeleton, the sea anemone model chosen for this study is unanimously recognized to be very similar to corals, to which we can therefore extrapolate our experimental results. This general introduction will thus focus mainly on corals, detailing their biology and the threat they are facing, while the characteristics and advantages of our anemone model in the study of such organisms will be described later in chapter 2.

Coral Reefs

Since Darwin and his description of these shallow water structures as "oases in the desert of the ocean", coral reefs have kept raising curiosity amongst people. This could be due to what struck Darwin the most, something he described as a paradox: the richness and diversity of such structure even so surrounded by water that contains hardly any nutrient. How can such richness and biodiversity sustain in such depleted waters?

Origins

This assertion whets even more ones curiosity considering the fact that, according to fossil records, coral reefs have a long successful story: being with other kind of reefs a major source of evolution and diversity throughout geologic times [199]. Apparition of "modern"

coral reefs as we can admire them today was however a long process marked by distinct steps.

While the fall of atmospheric level of carbon dioxide and the consequent saturation of calcium carbonate in the oceans have early led some marine species to build hard skeleton and premises of reefs, the first bioconstructed structures that can be seen as coral reefs came a few million years later. In fact, ancestors of reef building corals made their first appearance in oceans during the Ordovician (488M years ago) while terrestrial plants were only taking their faltering steps. They were then erected by representatives of the orders Tetracoralla (or Rugosa) and Tabulata, which are now extinct, most probably due to the Permian Crisis [353]. It's not until the late Middle Triassic (about 237M years ago) that today's "modern" corals, known as scleractinians, can be found in fossils records. They have supposedly evolved from an anemone-like soft-bodied ancestor and developed calcification under the pressure of changing environmental geochemical conditions, especially carbonate balance and CO₂ concentration. Such corals, however, were not yet the prolific builders that populate today's oceans as they were still lacking their most intriguing and important characteristic: their symbiosis with the *Symbiodinium* algae. On the basis of multiple factors such as their size and shape, their corallite integration, their annual growth bands or their isotopic composition, some scientists concluded that this symbiosis appeared relatively quickly. Indeed, many coral seem to already fulfill symbiotic traits during the Late Triassic. Scleractinians subsequently survived many environmental perturbations and major extinction events that shaped their diversity, such as the K/T mass extinction, and started to progressively colonize the oceans around the globe during the whole Phanerozoic [353]. Today's oldest coral reefs however rarely exceed 10000 years old.

Repartition

The peculiar nature of coral reefs rapidly gathered attention among the scientific community and the aforementioned reef paradox didn't remain a mystery for long. Observations made during pioneer studies quickly revealed their symbiotic nature, determining in the same manner their ecological needs and geographic repartition. Indeed, as we will see later, the symbiotic algae, although not the only symbiotic partners of corals [126, 204, 289], are the main factor involved in coral reefs unbounded growth and are therefore the key of their localization. The vast majority of algae-bearing corals, qualified for the first time of “hermatypic corals” by Wells in 1933 [399], can only be found in warm waters in which the temperature averages near the calcification optimum of 25°C to 27°C. They are therefore mostly restricted between the tropic lines but their repartition can be even more accurately defined by the 20°C isocrymes (imaginary lines connecting the same mean coldest temperature). Hermatypic corals also need clear oligotrophic waters ($N < 2 \mu\text{mol/l}$, $P < 0,2 \mu\text{mol/l}$), which, as well as for their depth limit of about 50 meters, is a consequence of the algae imperious need for sunlight. This excludes the presence of coral reefs in the vicinity of major river mouth carrying lots of sediments and decreasing drastically water clarity. Upwellings with their cold and nutrient-rich water brought from the depth also make nearby water unsuitable for coral growth. Coral reefs are consequently absent from area such as the West coasts of Africa and South America. Their vast majority is in fact found in the Atlantic (mostly Caribbean) and the Indo-Pacific region, which includes the Red Sea, the Indian Ocean, the South-East Asia and the Pacific Ocean. While the most famous reef is arguably the Great Barrier Reef off of the coast of Queensland in Australia, hotspots for coral diversity are located a bit northern, in South-East Asia, mostly in waters bathing the coast of Malaysia, Indonesia and Philippines (Fig. 1). Structures

adopted by coral reefs in these regions are variables but are commonly divided in three main categories: the fringing reef, the barrier reef and the atoll. These three reef types are in fact different evolution steps of the same reef over geological times: The fringing reef receding seaward to form a barrier as the island subsides and finally disappears in the water only leaving the ring of the atoll. Every other kind of reef that does not fit those three may be referred to as patch reef but can also be classified into other occasional categories such as apron reef, bank reef, ribbon reef, table reef, habili, microatoll, cays or seamount.

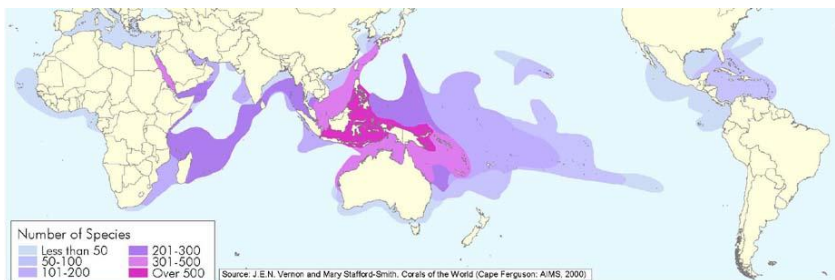


Figure 1 | Coral reef distribution and number of species. (from Veron, Corals of the World, 2000)

Value

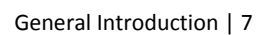
As mentioned above, coral reefs have always been a major source of biodiversity. They are still living up to this reputation as they are shelter to a number of identified species reaching between 100 000 and 150 000. The total number of species is however estimated to be between 500 000 and 2 millions. This means that we still only have an overview of coral reef diversity and that the number of species, which are yet to be identified, could be as high as 80% of the total. Nevertheless, coral reef value is not limited to this naturalist romantic view, seeing them for what they represent as natural heritage. They also have a more tangible and economical value that may be their

main asset into our economy-governed world. To the countries they border the coasts, coral reefs give many services like coastal protection during storms, touristic attraction or direct valuable resources. The number of people living in less than 100 km of a reef is estimated to be as high as 500 000 000. Their economical function goes even way beyond as they are also shelter to larvae and juveniles of over one quarter of all marine species. Therefore, they are not only an important resource for the 101 countries harboring reefs near their coastlines but an essential basis for every people and country relying on the fishing industry. Even though the difficulties of attributing a financial value to the “goods and services” given by coral reefs, an estimation made in 1997 by Costanza and colleagues ranged it to over 6000 USD hectare⁻¹ per year [65]. This value becomes head-spinning once summed by their estimation of the total area covered over the world, coral reefs being then worth 375 billions USD annually to mankind.

Those facts talk for themselves, coral reefs deserve and need to be protected. Today, as global warming scenarios get more and more precise, the threat on them is getting inevitable, leading some specialists to unfortunately consider their upcoming extinction [163, 164, 167]. The urge on scientists to help preserving this natural heritage against human impact has never been so high. Every effort grasping pieces of knowledge about the functioning of this ecosystem is worth being made. Especially regarding a better comprehension of the mechanisms involved in the balance of the coral symbiosis with, in line of sight, the elucidation of the symbiosis breakdown known as coral bleaching. This thesis takes interest on the often-unheeded events that happen following the said bleaching, especially on the histological level. Questions such as “Are there any modifications within the host after the loss of its symbiotic algae?” or “What is the dynamic of symbiosis recovery?” were guidelines during the whole

course of this study. In order to ease the comprehension of themes that will be discussed in the following chapters, the rest of this introduction will focus on the cnidarian biology, with a particular emphasis on their histology. We will then review the current knowledge concerning the symbiosis establishment, its regulation and, most of all, its breakdown leading to coral bleaching as well as its eventual recovery.

1



As said before, coral reef builders, which we commonly call stony coral due to their hard external skeleton, are members of the order Scleractinia, sometime called Madreporia. They are, along with anemones, which belong to the Actinaria order, members of the Zoantharia class, which is further included into the Anthozoa subphylum. Anthozoa, together with Medusozoa to which belong jellyfishes, compose the phylum Cnidaria (Fig. 2). Scleractinians most probably evolved from soft-bodied anemone-like ancestor. Phylogenetic reconstructions based on their microstructures as well as those based on fossil records tend to give scleractinians a polyphyletic origin [353]. However, modern genetic analyses tend to classify them into a monophyletic group further subdivided into two main branches separating “robust” or massive corals and “complex” or branched corals [198].

Ontogeny

Of the 1400 known extant species, 60% are colonials and composed of a high number of polyps, connected to each other and tightly attached to their underlying skeleton [15]. They can reproduce either by clonal ways, budding new polyps and therefore extending the colony, or by sexual means. Corals can be organized into four groups depending on their way of reproduction. They can either have two distinct sexes and be gonochoric or be both male and female and therefore hermaphrodite. Both those types can also be further subdivided into two modes of fertilization. Fertilization and formation of the larvae can either occur in the water column after release of gametes by species that will therefore be called “broadcast spawners” or into the gastric cavity of the polyp for fewer species called “brooders” [15, 367]. Broadcaster spawners tend to have a large colony and reproduce once a year during a short period while brooders are more likely to form small colonies producing larvae

multiple times each year [367]. Release occurs during the night for both types of fertilization with a higher settlement and survival rate of the larvae released by brooders before dawn.

Following fecundation, the newly formed coral zygote follows the classical stages of development. After the blastula stage and a stage similar to the nutritive stage of metathozoans in which it segregates nutrients in an inner nutritive layer, the embryo starts its gastrulation. This phenomenon, leading to the formation of the endoderm, is mainly provoked by tissue invagination but also by the action of individual epithelial cells that lose their morphology and migrate into the blastocoel. This process ultimately leads to the formation of a larva called planula. A recent study demonstrated that, during embryonic development, a part of cnidarian nervous system takes shape following an axial way and a serially repeated pattern [147]. This trait is similar to bilateralians and was probably inherited from a common ancestor to which the phylogenetic relation is still detectable during ontogeny. The separation between cnidarians and bilateralians becomes even more tenuous in the light of recent findings undermining the long-lasting concept of cnidarians constituted of only two tissue layers. These findings show that they may in fact harbor a third more discrete layer that can be seen as a mesodermal component [347].

Anatomy

Once completely formed, the planula develops numerous adhesion-committed cells, called spirocytes, to its aboral region and attaches itself to the substrate before undergoing its metamorphosis into a fully functional polyp [282]. The polyp is radially symmetrical and is composed of a column, usually ranging a few millimeters in diameter, topped by various numbers of tentacles surrounding the oral disc which opens in its middle by the mouth. The mouth, which

also serves as anus, is followed by the pharynx and leads to a cavity called the coelenteron or gastric cavity where the extracellular digestion occurs. This cavity extends into the tentacles as well as in the layer of tissue connecting the polyps of the colony (coenosarc). The water in the coelenteron shows specific physicochemical proprieties that differ greatly from the surrounding water. Those parameters may vary following a diel pattern and include O_2 and nutrients concentration, pH and alkalinity [5]. The coelenteron is divided by mesenteries which are only six in smallest polyps but are further separated by secondary, tertiary mesenteries, etc, in larger ones. Mesenteries can reach the pharynx and be complete or have their edges free in the coelenteron. This edge expends into a trilobed structure called the mesenterial filament which serves in digestion and water circulation. Those filaments extend at their basal extremities into threadlike appendices called acontial filaments which serve in defense mechanisms as well as in prey capture and extra-oral digestion processes. Both filaments harbor specialized cells such as nematocytes, mucocytes or other gland cells that we will describe later. While the colony grows, a thin layer of tissues called the coenosarc appears to connect the polyps' coelenterons to each other, allowing exchange of nutrients and their homogenous repartition thanks to a current produced by ciliated cells [290].

Once settled, the young coral quickly starts building its exoskeleton called *corallum*. This skeleton is made of aragonite ($CaCO_3$), a carbonate mineral of the same composition as calcite but differing in its crystalline structure. The skeleton is composed of two major parts: the *corallites* and the *coenosteum* (Fig. 3). The *coenosteum* is the porous heap of aragonite that can be seen as the body of the skeleton while the *corallites* house the polyps and present a more specific structure. *Corallites* are composed of a basal plate called *sole* and a cylindrical wall called *theca* which protrudes *septa*

within the mesenteries. As the colony extends, the polyps build higher floors to their *corallites* and the coral skeleton thickens and grows in its wide and characteristic variety of shapes. Skeletal differences between species range from different levels, from the *corallites* itself with some species showing fused polyps, to the whole colony which can be encrusting, massive, branched or table-like. Individuals of the same species can even show major structural differences depending environmental conditions such as current, turbidity or bathymetry [277].

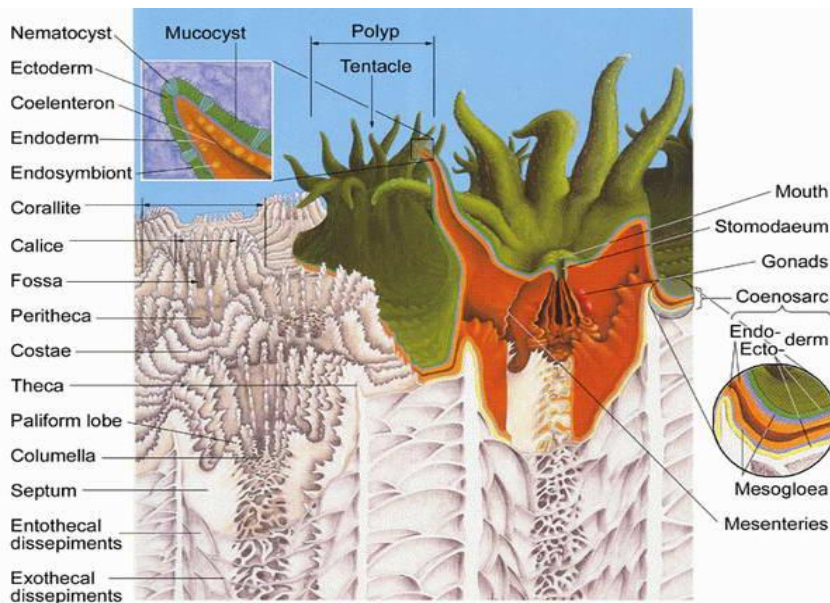


Figure 3 | Anatomy of the coral polyp and its skeleton. (modified from Veron, 1986)

Histology

As said earlier, corals, as for every cnidarians, are essentially composed of two tissues, the ectodermis and the gastrodermis. The mesoglea, a gelatinous matrix populated by only a few cells, separates these two tissues.

Ectodermis

The ectodermis covers the external surface of the polyp and the colony as well as the surface in contact with the aragonite skeleton. It is composed of four principal types of cells: the epitheliomuscular cells, the neuronal cells, the cnidocytes and the mucocytes.

Most of the surface of the ectodermis is composed of **epitheliomuscular cells** that rest against the mesoglea and form a prismatic epithelium. Unlike in traditional prismatic epithelium, epitheliomuscular cells extend into two, three or more basal extensions. Every successive extension in the column and the tentacles is connected to each other and contains smooth myofibrils that form a muscular longitudinal layer [24]. This layer allows contraction of the polyp and retraction in its *corallite* to take shelter and avoid predators or harsh environmental conditions. It also participates in the movements of the tentacles to the mouth during feeding [174]. These cells can also harbor cilia that create movement of the mucus layer covering the animal, either to bring food particles to the mouth or to clean the ectodermis from sediments. Epitheliomuscular cells are also believed to be the source of the other types of cells found in the ectodermis, whether during growth or regeneration processes. Recent observations made on the sea anemone *Nematostela vectensis* highlighted the plasticity of these cells and their ability to dedifferentiate and produce the other cell phenotypes [134]. Epitheliomuscular cells cannot be seen as stem cells *per-se* but are believed to give birth to all other cell lineages composing the ectodermis [134]. They are therefore supposed to underlie the regeneration processes occurring in the ectodermis observed during experimental lesions on *N. vectensis* [291] or during regeneration in the solitary *Fungiid* corals [207].

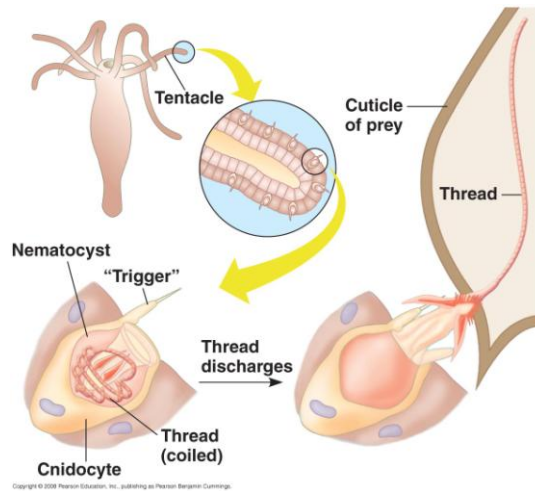


Figure 4 | Structure and functioning of the cnidocyte. (Copyright B. Cummings, Pearson Education, 2006)

Neuronal cells are represented in the ectodermis by two different cell types: the sensory cells and the nerve cells. Sensory cells are especially abundant in tentacles, conferring them their tactile faculty. Their apical region is elongated and forms either a bristle or a sphere while their basal pole connects to nerve cells through a variable number of neuronal processes. Some sensory cells can be found invaginated within epitheliomuscular cells. Nerve cells run at the base of the ectodermis, next to the mesoglea. They are similar to multipolar neurons found in other metazoans. Nerve cells also make neuroglandular synapses with mucous cells or zymogenic cells sometimes found in the pharynx and allow a nervous control over their secretions [402]. Nerve cells also make multiple connections with different kind of cnidocytes therefore modulating their discharge [400, 401].

Cnidocytes are the most characteristic and exclusive feature of cnidarians. Those even inherited their evocative name from it, *cnide* (κνίδη) meaning nettle in ancient Greek. They are very specialized

cells that can be found throughout the ectodermis and especially in the tentacles where they can be grouped in batteries. They are either lodged between epitheliomuscular cells or invaginated within them. They are round cells with the nucleus occupying a basal position while the rest of the space is taken by the *cnidae* or cnidocyst. The *cnidae* is a single-use evertting organelle having the form of a tube. It is coiled within a capsule derived from a large post-Golgi vesicle that has undergone very specific maturation processes [286] (Fig. 4). In Anthozoans, a ciliary cone complex present on the apical pole of the cnidocytes triggers the discharge of the *cnidae* following chemical and mechanical stimuli [197]. This discharge can also be triggered by a nerve impulse in order to coordinate multiple cnidocytes. Ensues a rapid change in the capsule permeability with the opening the apical flaps and an explosive eversion of the tube thanks to osmotic movements. This osmotic pressure can then reach as much as 150 bars releasing the *cnidae* in only 700 nanoseconds; producing an acceleration of $5\,400\,000 \times g$ and a pressure on impact of 7,7 GPa. Cnidocytes present a very complex structure and have been the subject of many studies. They are composed of strong assemblages of specific proteins in order to sustain the titanic constraints they are exposed to [3, 9, 74, 414]. Variations in ornamentation of the *cnidae* lead to the distinction of approximately 30 different types of cnidocytes among cnidarians [197]. Those can be regrouped into three main categories. The first category, the most diverse and the most widely represented, includes *cnidae* involved in the hunting of live prey. Those are called nematocytes and are armed with a spike that can pierce their prey before injecting it with venom. This venom has variable proprieties such as neurotoxic, myotoxic, hemolytic or necrotic. The second category, called spirocytes, is characteristic of Zoantharia and is used either to entangle preys or to adhere to substrates. Their thread bears fine tubules that solubilize and form an adhesive net. Instead of the usual cilia complex, spirocyst-containing

cnidocytes bear circlets of microvilli receptors on their apical surface. The last category called ptychocytes regroups cnidocytes which *cnidae* contain simple, spineless, adhesive threads. They are exclusively present in Ceriantharia, solitary anemones of the Zoantharia class that use them to build the tube in which they are encased.

Mucocytes represent the fourth type of ectodermal cells. They are club-shaped cells that can present contractile basal extensions similarly to the epitheliomuscular cells. They can occupy a major proportion of the ectodermis, especially on its oral portion, with some species having mucocytes accounting for as much as 90% of their areal extent in some areas of tissue [42]. Their size, which ranges from 5 to 10 micrometers wide and up to 30 micrometers high, as well as their abundance, depends on the species considered and the localization within the animal [42, 138]. Their nucleus is situated in a basal position while most of their cytoplasm contains large granular inclusions that will ultimately be secreted into the water from a circular aperture situated the apical pole.

Mucus

The observed composition of mucus seems to be very variable. This could be due in part to the different methods of collection and analyze, the mucus undergoing quick modification upon release into the water. Furthermore the mucus could vary in its composition depending on the stress applied to induce its production [42] or the species studied [257]. Besides water, which accounts for an important fraction of mucus weight, the main organic component of the mucus, is however constant among all species and regardless the conditions. This component is the mucine, a highly heterogeneous glycoprotein of which the shape, size and charge determine the mucus viscoelastic properties (Fig. 5). The mucine is composed of two parts. The core first, which accounts for 20% the molecule weight (200-500 kDa), is

made of a variable number of tandem repeats of proline, threonine and serine (PTS-repeats). Variable numbers of PTS-repeats are linked together by cystein-rich domains and are flanked by multiple “von Willebrand factor” domains and “cystein knot” domains, thus completing the molecule core. The remaining 80% of the molecule consist of carbohydrates either O-linked to the serine and threonine residues or N-linked to flanking domains. Those sugar side chains are composed of 2 to 20 monosaccharides and can be linear or branched. They can be constituted of sugar such as mannose, fucose, arabinose, galactose, N-acetylgalactosamine and N-acetylglucosamine. The extremities of the side chains can be sulfated and/or contain sialic acid terminals. This confers the mucus its characteristic polyionic proprieties, which are crucial for its hydration. Another major characteristic of the mucine molecule is the ability of its cystein residues to form disulfide bonds thus enabling formation of dimers and subsequent polymerisation [42, 52, 257]. The release of the mucus on the surface of the coral seems to be under nervous control [402], corresponds to the holocrine mode of secretion and therefore leads to the death of the mucocyte. Nevertheless, the dynamic of their development and their turnover rate is still unclear [42]. This is contrasting considering the numerous and important roles they play not only for the coral but also for the whole reef ecosystem.

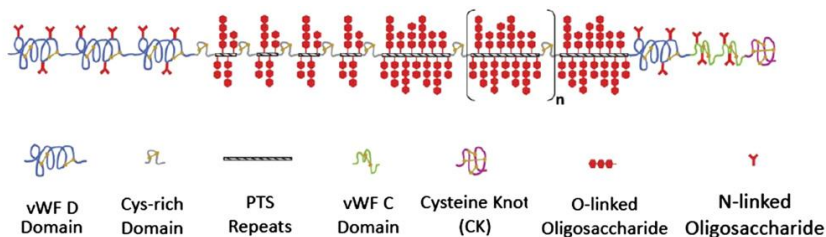


Figure 5 | Structure and components of the mucine monomer. (modified from [52])

The layer of mucus secreted on its ectodermal surface has multiple functions for the coral. The most evident one is, as mentioned earlier, the ability of mucus to trap food particles and bring them to the mouth thanks to ciliary movement [42]. This function is especially important for corals devoid of tentacles [342]. The mucus layer covering the coral also confers it protection against UV radiations thanks to a variety of UV-absorbing compounds regrouped as mycosporine-like amino acids (MAA's) [42]. This is of major importance in their resistance to light-induced bleaching, a threat of great concerns as we will see later.

UV protection can also be achieved thanks to UV-absorbing bacteria living in the mucus layer [309]. In fact, a very diverse bacterial flora lives within the mucus. Its composition may vary depending environmental conditions [202] but the major representatives are α - and γ -proteobacteria [126, 202]. Many of them play crucial roles in the coral biology and can be seen as symbiotic partners. Coral health can indeed be tributary to this probiotic bacterial flora as it can control colonization by opportunistic pathogens implicated in disease or bleaching event. Recent studies even suggested that this bacterial flora could stimulate coral immunity [126, 208, 311, 313, 321]. Bacteria in the mucus layer can also be seen as a supplementary heterotrophic food source accompanying food particles into the mouth of the coral.

The list of mucus benefices to the coral goes on as it also plays essential roles in its protections against desiccation during spring tides, against sediment smothering thanks to ciliary movements, against pollutant poisoning by limiting their penetration or against wave damage thanks to its surfactant and lubricant proprieties [42].

Once released into the water the coral mucus keeps playing important functions on the ecosystem level. Its dispersion from the mucocytes relies on the current as well as its intrinsic rheological proprieties, which also controls its exudation, hydration and swelling [42]. Upon release from the mucocytes, more than half of the mucus (56% to 80%) immediately dissolves into the water and feeds planktonic bacteria [404]. The threads and particles released from the mucus layer into the water carry with them particles of once suspended organic matter that accumulated onto them while still attached to the coral. While in the water column, mucus aggregates keep trapping suspended particles and triple their organic carbon and nitrogen content [404]. This makes the coral mucus a formidable energy carrier and a major input source into the trophic system of coral reefs. This particulate organic matter (POM) released by corals has already been documented for multiple species and seems to be constant throughout the year [272, 404]. If macroscopic animals can ingest some part of this POM, most of it reaches the sediment rather quickly and therefore relatively closely to its coral source (less than 5 meters)[52]. Once on the permeable calcareous reef sand, aggregates can either be directly eaten by benthic animals or be degraded by the microbial fauna living in the sediments providing 10% to 20% of the total carbon provided to the sedimentary community [404]. The turnover into nutrients is relatively fast, 7% of the carbon being transformed in one hour. Those nutrients can then enhance the primary production of planktonic autotrophic organisms on which can rely the food chain of the coral reef [52, 105, 404]. Considering these facts, it appears that any event inducing modifications into mucocyte number or mucus production by coral would have a strong impact not only on the coral itself but on the whole reef ecology.

Calicodermis

The description of cells given above concerns the ectodermal layer of the coral that is in contact with the water column. The ectodermal layer adjacent to the skeleton and responsible for the production of its aragonite component is however much different. This specialized tissue is called calicodermis or calicoblastic epithelium. It is devoid of cnidocytes and mucocytes and its sole function resides in the deposition of aragonite crystals through processes regulated by its dedicated **calicoblastic cells**. Its surface is also punctuated by anchoring cells or desmocytes that were formed on the aboral pole of the planula larvae during its settlement. They are tightly attached to the coral skeleton by desmosome-like extensions [71, 137].

The mechanisms by which the calicoblastic cells progressively construct the mineral skeleton are multifaceted. First of all they secrete a colloidal gel matrix called organic matrix into the medium between their apical membrane and the skeleton. This extracellular calcifying medium (ECM) is therefore filled by an organic framework that greatly enhances the deposition of calcium carbonate crystals [71, 136]. The calicoblastic cells also harbor many mitochondria that not only produce energy but also carbon dioxide which is a source of carbon for calcification. The important amount of energy produced is required for the functioning of the multiple pumps and transporters situated on the apical membrane of calicoblastic cells. They allow the cells to precisely control the ionic composition and pH of the ECM. This control is crucial to the growth of the skeleton as the more alkaline pH measured in the ECM (often 0.2 to 0.5 unit above surrounding seawater pH) helps producing bicarbonate anions by shifting the equation of dissociation of carbonate anions to the right ($\text{HCO}_3^- \rightleftharpoons \text{CO}_3^{2-} + \text{H}^+$). This has the effect of increasing the saturation

state of aragonite (Ω_{arag}) and allows the spontaneous precipitation of calcium carbonate (CaCO_3) [71]. Finally, the calcicoblastic cells also produce carbonic anhydrases, specific enzymes that once liberated into the ECM play an important role in the interconversion of inorganic carbon species [395].

As mentioned earlier, the symbiotic algae also play a major if not essential role in the high rate of calcification observed in reef-building corals. This was confirmed by studies showing a drastic reduction of the calcification rate following inhibition of photosynthesis within these algae [384]. This phenomenon of algal stimulation, known as light enhanced calcification, boosts CaCO_3 production up to tenfold; a production that therefore outweighs degradation processes such as wave erosion and allows the reef to expand. This increased calcification can even produce some sort of positive feedback for the algae, the structure of the skeleton produced by some corals being able to increase their light absorption [96]. The way by which symbiotic algae enhance calcification is however still poorly understood as both photosynthesis and calcification compete for available inorganic carbon. A recent hypothesis proposes that such high rates of calcification and photosynthesis are achievable thanks to the coral morphology which allows the compartmentalization of these two processes [185]. Translocation of energetically rich photosynthates from the algae located in the gastrodermis, as we will see later, could also fuel mitochondria within the separated calcicoblastic epithelium and enhance their performance. However, questions still remain concerning the complex movement of ions such as protons and carbonates which may either diffuse or be actively transported. Many studies have recently proposed complex ion fluxes and pathways, especially in the coelenteron and the coenosarc where the lumen between the two tissue bilayers may play an important function [8, 41, 185, 270]. There is however still much to understand

before getting a full picture of the mechanisms involved in the amazing calcifying capacity of corals.

Mesoglea

Beneath the ectodermis lies the mesoglea, an extracellular matrix mainly composed of collagen and proteoglycans [339]. While it has always been considered to be barely anhistic and almost deprived of cells, hence not representing a third type of tissue to the organism, more and more studies have pointed out the fact that it may be an old archaic form of mesoderm [347]. Indeed, in most cnidarians, anthozoans included, muscle cells clearly separated from both epithelia as well as wandering isolated cells can be observed within the mesoglea. While muscle cells are organized into bundles and have obvious contractile functions used for motility, lone cell functions are much more debated. These cells, called **amoebocytes**, are often compared to interstitial cells found in hydrozoans and extensively studied in *Hydra*. Whereas interstitial cells stem-like role in growth and regeneration processes has been clearly indentified [36, 39, 349], the function or precise identity of amoebocytes is much more vague. Their most arguable function is their role in immunity and inflammation following wounding or pathogen infection. In these cases, amoebocytes may show phagocytic capacity or secrete connective mesogleal fibers [134, 259]. However, as no unambiguous description and characterization of amoebocytes has been done to date, it is impossible to elude the possibility that the term “amoebocytes” is in fact a catch-all term, referring to wandering cells from different origins and different degrees of specialization that are temporarily found within mesoglea. This could include migrating cells participating in growth and regeneration as well as innate immunity cells such as granulocytes [287]. This is an even more plausible

explanation considering the fact that amoebocytes have also recently been observed in both epithelia [134, 380].

Gastrodermis

The gastrodermis is the second tissue layer composing the body of all cnidarians. Its cellular composition is fairly similar to the ectodermis but lacks cnidocytes and sensory neuronal cells. The main cellular components of the gastrodermis are the **nutritive-muscle cells**. They are the pendants of the epitheliomuscular cells found in the ectodermis but differ in some aspects. They are usually ciliated and their contractile extensions, which are less developed, harbor contractile fibers orientated perpendicularly to those of the ectodermis and the axis of the body column. They thus form a circular muscle that allows sphincter-like movements, especially in the mouth region to allow its closing. Contraction of those fibers is controlled by nerve cells underlying the tissue in the same way as in ectodermis but in a fewer number [24]. Similarly to epitheliomuscular cells, nutritive-muscle cells show an important plasticity and are therefore believed to ensure growth and regeneration. They are able to dedifferentiate and give birth to the other phenotypes present in the gastrodermis but are also the putative progenitors of cells observed in the mesoglea. More importantly, they are at the origin of the germ line precursor cells that will mature into gametes [134]. The last function of the nutritive-muscle cells is, as their name suggests, to phagocyte nutritive particles in order to complete their assimilation after their initial degradation in the coelenteron.

Enzymes liberated by zymogenic **glandular cells** operate this extracellular digestion. These cells have a similar appearance to the mucocytes, also present in the gastrodermis. Their nuclei occupy a basal position while the apical region of their cytoplasm is filled with vacuoles containing the zymogen molecules. Both mucocytes and

zymogenic cells harbor an apical cilium surrounded by microvilli [402]. While the secretion mode of gastrodermal mucocytes is likely the same as in the ectodermis, zymogen liberation has not been clearly documented yet but could be operated through exocytosis and hence correspond to the merocrine mode of secretion.

Last but not least, the most characteristic and by far the most studied cells of scleractinian gastrodermis are the **symbiotic host-cells** containing the *Symbiodinium* algae. These cells are specifically simplified and harbor usual cytoplasmic components except for one large symbiotic vacuole called symbiosome. This algae-containing symbiosome occupies most of the cytoplasm, squeezing the nucleus in a basal position and giving the cell a round shape that protrudes into the coelenteron. Whether symbiotic host-cells are readily competent before algae acquisition has not been established yet but is quite unlikely. The most plausible explanation is that gastrodermal nutritive-muscle cells undergo transdifferentiation into symbiotic host-cells following algae phagocytosis [71]. Not every scleractinian is symbiotic and therefore possesses this kind of cell. This character has been lost and/or gained multiple times during the phylogenetic history of scleractinians and is therefore polyphyletic [22]. Today, for the 1314 known species only 48,2% of the genera and 49% of the species harbor *Symbiodinium* within their gastrodermis [353].

Symbiodinium

Phylogeny

To understand the mechanisms underlying any symbiosis establishment and breakdown, it is first necessary to have a good knowledge of each partner involved. The cnidarian host being amply described, we shall now bear our attention to the *Symbiodinium* algae. *Symbiodinium*, commonly referred to as zooxanthellae, are unicellular algae, members of the Dinoflagellata phylum wherein they belong to the Dinophyceae Class and to the Suessiales Order. Although zooxanthellae were once all considered as members of a single pandemic specie, *Symbiodinium microadriaticum* Freudenthal (1962), precursor work of Rowan and coworkers using molecular techniques revealed a much more complex phylogenetic organization [331, 332]. Recent molecular and genetic analyses, based upon variation of nuclear ribosomal DNA (18s, ITS and 28s rDNA), chloroplast 23s rDNA and other specific sequences such as HSP90 or psbA non-coding region, have revealed that the genus *Symbiodinium* is in fact divided into nine large clades (A to I), each comprising multiple strains or species [60, 173, 216, 220, 303, 322]. Curiously, this evolutionary radiation seems to be relatively recent compared to the acquisition of symbiosis by scleractinians. Phylogenetic reconstructions showed however that their first diversification from a common ancestor happened only about 50 million years ago during the Eocene [218, 304].

Habitats

Today, the ability for *Symbiodinium* to establish symbioses is not restricted to reef-building corals but also involves a variety of other cnidarians (octocorals, soft corals, sea anemones and jellyfish) and some representatives of the Platyhelminthes, Mollusca, Porifera,

Foraminifera and ciliates [53, 354]. Several studies also report the presence of free-living *Symbiodinium* in the water column, within sediments [4, 53, 59, 142, 159, 246, 248, 305, 368, 409] and in association with macroalgae beds in the vicinity of coral reefs [306]. Nevertheless, the density distribution of free-living *Symbiodinium* seems to be highly heterogeneous between and within reefs [246]. While every clade could theoretically be found in the water column upon release by its host, not all of them seem in fact suited for this environment. *Symbiodinium* belonging to clades A and B are the most commonly found in free-living state while analyzing samples of the water column and sediments [60, 159, 368] but specimens belonging to clade C, D, E, F, G and H have also been detected and often reflect the dominant type found in symbiosis within areas of collection [142, 248, 368, 409]. For some of them the water column would then represent a transitory niche upon release by its host and before establishment of a new symbiosis.

Lifecycle

While in its free-living state, *Symbiodinium* adopts the lifecycle common to most of the dinoflagellates. It alternates between two haploid stages [337]: a spherical stage or coccoid and a smaller flagellated and mobile stage called mastigote (Fig. 6). So far, *Symbiodinium* have only been reported to multiply by asexual means, although sexual reproduction is supposed to occur. Its division rate in culture is about 1-3 days and follows a diel pattern, the mother cell releasing two mastigotes as the light period starts [115]. The cell structure of *Symbiodinium* is quite typical to the genus dinoflagellates with its motile form wearing two perpendicular flagella. One notable difference of this stage is the absence of cellulosic plates or armor. Its larger coccoid form, which is not to be confounded with a vegetative cyst, ranges from 6 to 13 micrometers and is enclosed within a thick

cellulosic cell wall. In this stage, *Symbiodinium* is also characterized by a large single, peripheral, reticulated chloroplast bounded by three membranes [79, 119]. Although *Symbiodinium* are mixotrophe and therefore able to feed heterotrophically [180], they rely essentially on the chloroplast and photosynthesis for their energy incomes.

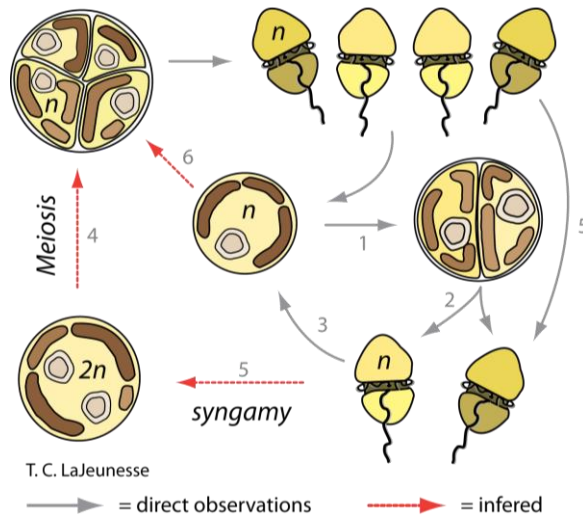


Figure 6 | *Symbiodinium* life cycle. Mitosis (1) produces two mastigotes (2) that can return to the coccoid form (3). Tetrads result from meiosis (4) or two successive mitosis (6) and produce four mastigotes. Sexual reproduction is believed to occur during the mastigote stage (5).

Photosynthesis

A brief reminder of the photosynthesis basics seems therefore adequate. Photosynthesis is divided into two main processes, the light and dark reactions. In the light dependent reaction, photons are absorbed by chlorophyll pigments of the antenna to which they transfer their excitation energy. This energy is transmitted to the reaction center where it either induces charge separation and produces an electron or returns to the antenna to be lost as heat or fluorescence. Thanks to pheophytin and quinone molecules, the newly

produced electron enters then the electron transport chain where it will ultimately produce NADP^+ and NADPH. The chlorophyll molecule regains its lost electron through the lysis of water molecules which also produces dioxygen and a proton. This proton, together with another one produced by the electron transport chain creates a gradient across the chloroplast membrane that enables production of ATP by an ATP-synthase enzyme. The dark reactions use the newly produced ATP and NADPH as well as CO_2 to produce C3 sugars. This process involves the RuBisCO enzyme and a series of reaction called the Calvin-Benson Cycle.

Clades

Within the nine *Symbiodinium* clades listed today, all but the clades E, H and I have been identified within a scleractinian host [60, 303]. Nevertheless, clades A, B, D and especially C are the most common ones within scleractinians while clades F and G are more anecdotic and are found only in modest numbers. Clades F, G, H and I are meanwhile mostly established within foraminifera [60, 354]. The four major clades living in symbiosis with scleractinians can be found all around the globe and can be present in different combinations within the same coral colony. Clade C, however, is the dominant one across the Indo-Pacific region while clade B is the most encountered in the Atlantic [217, 218]. The origin of this disparity could be found within the field of phylogeography with oceans separation and allopatric speciation events favoring different clades in different places. Today however, the dominance of some clades or subclades in some places and the niche partitioning within some habitats is most probably related to their competitive advantage over others given their ecological proprieties and environmental conditions [64, 217, 218, 334]. Indeed, while some subtle morphological differences such as cell size can be observed within clades, those differ mostly by their

biochemical or physiological characteristics such as MAA production, thylakoids membrane composition or host infectivity [76, 77, 216, 372].

Clade A for example is relatively different from others and has been described as an opportunistic or even parasitic clade [234, 260, 357, 377]. This can be in some ways related to the ancestral position of the clade A in *Symbiodinium* phylogenic tree and its close proximity with its sister group apicomplexans, well known for their parasitic lifestyle [234]. *Symbiodinium* of the clade A rather infect host in suboptimal health state, releasing less fixed carbon and hence being less beneficial to them. This ancestral clade has seemingly followed a different evolutionary trajectory and is probably better adapted to the free-living state as suggests its ability to outcompete other clades in cultures [304, 357]. On the other hand, members of the clade A have been reported to possess an enhanced capacity to use alternative photosynthetic electron-transport pathways as well as pronounced ability to dissociate its antenna complex of the photosystem II. They are therefore less subjected to damages in environment characterized by high light intensities and could then be beneficial for their hosts in such environments [314].

In fact, most of the differences between clades rely on their ability to cope with diverse environmental conditions and abiotic factors [357]. While some studies have used *in vitro* cultures of *Symbiodinium* to analyze the physiological variations between clades, especially concerning their photosynthetic apparatus [76, 314, 371], most observations have been made on symbiotic hosts and extrapolated to the clades they contain. Early observations of differential clade distribution among a same colony [329, 330, 381], or along the depth gradient for example [178, 378], revealed their discrepancies regarding light tolerance. Most of the recent studies,

however, focus the tolerance of some clades against elevated temperature and therefore against bleaching. Some pointed out differences regarding heat resistance between different subclades within clade C [108, 335] but the majority of the publications are related to the **clade D**. *Symbiodinium* belonging to this clade seems indeed to have an increased capacity to cope with elevated temperatures [328]. Studies already highlighted the outcompeting capacity and the opportunist behavior of these algae, which sometimes gain prevalence within host that previously suffered from heat-induced bleaching [219, 375]. They seem also able to confer their host with an increased resistance to such noxious events [26, 260, 284, 355] or infect aposymbiotic juveniles with greater success when exposed to high temperature [2]. There are however some drawbacks with clade D members being almost always suboptimal for their hosts, causing a decrease in their growth rate and reproductive capacity compared to conspecific specimen bearing algae of the clade C under normal conditions [187, 188, 245, 355, 357]. This decrease seems to be linked to a lower photosynthetic efficiency and a lower lipid store of *Symbiodinium* belonging to the clade D. Their tolerance to heat is indeed likely to be related to a lower maximum relative electron transport rate as well as a lower chlorophyll-a content [188]. This explains why the clade D is able to outcompete the clade C during water temperature anomalies and bleaching events but never achieve to establish its predominance once environmental conditions returned to normal.

Clade C and **clade B** *Symbiodinium* are, as said earlier, the most commonly found within scleractinian corals. This could be due to their relative flexibility regarding to environmental conditions [196] but more simply, and especially concerning clade C, because they are the most beneficial for their host's fitness, releasing the highest amount of photosynthesis derived carbon [234, 357]. Clade C is particular in

the sense that it seems almost strictly restricted to its symbiotic way of life with scleractinians. This restriction to a specialized environment is even more accentuated by the large number of hosts transmitting clade C *Symbiodinium* in a vertical manner, directly from the mother colony to the larvae. This promoted evolution within clade C, that harbors now a subcladal diversity equivalent to the diversity observed within other genus of dinoflagelates [354, 357].

Symbiosis¹

As we can see, every clade has its specific characteristics and confers its host with variable fitness. Observations like these, underlying different outcomes for the host depending on the clade and the environmental conditions, lead some researchers to propose the existence of a continuum of relation status between both partners. This continuum would extend from pure mutualism to plain parasitism by the algae with a relative position depending on the environment and the clade but also on the host [234]. Vertical transmission and obligate symbiosis, as observed with many representatives of the clade C, favoring mutualism and symbiont effectiveness for its host while horizontal transmission and facultative association, as mainly observed for clade A, favoring parasitism. Still, none of the clades or subclades can be seen as pure mutualists or parasites but as occupying a subtle position between those two extremes. Understanding of the relation between coral hosts and algal symbionts gets even more convoluted as recent studies have even hypothesized that the host could be itself a parasite. Corals could indeed be actively farming *Symbiodinium* and would then choose with which to establish the most advantageous symbiosis [407]. Such an active or even exclusive participation of the host in the symbiosis is supported by studies showing that symbiosis fitness not only relies on the symbiont and environmental conditions but also on the host itself [145]. This leads to the idea that the host can operate, at least in part, a choice over its dominant symbiont composition, relegating other algal populations, which are almost always present [19, 261, 356], to a minority fraction. This choice may obviously depend on *Symbiodinium* availability but also on the host physiological characteristics in

¹ Parts of the following section have been published as a review article in Journal of Experiment Marine Biology and Ecology. (cf. [118])

response to environmental conditions. This multitude of factors to be taken into account makes it very tricky to understand the reason why some clades or subclades are present in some corals within some locations. Cladal composition could indeed vary between different geographic sites at global but also local scales, sometimes even showing some form of endemism [123, 374, 383]. Monitoring showed that, within these particular locations, the symbiont composition inside different coral taxa or even colonies seems however to be stable as long as environmental conditions stay steady [144, 301, 323]. Yet, its reaction to environmental stress and its eventual switch to a composition dominated by another clade are not unanimously recognized and may be species specific [18, 143, 374, 375]. This hypothesis will be addressed later, when we discuss the subject of coral bleaching.

Transmission

Various degrees of cladal composition specificity exist among coral species [17]. Such specificity can be observed early during the life of corals. This is especially true for species that acquire their symbionts through vertical transmission. In this case, algae are transmitted from the mother colony to its egg or brooded planula larvae prior to release [28, 29]. Interestingly, during early stages of development, algae can be found within both ectodermis and endodermis [175, 416]. This suggests an initial, and later lost, competence of ectodermal cells to host algae. The developing larvae perpetuate then the association present within its species local population. This mechanism is however a minority, representing only 15% to 20% of hermatypic scleractinian coral species with particular occurrence within brooding species. The vast majority of corals rely on horizontal transmission where aposymbiotic larvae recruit new algae from the surrounding water [15, 99, 318, 326, 345, 367]. Larvae or

juveniles of these coral species are able to initially associate with either homologous (same as adult colony) or heterologous clades of *Symbiodinium* [1, 51, 67, 153, 245, 325, 345, 398]. Such early association has also been reported in cultured gorgonians and sea anemones [61, 73]. However during the following months, the initial distribution of symbionts progressively adjusts to the most common distribution found in adjacent adult colonies [1, 61, 140, 245]. The host seems indeed able to finely tune its symbiont composition thanks to alternative discharge mechanisms [410]. This corroborates results showing that the pattern of transmission has only little effect on the *Symbiodinium* composition of some coral species [382] and suggests the presence of specific mechanisms to favor the development of an optimal relationship between the coral and a specific symbiont type.

Acquisition

During this so-called horizontal transmission, many studies showed that larvae acquire their symbiont during a nutritional process [153, 160, 325, 326, 345, 346, 398]. Although symbiont infection through feeding is probably the major acquisition mechanism, it could also be facilitated by active mechanisms deployed by *Symbiodinium*. Indeed, as described earlier, *Symbiodinium* cells interchange between a coccoid form, which is always the one encountered when endosymbiotic, and a motile zoospore. The motility of the flagellated *Symbiodinium* is regulated in a diel-cycle and cells are active under illumination [66, 111, 115, 228, 408]. During that period, *Symbiodinium* seems able to swim in an oriented manner, thus showing phototaxis [169] and/or chemotaxis [110, 292]. However, a potential host-seeking behavior remains limited by the flow velocity and the proximity of a host [293]. Finally, the success of *Symbiodinium* acquisition strongly depends on environmental conditions. In the same manner as during bleaching, which will be extensively discussed

later, high temperatures accompanied by elevated light irradiance have been demonstrated to drastically impair algae uptake. *Symbiodinium* concentration in juvenile corals falls from 87% at 28°C and high light to 8% at 31°C [2].

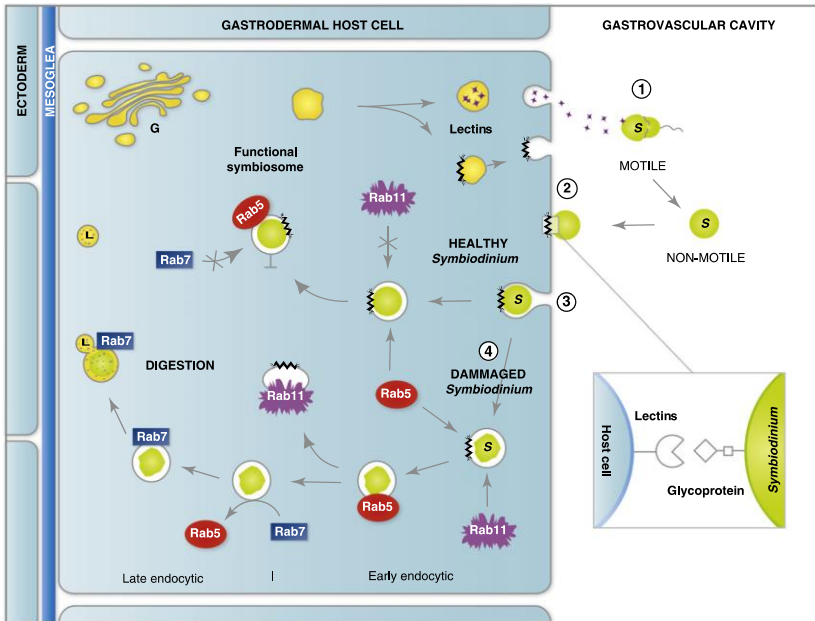


Figure 7 | Schematic illustration of *Symbiodinium* infection of a cnidarian host cell. Motile or non-motile (cyst) *Symbiodinium* enter the gastrodermal cavity of the host. **(1)** Lectins secreted by the host cell induce motile algae to progress to the cyst stage. **(2)** Contact and recognition are mediated by other lectins present on the host cell surface and glycoproteins on the surface of the non-motile *Symbiodinium*. **(3)** *Symbiodinium* are phagocytosed and oriented to the early endocytic compartment. **(4)** Healthy *Symbiodinium* end in a functional symbiosome while damaged *Symbiodinium* are digested by fusion with lysosome after transiting through late endocytic compartment. Rab5, Rab7 and Rab11 are respectively involved in the early endocytic compartment, late endocytic compartment and endosome recycling. G: Golgi apparatus, L: Lysosome, S: *Symbiodinium*.

In order to establish the symbiosis, both partners first have to recognize each other. Recognition mechanisms could be similar to the winnowing mechanism, which consists in a multi-step process involving both partners, that was first described by Nyholm and

McFall-Ngai [278] for the symbiotic relationship between the bobtail squid, *Euprymna scolopes*, and its bioluminescent bacterium, *Vibrio fischeri*. These mechanisms necessarily take place before, during, and after contact with *Symbiodinium* and its internalization, leading ultimately to the formation of a stable mutualistic relationship [71]. Recognition mechanisms between host cells and algae seem to be necessary, at least at some point, during vertical or horizontal transmission. Indeed, while it is quite obvious for horizontal transmission, Marlow and Martindale [251] showed that recognition events could also be involved during vertical transmission in *Pocillopora meandrina*.

According to Weis [394], among others, the onset of the infection by *Symbiodinium* involves the same mechanisms as those acting in the recognition of pathogenic organisms (Fig. 7). For many eukaryotes, the innate immune system implies the production of pattern recognition receptors (PRRs) able to recognize and bind to specific conserved components of microbe cell walls (carbohydrates, proteins, lipids; [200]). Among those PRRs, lectins (carbohydrate-binding proteins; [139]) are widely distributed in most classes of living organisms and are thought to play an important role in various symbiotic associations (e.g.: [50, 161, 264]). To date, lectins have been described in, at least, two cnidarians classes (Hydrozoa and Anthozoa) and have been reported in five scleractinians, including *Acropora millepora* [213], *Montastrea faveolata*, *Oculina patagonica* [155], *Pocillopora damicornis* [385] and *Ctenactis echinata* [183]. Several studies have thus examined the implication of lectin/glycan interactions in the recognition process between cnidarians and *Symbiodinium*.

Lin et al. [243] identified ten different proteins on the cell surface of symbiotic dinoflagellates. Among those figured five glycoproteins with two kinds of terminal sugar residues: mannose-mannose and

galactose- β (1-4)-N-acetylglycosamine. Moreover, cell surface glycan profiles of cultured *Symbiodinium* seem to be stable throughout their life history, indicating that *Symbiodinium* may maximize the potential for host recognition by retaining recognition molecules throughout their vegetative growth [247]. The importance of these membrane-bound glycoproteins during the infection of the sea anemone *Aiptasia pulchella* with *Symbiodinium* has been demonstrated by masking or removing cell surface glycans of the dinoflagellate. Indeed, *Symbiodinium* incubated with either trypsin, α -amylase, N-glycosidase, O-glycosidase or with different kinds of lectins, in order to remove or mask carbohydrate groups, showed a significant decline in the infection rate of an aposymbiotic host [243]. Similarly, *Symbiodinium* treated to digest or mask α -mannose/ α -glucose and α -galactose residues failed to efficiently infect larvae of the fungiid coral *Fungia scutaria* [405].

These membrane-bound glycoproteins could play a role in *Symbiodinium* recognition by acting as “markers” that could be bound by various types of lectins. **SLL-2** is one of these lectin-type proteins. This (N-glycosylated) galactose-binding lectin, isolated from the octocoral *Sinularia lochmodes*, is preferentially localized not only in nematocysts but also on the cell surface of *Symbiodinium*, whether they are present within a host cell or in the coelenteron [181, 184]. The most surprising propriety of SLL-2 is its ability to induce the switch of flagellated and motile *Symbiodinium* cells to a non-motile coccoid form still able to divide [205]. Such physiological transformations may favor a condition more suited for the establishment of symbiosis. SLL-2 action on *Symbiodinium* seems to present more than a simple carbohydrate binding property as shown by the lack of algal modification in the presence of proteins showing similar properties as SLL-2. Koike et al. [205] suggested that SLL-2 could be stored within nematocysts and released into the gastrodermal cavity in the

presence of micro-algae. It would then bind to glycolipidic residues present on their surface [182]. Response of these algae to SLL-2 could therefore act as a first screening process, modifying algal physiology to favor further interactions with the host or causing damages to micro-algae unsuitable for symbiosis. Recently, CecL discovered in *Ctenactis echinata* showed effects on cell transformation highly analogous to those of SLL-2. It seems that CecL lectin also has the ability to temporarily suppress the rate of cell division without affecting cell viability and thus, can regulate *Symbiodinium* density in the coral gastrodermis, where only a limited number of algae can be accommodated [183].

Another lectin has been identified in *Acropora millepora* [213]. Named **millectin**, this lectin possesses a Ca^{2+} -dependent carbohydrate-binding site that preferentially binds to mannose and similar sugars. According to immunohistochemical analysis, millectin is localized in nematocysts present in the epidermal tissue [214]. This protein is phylogenetically close to collectins, which play a key role in vertebrate innate and adaptive immune responses. Millectin expression is up-regulated in response to lipopolysaccharides and peptidoglycans. Moreover, it has the ability to bind to both gram + and gram – bacteria (including *Vibrio coralliilyticus*, a coral pathogen) and to various clades of *Symbiodinium* (C1, C2 and A2) *in vitro* and *in vivo* [213, 214]. When bound to pathogens, millectin prevents their dispersion into the host and induces opsonisation for phagocytosis and destruction. Finally, numerous millectin isoforms showing amino acid substitution sites in close proximity to the binding site have been identified. This vast diversity of millectins suggests a probable appearance of some recognition specificity and a role of millectins in the winnowing process [213].

Recently, the sequence variation of a putative coral immunity gene, *tachylectin-2*, has been investigated in the coral *Oculina patagonica* [155]. **Tachylectin-2** was originally isolated from the Japanese horseshoe crab (*Tachypleus tridentatus*) and has been demonstrated to possess anti-microbial activity [281]. Nevertheless, a role for Tachylectin-2 in the symbiosis of coral species has not been confirmed yet.

Lectins also appear to play an important role under stress conditions. Indeed, the downregulation of the transcriptional expression levels of two C-type lectins has been observed in aposymbiotic larvae of *A. millepora* [324] and nubbins of *Pocillopora damicornis* exposed to thermal stress (*PdC-lectin*; [385]). Conversely, the same gene has been shown to be up-regulated when exposed to the pathogen *Vibrio coralliilyticus* under virulent conditions [386]. These contrasting results underline the complexity of these lectin/glycan interactions and the tenuous link between the coral physiological response and the establishment of the symbiosis.

Lectin-type proteins are not the only molecules involved in mutual recognition between host and symbiont; other proteins have also been reported to be over-expressed in the presence of symbionts. These include **AtSym-02**, a glycosylated membrane protein identified in *Acropora tenuis* [412], and **Sym32** identified in *Anthopleura elegantissima* [315]. AtSym-02 may belong to the fasciclin-I (*fasI*) gene family, also known to be involved in cell-cell recognition mechanisms [412]. Sym32 can be found both in anemone protein homogenates and bound to membranes. Sym32 is also expressed more in anemones infected by *Symbiodinium* than in those hosting green algae (*Zoochlorellae*), leading the authors to hypothesize that the symbiont has the ability to control the expression of some host genes [315].

Establishment

After the recognition step, symbionts are internalized by a phagocytic process conducted by host cells, probably through the formation of a lectin-binding complex. Then, a wide range of other cellular processes are necessary for the maturation of *Symbiodinium*-containing phagosomes into functional symbiosomes able to avoid fusion with the host endolysosomal system during endosymbiosis [55, 114]. Indeed, while normal phagosomes mature by fusing with lysosomes, early observations showed that healthy *Symbiodinium*-containing symbiosomes did not reach that stage [114], suggesting the existence of host and/or symbiont-specific molecules involved in mechanisms maintaining the symbiosis once established. Parts of these mechanisms, such as modulation of TGF β synthesis, could be similar to those employed by some parasites to promote host tolerance [75, 344], while others could be specific to this symbiosis.

Although the mechanisms of symbiosome membrane formation are still poorly understood, some evidence in *Aiptasia pulchella* indicates that ApARF1 and ApRab genes could be involved in this particular process. ApARF1 is homologous to ARF1, a member of a family that regulates intracellular vesicle transport and its gene expression appeared reduced in symbiotic anemones [56]. However, further investigation is needed to clarify apARF1 function in the establishment of symbiosis. The role of ApRab seems more convincing. ApRab shows similarities with Rab family members, coding for small GTP binding proteins found in many vertebrates, where they assume the regulation of vesicular trafficking, membrane fusion and also the biogenesis and the function of membrane-bound organelles [171]. To date, four of these ApRab proteins have been shown to play a role in the establishment of symbiosis.

ApRab7 usually participates in late acidic endocytic and phagocytic pathways [387]. However, in *A. pulchella*, ApRab7 is excluded from symbiosomes containing either resident or newly internalized *Symbiodinium*. ApRab7 can also be found on phagosomes containing heat-killed or PSII-impaired (DCMU-treated) *Symbiodinium*, where it probably promotes their maturation and fusion with lysosomes [55].

ApRab5 appears before ApRab7 and is present in the early endocytic and phagocytic compartments where it can promote their fusion [23]. In *A. pulchella*, ApRab5 localizes on symbiosomes containing healthy algae and is absent from symbiosomes in which algae have been damaged by heat or DCMU treatment. These observations suggest that active retention of ApRab5 by *Symbiodinium* participates in their persistence in the host, possibly by preventing ApRab7 binding. Indeed, these two proteins never co-localize in *A. pulchella* vesicles [54].

ApRab11 acts during endosome recycling processes, a necessary step towards maturation [415]. In *A. pulchella* ApRab11 can be located on phagosomes containing damaged symbionts but is absent from those containing healthy ones [57]. By actively excluding ApRab11, the authors observed that *Symbiodinium* interferes with the vesicular recycling process and thus prevents the maturation of their symbiosome and its fusion with lysosomes.

ApRab3 is the last of the ApRab family members identified in cnidarians. It appears to be preferentially localized in the compartments of the biosynthetic pathway including both the Trans Golgi Network and a subpopulation of secretory vesicles. In *A. pulchella*, symbiosome formation seems to involve interactions with ApRab3-positive vesicles. However its function in the symbiosis

establishment remains to be determined, as phagosomes containing either healthy or damaged *Symbiodinium* or even latex beads progressively accumulate ApRab3-specific labeling [171].

The differentiation of phagosomes into functional symbiosomes obviously requires some particular cellular developments in order to maintain the endosymbiosis and to optimize mutual exchanges. Indeed, the analysis of symbiosome structure conducted on *Aiptasia pallida*, using transmission electron microscopy and immunological techniques, revealed a membrane complex composed of a single host-derived outer membrane and a multilayered inner membrane originating from the algal symbiont [388, 389]. The presence of symbiont thecal vesicles *in situ* suggests that this multilayered membrane could be the result of a continuing delayed *in situ* ecdysis cycle [388]. Anyway, the formation of this kind of structure requires some regulation by the cytoskeleton of the host cell and its symbionts, allowing normal host cell physiology [295]. Moreover, proteomic analyses of isolated symbiosome membranes conducted on *A. pallida*, highlighted the presence of proteins highly similar to GPCR family proteins. These receptor-like proteins are distributed to symbiosome surface and should be involved in various cellular responses and modulation of host gene expression [295]. The presence of ATP synthase complexes and ABC transporters on this structure demonstrates the pivotal process of molecular transport during the mutualistic association [295].

Exchanges

Indeed, the symbiosis is considered to be optimally functional when a significant amount of photosynthates generated by *Symbiodinium* is transferred to the host cells. Once in symbiosis, the symbiont provides up to 95% of its photosynthetic products (glycerol, glucose, amino acids or lipids) to the host [250, 265, 267], thus

contributing massively to its energy demands [128]. In many species, half of this translocated carbon is used for mucus production [404]. Lipid droplets could play an important role in these transfers as shown by the light induced rhythmicity observed in their formation and localization [58, 294]. A significant part of the photosynthetically fixed carbon is translocated through processes controlled by host release factors (HRFs) which are yet to be clearly identified [411]. HRFs are believed to divert surplus of assimilated carbon from the synthesis of storage compounds to translocated compounds. Interestingly, if the heterotrophic incomes of a coral host become scarce, this leads its nitrogen-limited symbionts to allocate more carbon to storage and therefore to its starving host [72]. Contrastingly, translocation of photosynthates could also be induced by the symbiont. This hypothesis, known as phagosome arrested hypothesis (PAH) states that *Symbiodinium* stays hidden in its host cell by mimicking a constant prey digestion by the endosomal machinery through perpetual release of carbon based nutrients [157]. Regarding the amount of transferred molecules, especially glycerol, osmolarity within the host cell is susceptible to many changes; its fine regulation has recently been pointed out as a key factor in symbiosis regulation. While increase in osmolarity has been shown to enhance release of photosynthates from algae [364], its decrease, due to photosynthesis failure for example, could lead to hyperosmotic stress and ultimately to symbiosis breakdown [253]. Finally, as said earlier, high O₂ concentration due to *Symbiodinium* photosynthesis also helps in maintaining the high ATP level needed for the calcification process [185].

In return for these services, the host ensures protection to *Symbiodinium* and provides a source of inorganic nutrients (CO₂, NH₃ and PO₄³⁻)[411]. Carbon dioxide transfer to the algae seems particularly crucial considering its low concentration in seawater and

the poor discrimination of dinoflagellates RuBisCO II between O_2 and CO_2 . The host addresses this issue not only with CO_2 production by respiration processes but also thanks to mechanisms concentrating it from the surrounding water. These mechanisms mostly involve various isoforms of the carbonic anhydrase (CA), an enzyme operating conversions between HCO_3^- and CO_2 , which is upregulated in symbiotic cnidarians [120, 397].

Regulation

Under normal conditions, *Symbiodinium* densities in symbiotic corals reach a steady state wherein neither partner outgrows the other [266]. The maintenance of this dynamic equilibrium suggests the existence of intrinsic and environmental factors that can potentially regulate algal density pre- or post-mitotically [168, 269]. This regulation involves a variety of mechanisms, like the limitation of algal nutrient supply [101], the digestion of algae [195, 269, 376], the expulsion of excess or dividing *Symbiodinium* [13, 168, 195], the accommodation of excess algae by division of host cells [376] and possibly the production of growth inhibiting factors [352]. This fine regulation of algal density also occurs on daily [109, 166, 195, 361] and yearly bases showing, for example, seasonal variations [43, 100, 109, 360]. Expelled algae can then be recruited by gastrodermal cells or released to the ambient environment. Symbiotic algae that are released every day to the external environment, either through density regulation mechanisms [166, 361] or through survival of corallivorous species digestion [12, 262], may contribute to the maintenance of a free-living *Symbiodinium* population.

Bleaching

1

Under atypical or extreme conditions however, this seemingly stable equilibrium often suffers severe impairments that ultimately lead to its collapse. This phenomenon, revealing the coral white skeleton beneath its algae-depleted tissues, is known as coral bleaching (Fig. 8). This sadly famous incident is the central element of a majority of recent coral-related studies, including this one. The main reason of this infatuation is, as said earlier, the upsurge of scenarios based on climate change predictions considering a baneful destiny for coral reefs. The urge for a better understanding of coral bleaching is even more strengthened by the increased number of field observations witnessing its occurrence all around the world. The change is underway and the threat needs to be taken seriously. Since the first alarming observations of coral bleaching, their subsequent death and the species disappearance in Panama following a warming event in the early 80's [132, 133, 223, 236], reports of bleaching keep accumulating. Whether they come from the Caribbean region [81, 84, 122, 125, 327], the Pacific Ocean and the neighboring South-East Asia [30, 47, 130, 186, 279] or the coasts of the Indian Ocean [44, 221, 237, 255, 256], they all give the same feeling of anxiety and alarmism reporting reduction of coral health, cover and architectural complexity accompanied by modifications of the benthic community.

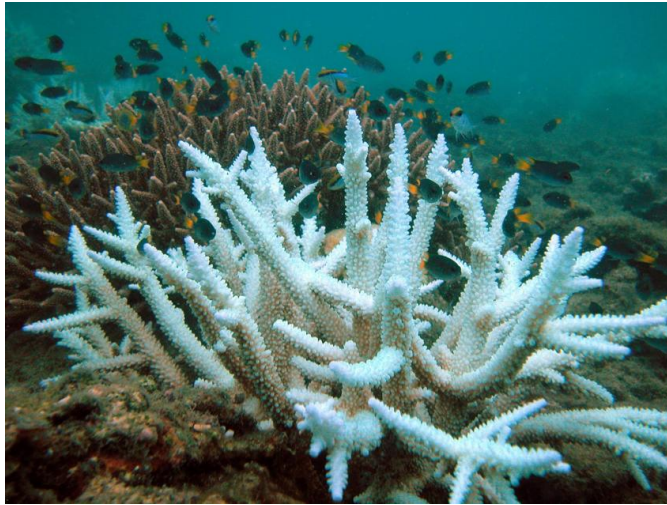


Figure 8 | Illustration of a bleached *Acropora* sp. (From en.wikipedia.org)

Causes

Coral bleaching has been rapidly linked to elevated sea surface temperatures [141] but the concomitant cellular responses to this abiotic stress have been slow to be elucidated and are still not totally understood. Scientists particularly struggle to understand the respective responsibilities of each partner (coral, algae and bacteria) in the collapse of the symbiosis. Many fingers are however pointed toward *Symbiodinium* and its photosynthetic apparatus. This is legitimate considering studies demonstrating a link between algal density and symbiosis susceptibility to light and/or thermal stress in cultured cell aggregates [275], planula larvae [274] or adult colonies [68]. Every investigation undertaken to identify these intimate links between *Symbiodinium* and bleaching leads to the same culprits: photosynthesis dysfunction and reactive oxygen species (ROS) production [83, 179, 298, 351]. Subtle distinction can then be made concerning the different effects and relative responsibilities of light and high temperatures here. Sunlight could be considered, with the

energy it carries, as the source of the ROS, the toxic compounds that lead to bleaching, while elevated temperature is the cause of impairment within the photosynthesis apparatus that ultimately leads to this production of ROS. High UV radiations could also induce some deterioration of photosynthesis components and have been subject of a few studies in the past [21, 230, 233, 235]. UV could especially induce reduction in chlorophyll synthesis and exacerbate effects of visible light and heat on photosynthetic apparatus. This abiotic factor is however barely affected by climate change, thus relegating its implication in increasing coral bleaching events as a facilitating factor but not a main cause such as elevated temperature.

Within chloroplasts machinery of *Symbiodinium*, photosystem II (PSII) is the most sensitive component to light and therefore the main siege of the degradations leading to ROS production. It can suffer from multiple sorts of damages that often result in a process called photoinhibition, a drastic reduction of photosynthetic capacity. Several studies have pointed out the role of the D1 protein during heat related photoinhibition in *Symbiodinium*. The D1 protein plays a central role in PSII functioning but is also its most vulnerable component: it is very sensible to heat and can be easily destabilized. An active repair mechanism fortunately exists but often fails to cope with the high rate of D1 degradation occurring during high water temperature events. Some experiments even showed that D1 repair mechanism could itself suffer from heat. Following D1 degradation, excitation energy starts backing up and finally leads to photoinhibition. This is usually translated by a drastic reduction of photosynthesis efficiency, measured by its maximum potential quantum yield (F_v/F_m) [369, 392, 394]. Similar effects can be observed with chemicals such as DCMU, an herbicide blocking the PSII electron transport chain [190]. Elevated temperatures seem also able to impair the dark reactions of the photosynthesis, altering CO_2

fixation mechanisms through degradation of the Rubisco [192, 230, 351, 394]. Similar results have been observed in the symbiosis between *Symbiodinium* and giant clam (*Tridacna gigas*) [227]. The consequent limitation of ATP and NADPH consumption leads once again to a backup of excitation energy within the PSII and its photoinhibition. Finally, the lipidic structure of the thylakoid membranes represents a third site of damages from heat within *Symbiodinium* chloroplasts. This results in an energy uncoupling of electron transport in both photosystems and prevents electrons generated from taking part in ATP and NADPH production [351, 372, 394].

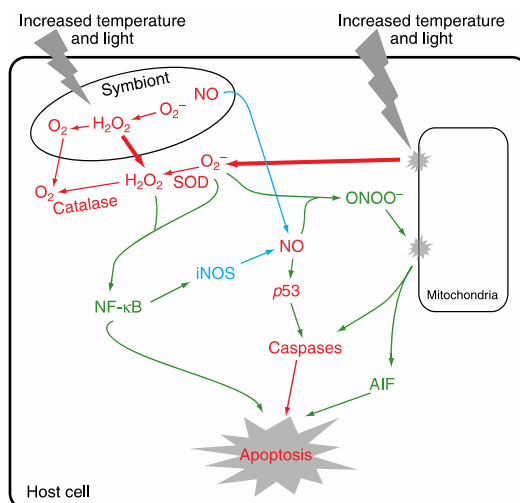


Figure 9 | Oxidative stress mechanisms responsible for bleaching. (from [394])

Mechanisms

All these deleterious mechanisms lead to the same thing: an accumulation of electrons within the chloroplast of *Symbiodinium*. Instead of reducing $NADP^+$, these electrons react with O_2 and pigments to form two ROS species: respectively the superoxide anion (O_2^-) and the highly reactive singlet oxygen (1O_2). At first, the algal cell

tries to cope with this ROS production, alleviating their effect with the help of detoxification enzymes such as the superoxide dismutase (SOD) and ascorbate peroxidase (APX). It is then important to note that the less damaging H_2O_2 produced by SOD is likely, in presence of Fe^{2+} , to transform into the hydroxyl radical ($\cdot\text{OH}$), the most reactive ROS. These detoxification mechanisms set in place by the algae will however be eventually exceeded. Accumulation of ROS will then engage a positive feedback loop, degrading pigments and reacting with D1 proteins and photosynthetic membranes to further damage the photosynthetic machinery [192, 231, 372, 394].

ROS produced by the algae eventually diffuse into the cytoplasm of the gastrodermal host cell. From there, things get a bit more complicated (Fig. 9). A direct response to the stress from the host cell itself becomes indeed difficult to discern from its reaction to the diffusion of ROS from its symbiont. This is particularly true regarding the ROS produced by the host and their addition to those coming from the algae. These come from the host mitochondria, which are also susceptible to damages from the heat [89]. All these ROS elicit the establishment of an antioxidant response by the host cell. This consists in the production of different types of SOD, as well as catalase (CAT), ascorbic acid, carotenoids and mycosporine glycine [14, 238, 258, 317]. Similarly to the algae, this response quickly becomes overwhelmed by the amount of ROS, still accumulating in the cytoplasm, and fails to prevent their oxidizing nature to cause damages to the DNA, membranes and proteins of the host [231, 232, 316]. A recent study even suggests that the impact of ROS could even go beyond the collapsing cell membrane and diffuse into its extracellular environment to affect surrounding cells [338].

ROS may however not be the only molecules involved in the cellular bleaching cascade. Many studies have demonstrated that

nitric oxide (NO) also plays an important role in the induction of the symbiosis collapse. Origins of this molecule could be multiple. It could, in part, diffuse from the algae where high temperatures have been demonstrated to induce its production [40]. NO is also believed to be synthesized in high concentrations within the host cell in response to heat stress. This production involves the activation of the transcription by NF- κ B of the gene coding for the inducible nitric oxide synthase (iNOS)[296, 394]. Nitric oxide would then activate apoptosis pathways either by direct action over p53 or through damages made to the mitochondrial membrane by its derivate peroxynitrite (ONOO⁻), which forms in reaction with superoxide [154, 231, 296, 394]. Finally, some studies demonstrated that calcium concentration seems to play a significant role in bleaching. They showed that heat stress increases its cytoplasmic concentration and that bleaching could be inhibited using calcium chelator or ionophore to reduce its extra- and intra-cellular concentration [102, 176].

Countermeasures

This list of mechanisms, although trying to be as exhaustive as possible, probably does not represent what exactly happens within every coral gastrodermal cells during bleaching. Not all of these mechanisms may occur but different combinations may or may not cross the threshold that will elicit breakdown of symbiosis. Moreover, multiple studies have shown that coral response to a defined abiotic stress may vary depending on the host species [33, 112]. This could be related to their different intrinsic abilities to cope with cellular stress. Indeed, the host cells often deploy a large panel of molecules trying to overcome or alleviate the perturbations happening in its cytoplasm. The type and concentration of these molecules may vary between different species, explaining their different susceptibility [14, 112]. Aside from the antioxidant system, another main strategy consists in

the production of fluorescent pigments (FP's) that dissipate high-energy light radiations. The different production capacities of these pigments between coral species are very likely to define, in part, their variable susceptibilities to bleaching [14, 287]. The aforementioned MAA's are also found within the host cytoplasm where they play a crucial role absorbing UV radiation and dissipating their energy without formation of ROS. The shikimic acid pathway that leads to the formation of such molecules excludes their possible production by the host cell. MAA have thus either to come from heterotrophic feeding or from translocation from the algae. Even if the algae also benefit from the highest MAA concentration found in its host cytoplasm, which partner of the symbiosis control their complement and distribution is still largely unknown [14, 120]. Finally, a last known strategy of the host to alleviate the cellular stress and escape bleaching consists in the upregulation of heat-shock proteins (HSP's). These are molecular chaperones that help maintaining proteins tertiary structures and functions during stressful conditions. Their increased production seems to be related to the heat-related buildup of calcium concentration within the host cell [14, 102].

Algal loss

All these procedures may however not be sufficient to prevent the host cell from crossing the threshold that will engage it into bleaching. Defining this tipping point and the succession of events that follows therefore represents important steps in the understanding of the coral-bleaching phenomenon. Such is not an easy task as many mechanisms are evoked to explain the disappearance of algal coloration. While some studies have shown that visual bleaching of coral can be sometimes related to a diminution in chlorophyll concentration [370, 394], scientists agree that disappearance of *Symbiodinium* from the host gastrodermis is the main cause of coral

bleaching. How this happens has been largely debated and the mechanisms proposed in the literature can be organized in two categories: those that do not imply the loss of the host cells and those that do (Fig. 10).

Host-cell persistence

Mechanisms involving persistence of the host cell might only take place during mild environmental stress or before damages provoked by ROS production become too severe. Such mechanisms are most often related to *in situ* degradation of the algae as reported by microscopic examination performed on corals [6, 45] and sea anemones [88, 93, 116, 362]. This degradation is believed to result from two possible mechanisms. The first mechanism involves damages caused by ROS, such that they induce the death of the algae. This death can occur by means of programmed cell death (PCD) or apoptosis with the algae showing typical cellular patterns such as a condensed nucleus, crenated membrane and condensation of organelles and cytoplasm [88, 93, 116, 362]. ROS damages to the algae cellular components can be so harsh that the PCD cannot be established and the death occurs in an uncontrolled manner. The algae therefore die showing classic necrosis features that are pyknotic chromatin, vacuolization, breakdown of cellular structures and rupture of the membranes [88, 92, 93, 116, 362]. The second mechanism of algal degradation implies active degradation by the host cell and digestion of the remains. This mechanism could be spontaneously initiated following the impairment of the ApRab pathway manipulation by the algae. This one can therefore no longer prevent maturation of its symbiosome into a phagosome and lysis of its component by enzymes contained in lysosomes. It could also involve autophagy, a catabolic pathway responsible for the recycling of organelles as well as other cellular key functions. In this case, it is

sometimes called symbiophagy and actively participates in algal *in situ* degradation by promoting fusion of the phagosome with lysosomes [85, 91, 152]. Other scenarios sparing the host cell during algal expulsion have been proposed but are not unanimously accepted. Those were rarely described in the literature and include algae exocytosis [358] and the pinching off of a portion of the host cell cytoplasm [336].

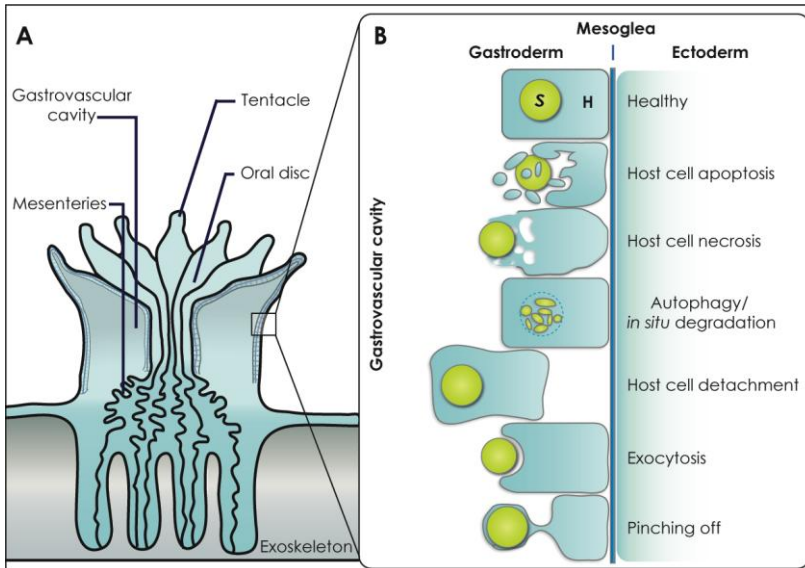


Figure 10 | Host-controlled cellular processes involved in *Symbiodinium* loss. A. Schematic representation of the coral polyp with its tissular organization. **B.** Illustration of the mechanisms believed to be involved in the release of *Symbiodinium* from its host cell. S: *Symbiodinium*, H: Host cell. (Adapted from [127, 394])

Host-cell loss

Although it could only concern severe cases of bleaching-related intense stress [363], many studies reported that loss of algae during bleaching is often accompanied by the loss of their host cells. Two scenarios are responsible for this loss, both happen consequently to major disruptions of the host cell homeostasis. Perturbation of the

calcium cytoplasmic equilibrium leads to the collapse of the cytoskeleton and perturbations of the cell adhesion. The host cell detachment that follows has often been described as a cause of *Symbiodinium* loss [45, 127, 341]. The vast majority of recent studies, however, agree to say that *Symbiodinium* loss during bleaching is largely caused by the death of their former host cells. Cellular swelling as well as other cellular characteristics of necrosis can sometimes be observed [223]. Nevertheless, necrosis is likely related to extreme stress conditions while apoptosis seems to be much more frequent and is described in recent studies [92, 93, 154]. As evoked earlier, environmental stresses experienced during bleaching are believed to induce apoptosis following multiple cascades of events. Heat as well as ONOO^- and ROS can cause damages to host mitochondria and therefore induce activation of caspases and other pro-apoptotic factors. Nitric oxide also activates p53, a pro-apoptotic transcription factor that also activates caspases. Finally, NF- κ B, which is upregulated due to ROS concentration, can have a direct action over apoptosis activation [317, 394]. The host seems however to operate some control over this caspase-induced apoptotic pathway. Some studies showed fine regulation of Bcl-2 protein family members following bleaching induction of apoptosis [215, 299]. Authors thus propose that, following caspase activation, coral quickly modulates this response in order to limit apoptosis and prevent its progression to other cells to limit the damages and optimize its survival. Happening prior to bleaching, the suppression of the cell death cascade within these cells will allow them to serve as basis for later tissue regeneration [7, 373]. Moreover, apoptosis and autophagy seem to be interconnected as demonstrated by experiment showing induction of one while the other is inhibited [91]. A similar “see-saw” mechanism has already been reported in vertebrates during immune response to pathogens and parasites.

Altogether, these information combine to form the following hypothesis of what is happening during bleaching. Exposed to environmental stressful conditions (high light and temperature), the cnidarian gastrodermic host cell will experience diverse degrees of perturbations depending the intensity of the stress, the algal clade it contains and its cellular equipment, which vary depending its species. Consequently, the host cell will identify algae, which are already suffering and could be dying, as the source of the stress and will try to get rid of it. Depending the scale of the perturbation it has suffered, it will favor “soft” mechanisms such as autophagy before using apoptosis. However, if the stress is too strong, the host cell will ultimately lose control of its homeostasis and either detach or undergo necrosis.

Adaptative bleaching

After release from their former host cells, *Symbiodinium* accumulate within the gastrodermal cavity before being expelled in the form of aggregates embedded in mucus pellets. It is then important to notice that multiple studies have observed that most of these algae are not always condemned and seem to be able to recover from the stress and multiply in the water column [34, 158, 307, 308]. The expulsion by the host of these stressed but seemingly viable algae corroborates the adaptative bleaching hypothesis (ABH). According to this hypothesis, the host could use aforementioned mechanisms such as apoptosis to eliminated algae unsuited to environmental condition in order to establish symbiosis with better ones. Such radical way of proceeding has even been documented in the winnowing mechanism during symbiosis establishment in coral larvae [94]. The ABH was initially proposed by Buddemeier and Fautin [49] who considered bleaching as an opportunity for the host to be repopulated by new, stress tolerant, algae [103]. Mathematical modelization [391] as well

as evaluation of its basic assumptions [82, 201] further strengthened this hypothesis. This shift between two dominating *Symbiodinium* populations, which usually favors the thermally tolerant clade D, has since been described on multiple occasions whether following natural bleaching events [20, 189, 375] or transplantation induced bleaching [16]. Such adaptive response is also supposed to occur during mild environmental changes and in absence of bleaching. This more progressive modification, called symbiont shuffling, would be similar to bleaching in the mechanisms involved but would operate more progressively and therefore in absence of visual whitening effect [31, 280]. ABH is now accepted by numbers of scientists but some of them raise concerns about long terms effects of such new association and the need for more studies. As mentioned earlier, hosting of thermally tolerant symbionts lower the symbiosis fitness and therefore reduce the competitiveness of such corals if conditions return to normal [203].

Recovery

The ABH illustrates a specific case of algal density recovery from bleaching. Such recovery has been documented on cnidarians, whether in the field [113, 156, 377] or during laboratory experimental studies [32, 240], and can take from a few weeks to several months. The origin of the new *Symbiodinium* is however not clearly established as they could come from the environment or the multiplication of the remaining ones. Presence of new genotypes could then be explained by their new acquisition or by their initial low level that did not reach detection threshold [165].

Tissue regeneration

Recovery from bleaching does however not only depend upon reinfection by new *Symbiodinium*. During the weeks or months leading up to complete reestablishment of the symbiosis, the host will

have to face other hardships in order to survive. First of all, the coral will have to mend its injured tissues. As we just described, expulsion of algae is mainly linked to the loss of their host cells and subsequently involves damages to the host gastrodermis. Moreover, some authors reported that, during prolonged exposure to high intensity stress even the ectodermis could suffer some slight damages [7, 93]. It seems therefore obvious that the host suffers wounds that need to be treated in order to ease recovery, especially if the bleached gastrodermis now lacks competent host cells able to engage symbiosis with new algae. Studies addressing this matter are however very scarce, with only some studies evaluating re-epithelization after UV-induced damages [25] or impact of bleaching in wound-healing processes [252]. The processes reported in these studies involve successive phases that are similar to those observed in other invertebrates or vertebrates [288]. Some of them, such as amoebocyte penetration, proliferation of newly formed cells and their maturation, could also be involved during bleaching-related healing and could be relevant in the understanding of this process. Yet of great interest, this is nevertheless speculative as the evaluation of the regeneration processes occurring after heat-induced bleaching has never been addressed so far. The source of the new cells in particular, although possibly originating from dedifferentiation of epitheliomuscular and nutritive-muscle cells [134], is still to be determined.

Heterotrophy

The second, but not least, danger that hermatypic corals have to face after bleaching is the drastic reduction of their autotrophy-based carbon incomes. Whereas *Symbiodinium* was initially providing almost all of its energy demand, the bleached coral is now threatened by starvation [149]. Survival to bleaching is therefore logically linked to

its energy reserves, as shown by longer survival rate predicted for individuals harboring large lipid stores [11], as well as its ability to find a new source for carbon acquisition. Numerical models of symbiosis exchanges also suggest that heterotrophy could be crucial for the survival of the host and could potentially delay the onset of coral mortality [11, 150].

Multiple studies have already addressed the importance of heterotrophy for corals as well as the mechanisms implied. Feeding in coral involves both passive and active mechanisms. Passive mechanisms refer to simple diffusion of dissolved organic matter (DOM) from the water column. This essentially concerns carbohydrates but also includes uptake of ammonium and urea, both byproducts of animal metabolism that contain nitrogen, a crucial element for the *Symbiodinium* [174]. Active feeding mechanisms are meanwhile used for the capture of food particles and live prey. They can be elicited by either chemical or tactile stimuli and involves capture by tentacles, entanglement with mucus net and filaments or both [242]. A large variety of carbon sources participate in active feeding by cnidarians. They range from suspended detritic particulate organic matter [241] to the microbial and macroscopic fractions of the zooplankton. Active feeding is therefore assumed to be occurring mainly during the night, when zooplankton density at the depth of the coral is the highest [174]. Recent studies also showed that coral could vary its diet and sometimes be herbivore and feed on planktonic microalgae [224] or neighboring algal turfs [249]. Many morphological factors affect coral heterotrophic feeding ability. In contrary to what was initially thought, the size of the polyp has only little importance. The shape of the colony, branched corals being more efficient, as well as the size of the tentacles and the type of nematocytes are more determining factors in prey capture [174]. Some corals even developed specific morphological features like the multifunctional

digestive filaments described in *Mycetophyllia reesi*, a species lacking tentacles [135]. Similar types of acontial filaments are often used to initiate digestion of large preys before their penetration into the gastrovascular cavity [403]. Depending the species and the type of prey, the complete digestion, measured by total disappearance of the prey DNA, can then take up to ten days [225].

While being essential in aposymbiotic cold-water corals [273], heterotrophy has furthermore been demonstrated to provide benefits to symbiotic corals. In addition to its essential nitrogen contribution to *Symbiodinium* survival, it can also boost colony growth [285], tissue thickness [95] and calcification rates [86, 95]. Heterotrophic carbon incomes have however been demonstrated to reduce the percentage of photosynthetic carbon translocation from *Symbiodinium*, reducing it from 70% to 53% [379]. There also seems to be an even stronger link between heterotrophy and autotrophy as variations of heterotrophic capacity in response to environmental conditions have often been described. Such variations can be observed in relation with fluctuation of abiotic factors that are within the range of normal conditions. Heterotrophic capacity has, for example, been demonstrated to vary in a seasonal manner, being more important during winter [106]. Nevertheless, variations in heterotrophic feeding are more pronounced when the coral is exposed to abnormal conditions. Studies evaluating this have mainly focused on heterotrophic response to light regime variations. Some showed that heterotrophy increases following darkness exposure and subsequent diminution of photosynthetic capacity [172]. Other experimental results showed that, when exposed to shaded and turbid conditions, corals could increase their heterotrophic feeding and compensate for the reduced photosynthesis [10]. Field study of stable isotopes of C and N supports this and shows that corals are able to switch to heterotrophy and support both its own and *Symbiodinium* energy

demands during rainy seasons and low turbidity [366]. This ability seems however to be very species-specific [174].

Even though these findings suggest that heterotrophy can be a crucial element in the response of corals to bleaching [14], only few studies addressed this assumption so far. Most of them decided to focus on the role of heterotrophy on bleaching resistance and photosynthesis activity instead of the host energetics. They demonstrated involvement of heterotrophy in sustaining photosynthetic activity of PSII as shown by a lower decrease in maximum potential quantum yield, relative electron transport rate or oxygen production [37, 107]. This is further confirmed by study showing that food availability reduces pigmentation loss and increases coral survival during heat stress [62]. Although not completely understood yet, this alleviating effect could be related to the high amount of nitrogen needed for protein repair and synthesis of new PSII D1 protein [37]. Finally, only one study to date has assessed the variation in heterotrophic feeding following bleaching in terms of energetics. It however provides results of major importance [149]. In this study, multiple coral species were bleached by exposure to high temperatures. This has the effect of reducing the contribution of *Symbiodinium*-acquired carbon to animal respiration (CZAR) from over 100% to approximately 50%. Following this reduction, one of the species (*M. capitata*) showed a fivefold increase in its feeding rate and a subsequent striking augmentation in its contribution of heterotrophically acquired carbon to daily animal respiration (CHAR). This capacity, which like other corals initially ranged around 20%, was then as high as 100% and therefore able to cover all energy demands of the coral. *M. capitata* was therefore able to maintain high energy reserves contrary to other species, which only owe their survival to the depletion of these reserves. This could allow production of gametes following bleaching but, above all, could make the difference

during prolonged bleaching events and allow survival of species capable of such plasticity.

Whereas some coral species seem able to increase their heterotrophic capacity in response to bleaching, strategies involved in this process are yet to be identified. These could include augmentation of polyp preying activity, with a longer lasting protrusion time, or modification of histophysiological aspects like nematocyte number and mucus production. So far, information available on this matter is very limited and only concern mucocyte numbers and mucus production. Most of the studies evaluating this subject tend however to confirm the possible role played by mucus in heterotrophic shift. Generally speaking, they demonstrated an increase of mucus production [276, 406] or mucocyte number [223] during bleaching or seasonal elevation of sea surface temperatures [302]. This phenomenon seems however to be very variable and species-dependent with some corals showing opposite trends [132]. Despite these results, coral mucous response to bleaching is still largely debated and, though unlikely, can be seen as an uncontrolled reaction to stress. Nevertheless, could such a waste of carbon be possible in an already threatened host, especially considering the majority of mucus-allocated carbon initially came from *Symbiodinium* [42, 276, 379]? Aside heterotrophy, the multiplicity of the functions that mucus could play following bleaching events further legitimates the doubt on this eventuality and raises the importance of further investigations.

Objectives

As we have just reviewed, cellular regeneration and mucocytes production, although believed to play an important role in coral recovery, have been poorly addressed so far. The aim of this thesis is to gather information that will partially complement the lacunar knowledge concerning the histological modification occurring subsequently to bleaching. To do so, we decided to use the sea anemone model *A. pallida*. The usefulness of this model has been recognized for a long time now and its utilization participated in many of the aforementioned results.

The **second chapter** of this work will therefore explore the histological reaction of *A. pallida* to repetitive exposure to cold, a very potent bleaching inducer. It will also provide information on the validity of the histological staining methods used as well as first insights of the host response to algal loss. This chapter will finally address the circadian variations of DNA replication in healthy *A. pallida* in order to give an idea of the impact of light exposure on cell replication.

Techniques and model utility being tried and tested, the **third chapter** will then focus on the histological effects of environmental bleaching conditions, namely elevated temperatures conjugated with high solar irradiance. Exposed to such conditions, anemones will be subjected to evaluations of their cellular proliferation rate and mucocyte number. These measures, applied on the course of days and weeks until recovery to normal *Symbiodinium* densities, will provide new information on the regenerative process occurring following algal loss as well as first evidence of a hypothetical mucocytes proliferation and heterotrophic shift.

In the **fourth chapter** we will try to further investigate this heterotrophic shift theory and the links with energetic dependence to the algae. Bleaching will therefore be induced using chemicals that are believed to only affect photosynthesis, thus minimizing the impact to the host. This will be confirmed by the comparison of the cellular death between treated and untreated anemones. The results gathered in this chapter will consequently further strengthen the link between the loss of autotrophic energy incomes and the rate of proliferation as well as the mucocyte number.

The origin of the proliferating cells and the determination of their final phenotype will be the center element of the **fifth chapter**. Although origin and migration of newly formed cells have been extensively documented in *Hydra*, these elements have not yet been described in *A. pallida*. We will give first insights to this matter by evaluating the relative numbers of new cells in both tissues of the anemone after pulse incubation in proliferation marker and inhibition of mitosis. Some of the final phenotypes of these new cells as well as their putative increase following bleaching will then be identified by co-localization using different staining methods.

The **sixth** and final **chapter** of this thesis will serve as a general discussion of the gathered results. Using these results we will try to expand the actual knowledge concerning tissue regeneration and modification following *Symbiodinium* expulsion. We will also try to include the newly found information into the heterotrophic shift hypothesis and highlight the importance of mucus production and mucocyte number in this framework. We will finally discuss the questions left unanswered and propose some perspectives for future research.

Chapter 2

Cold Shock Response and circadian rhythmicity of EdU incorporation in *A. pallida*.

Introduction

The actual threat posed by climate change on coral reefs is currently urging coral scientists to improve their knowledge on cnidarian-*Symbiodinium* symbiosis. Whereas many research fields deserve interest, there is especially much to learn about symbiosis and bleaching on the cellular scale. In order to do so, many experts argued that coral science should learn from the history of biological science and note that lots of major discoveries have been made thanks to model organisms [396].

Some tropical sea anemones of the genus *Aiptasia* (usually *A. pallida* or sometimes *A. pulchella*) are unanimously recognized as ideal models for the study of the cellular processes involved during bleaching (Fig. 11). While the only drawbacks of using such models are due to their lack of skeleton, making them unsuited for studies of calcification or endoskeletal microbes, their advantages are multiple (Fig. 13). One of the most important advantages of *Aiptasia* is the ease with which it can be cultivated. Its ability to maintain rapid growth rate in standard aquarium conditions makes it a “pest-species” for some hobbyists but is essential for laboratory studies. Large clonal populations can then be quickly obtained [226] and used with multiple kinds of techniques as illustrated by the abundance of scientific

publications using *Aiptasia*. These techniques include genetic, molecular or biochemical analyses [124, 210, 297, 365] as well as different types of microscopic observations [89, 90]. The absence of skeleton, easing the use of such procedures, is therefore another major advantage of *Aiptasia* (Fig. 12). Yet, the main benefit of using *Aiptasia* as a model for the study of coral is to be seen in its relative tolerance to bleaching. Indeed, while many corals often die from bleaching, *Aiptasia* can be easily bleached and proves itself to be remarkably tolerant with a high survival rate of individuals deprived of their symbiotic algae. Such anemones can then be maintained bleached for months and be eventually reinfected in order to study the process occurring during this period [32, 396].

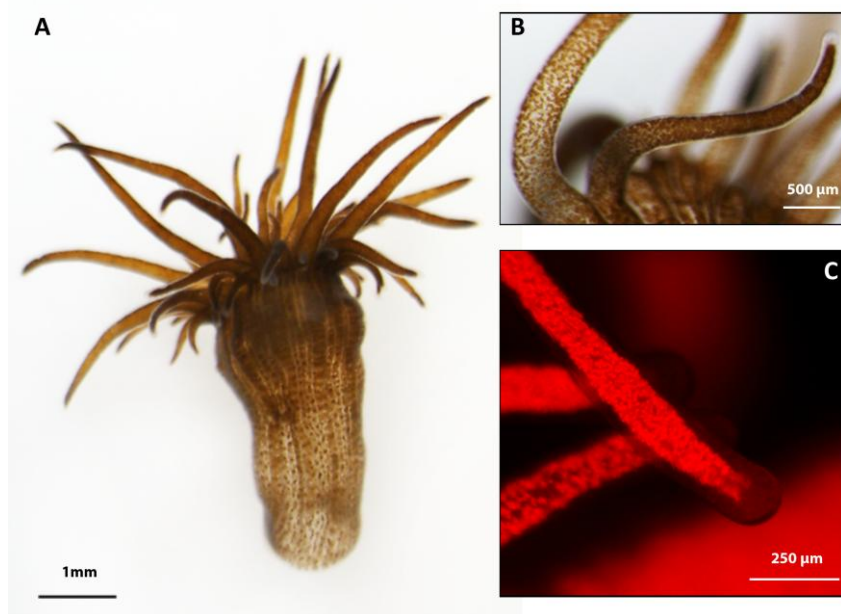


Figure 11 | *In toto* illustrations of the model anemone *Aiptasia pallida*. (A) Overall view shows the general distribution of *Symbiodinium*. **(B)** Closer view of some tentacles revealing the patchy distribution of brown *Symbiodinium* within the gastrodermis. **(C)** The lack of chlorophyll autofluorescence in the ectodermis of a tentacle tip confirms the gastrodermal distribution of *Symbiodinium* (arrow).

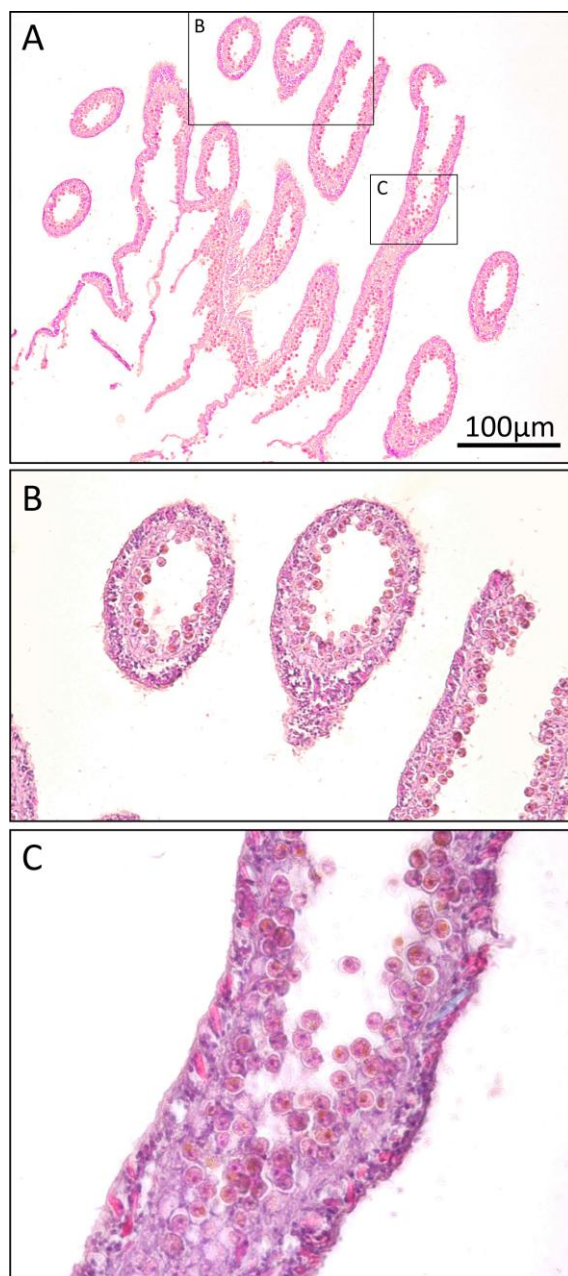


Figure 12 | Histological illustration of the model anemone *Aiptasia pallida* (H&E staining). General view (A) and close-ups of tentacles (B) and symbiotic algae (C).

There is still however much to learn about the *Aiptasia* model. While many studies are working on deciphering its genome, other macroscopic traits are somewhat neglected. This particularly concerns histological aspects that, although being extensively studied in other cnidarians such as *Hydra* [39] or the non-symbiotic anemone *Nematostella vectensis* [134], are poorly described in *Aiptasia* with only some ultrastructural studies [401, 402].

Table 3. Advantages of *Aiptasia* as a model system

Property	Corals	<i>Aiptasia</i>
Size	Large colonies of many polyps	Individual polyps of variable size (0.5 mm–1 cm oral disc diameter)
Growth rate	Slow	Fast*
Growth conditions	Finicky, require very specific growth conditions	Tolerant, hard to kill*
Availability	Mediocre to poor: hard to grow, hard to collect (protected; only in tropical oceans), different species in different parts of world	Excellent: can be grown in large numbers in any laboratory using artificial sea water with fluorescent lights and pet-store brine shrimp as food
Genetic uniformity	Variable	Clonal populations available
Accessibility of cells for microscopy	Poor because of calcium carbonate exoskeleton	Excellent: by examination of whole polyps or cells in macerates
Accessibility for biochemical and molecular biological procedures	Mediocre because of calcium carbonate exoskeleton	Excellent: whole anemones can be homogenized or extracted
Susceptibility to genetic methods, including RNAi and transformation	Poor, except for some larval systems	Uncertain but has potential (and some promising early results)
Tolerance of bleaching	Poor: typically dies after bleaching	Excellent: can be fully bleached, maintained for months, and re-infected

*These properties cause *Aiptasia* to be considered a 'pest species' by salt-water aquarists.

Figure 13 | Advantages of *Aiptasia* over corals as a model organism. (from [396])

One of the main elements of the histological characterization of *Aiptasia* would be the study of its cellular proliferation during normal and bleaching conditions. The use of 5-ethynyl-2'-deoxyuridine (EdU) incorporation, a recently developed technique, seems therefore to be ideal considering its ease of use, its novelty to this field and its proven effectiveness [48, 333]. EdU has been used as a marker of cell proliferation in multiple studies and in many different animals such as annelids [129], reptiles [300] and mammals [97]. This molecule and its use are very similar to those of BrdU but have some significant advantages. After incorporation within the cell DNA during the replication phase, this nucleoside analogue of thymidine can be detected thanks to a simple and quick "Click" reaction with a fluorescent azide (Fig. 14). The small size of this marker enables a good tissue penetration and allows *in toto* whole individuals staining. Moreover, the reaction involved (a copper(I)-catalyzed variant of the

Huisgen [3+2] cycloaddition between a terminal alkyne and an azide) allows the conservation of the DNA integrity. These are particularly appreciable compared to the BrdU revelation process, which involved antibodies recognition and acid catalyzed hydrolysis of the DNA.

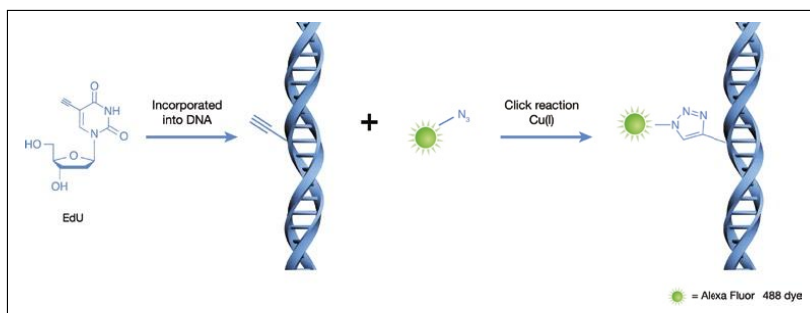


Figure 14 | Illustration of the "Click" reaction between the Alexa fluor 488 azide and the ethynyl group of the EdU molecule incorporated in the DNA. (from <http://www.lifetechnologies.com/>)

In this chapter we conducted a primary approach of the use of EdU incorporation in measuring cell proliferation in *A. pallida* either during normal conditions or after bleaching. In normal conditions, particular interest has been given to the evaluation of EdU incorporation depending the time of the day. The autotrophic symbiotic nature of hermatypic cnidarians and the possible diel rhythmicity of photosynthates transfer [58] suggest that cell proliferation may present a circadian variation. For this preliminary assessment of the effect of bleaching on cell proliferation we opted for the use of a cold-shock protocol in order to trigger *Symbiodinium* expulsion. This protocol was chosen for its simplicity and its recognized efficacy [358]. In addition to the evaluation of cell proliferation, we also conducted mucocyte staining using a mucus-specific fluorescent probe [302]. The subsequent results would give us a first insight in mucocyte density variation following loss of

Symbiodinium and would bring first clues about a hypothetical heterotrophic shift.

Material and Methods

Biological material

2 A few dozen of *Aiptasia pallida* were initially sampled in the Dubuisson public aquarium of the University of Liège. Specimens were then kept during several weeks in artificial seawater (Reef Crystals, Aquatic systems, France) in order to multiply and form a multi-clonal population of anemones. During this period, they were exposed to light following a daily cycle of 12 hours/day (from 7h00 to 19h00) at an intensity of 30-50 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. An electronic system (Dupla T-Control Delta, Dohse Aquaristik, Germany) was used to maintain the water at a constant temperature of $26 \pm 0.2^\circ\text{C}$. *Artemia* shrimps were fed to the anemones on a weekly basis, except during experiments.

Experimental treatment

In order to assess cellular proliferation, anemones were incubated in a solution of the thymidine analogue 5-ethynyl-2'-deoxyuridine (EdU; Invitrogen, Eugene, Oregon, USA) in artificial seawater. Preliminary tests were first conducted to assess the optimal concentration to be used. The ideal concentration having been fixed at 10 μM , this technique was then utilized for 2 different experiments, one evaluating the circadian variations of cell proliferation and the other measuring the effect of bleaching on this proliferation.

To study the circadian variations, anemones were grouped by 5 in 12 beakers and placed in an experimental tank reproducing the same conditions as in the stock aquarium. Each group was successively

incubated in EdU for 2 hours, thus covering a complete 24-hour cycle. After incubation each specimen was briefly anesthetized in a 1:1 solution of seawater and 0.37 M MgCl_2 before fixation in seawater containing 4% paraformaldehyde.

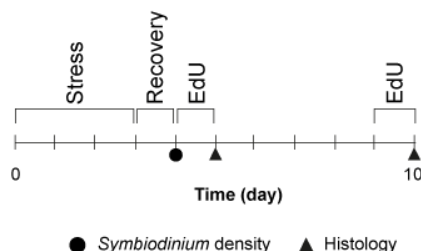


Figure 15 | Summary of the cold-induced bleaching experiment.

For the bleaching experiment, algae expulsion was induced by a cold shock treatment [358]. This treatment implied two successive exposures of anemones to a water at 4°C in the dark. The 2 exposures were separated by an interval of 20 hours during which the anemones were incubated in the dark at room temperature (20°C). Following the second exposure, the anemones were again incubated in the dark at room temperature for 44 hours. Once returned to stock aquarium conditions, the anemones were separated into 3 groups and allowed to recover for 24 hours (see Fig. 15). Anemones of the first group (N = 5) were fixed in a 30% formalin solution and used for evaluation of their *Symbiodinium* density. They were dried in absorbent paper and weighed using an analytical scale. They were then crushed in a glass potter with a precise quantity of filtered seawater. The solution was then subjected to several counts under the microscope using a haematocytometer. Using the weight of the anemones and the quantity of water used, we calculated the density of *Symbiodinium* per mg of fresh tissue. These results were compared with densities measured in healthy anemones collected from the stock aquarium on the same day (N = 5). Anemones of the 2 other groups were incubated

for 24 hours in EdU at different times after the recovery period. One group of these anemones (N = 13), together with control anemones freshly isolated from the stock aquarium (N = 10), was incubated directly after the recovery period. The remaining anemones (N = 15) were incubated 5 days after the end of the recovery. After incubation in EdU (48 hours and 7 days after the end of the stress treatment), all anemones were anesthetized with MgCl_2 and fixed in paraformaldehyde.

Tissue histology

Histological techniques were used to evaluate EdU incorporation as well as to identify and count mucocytes. Following fixation, anemones were dehydrated through a series of alcohol bathes with increasing concentrations of ethanol followed by 2 bathes in Neoclear® (Merck, Millipore International). Samples were subsequently imbedded in paraffin (paraplast Xtra, Sigma), cut into 5 μm -thick slices and mounted on silane-coated slides. After paraffin removal and re-hydration the slides were washed in Phosphate-Buffered Saline (PBS; 3.82 g/L $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$; 10.48 g/L NaHPO_4 in 0.45 M NaCl). The slides were then incubated for 10 minutes in a 3% solution of bovine serum albumin in PBS and 20 minutes in a 0.5% solution of Triton x-100 in PBS. After 3 more PBS washes, the slides were incubated for 30 minutes in the “Click-iT” revealing solution (Click-iT EdU Alexa Fluor 488 Imaging Kit, Invitrogen, Eugene, Oregon, USA). The slides were then washed 3 more times in PBS prior to incubation of 15 minutes in a 5 μM WGA (wheat germ agglutinin + Alexa 594, Invitrogen, Eugene, Oregon, USA) in PBS to label mucocytes. Finally, the slides were washed 3 last times in PBS, dried and mounted for microscopy (Vectashield + DAPI, Vektor labs, Burlingame-California-USA). Slides were examined using a fluorescence microscope (Nikon TE2000-U).

Countings and statistical analyses

Mean densities of EdU+ nuclei and mucocytes were evaluated using Nikon NIS software v3.1. Each value resulted from 5 counts made in transversal sections of tentacles randomly selected for each anemone. For the circadian experiment of EdU incorporation, nuclei counts were reported to the total area of the tentacle. For the bleaching experiment, these counts were reported to the tissue area of the ectodermis. The bleaching inducing algal loss and cell death in the gastrodermis, this method was therefore preferentially chosen to standardize our results. Diminution of *Symbiodinium* densities following bleaching was evaluated using Student t-test. Analyses of variance (one-way ANOVA) followed by Fisher's *post hoc* were used to compare EdU-positive (EdU+) nuclei and mucocyte densities between the bleached groups and the control group. Analyses of variance and Student t-test were both used to evaluate the impact of the time of the day and the presence of light in the number of EdU+ nuclei. These analyses were performed using Statistica v10.

Results

Diel variations of cell proliferation

Histological analyses highlighted variations of the number of EdU+ nuclei within healthy anemone tentacles (Fig. 16). Analyses of variance revealed that this variation is significantly correlated to the time of the day (ANOVA $F(11,48) = 2.95$ and $p = 0.005$). Exposure to light seemed to have no significant direct effect on EdU incorporation ($p = 0.13$). However, further analyses showed that the introduction of a 2 hours lag period, shifting the light period of one group in the data, reveals on significant impact of the light on EdU incorporation ($p = 0.0005$). The density of EdU+ nuclei rises then significantly at 9:00 and

stays high during the day before reaching its maximum at 19:00 and decreasing.

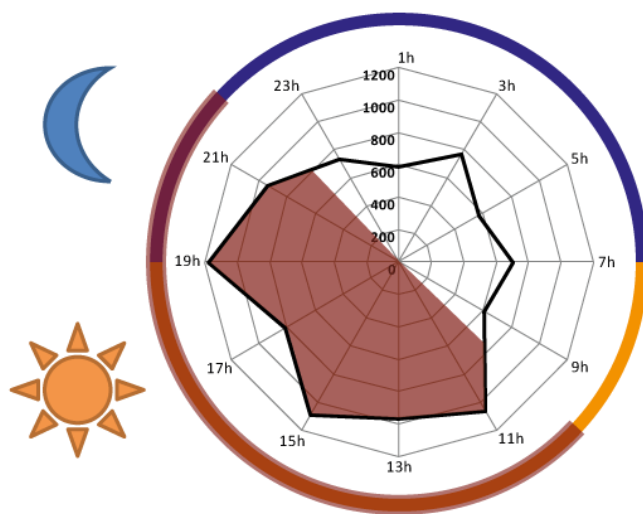


Figure 16 | Diel variations of EdU incorporation. The density of EdU+ nuclei within *A. pallida* tentacles increases significantly 2 hours after exposure to the daylight (red area) before returning to lower values during the night.

Cold Stress and *Symbiodinium* density

The cold stress treatment successfully bleached the anemones, as shown by their apparent loss of coloration. However, expulsion of algae only occurred during the 24 hours recovery period, anemones still showing their characteristic brown coloration after the 48 hours in the dark concluding the stress procedure. Control anemones showed a density of $620 \pm 8.1 \times 10^3$ (mean \pm S.E.M.) algae per mg of fresh tissue while this value dropped to $70 \pm 36 \times 10^3$ after the recovery period (Student t-test, $p < 0.0001$)(Fig. 17).

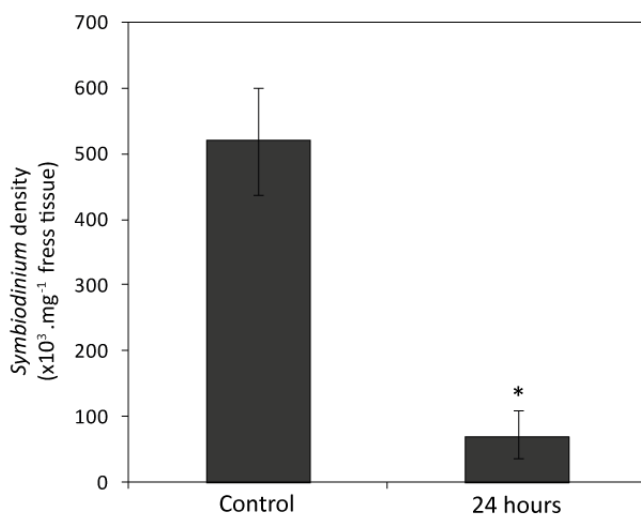


Figure 17 | Loss of *Symbiodinium* following cold-shock stress. *Symbiodinium* density (mean \pm S.E.M.) was lower in stressed anemones than in controls 24 hours after the stress. Asterisks represent values significantly different than controls (Student *t* test; $p < 0.05$).

Cell proliferation in bleached tissues

As for the study of circadian variations, EdU+ nuclei were observed in the tentacles of control anemones, within both the ectodermis and gastrodermis. However, this value differs greatly between the two tissues, with the ectodermis showing 20 times more EdU+ nuclei than the gastrodermis. The cold-stress treatment increased the cell proliferation in both tissues at both time points (Fig. 18A and B). The analyses of variance showed a significant effect of the stress in the ectodermis (ANOVA: $F(2,35) = 7.44$; $p = 0.002$) where the number of EdU+ nuclei rose from 1001 ± 80 nuclei/mm² before treatment to 1418 ± 75 after 48 hours of recovery ($p = 0.0005$). This value was still higher than controls after 7 days of recovery with 1289 ± 91 nuclei/mm² ($p = 0.024$). The effect of the cold-stress was even more pronounced in the gastrodermis ($F(2,35) = 10.89$; $p = 0.00001$) where the number of EdU+ nuclei rose from 42 ± 24 nuclei/mm² in

control anemones to 206 ± 22 nuclei/mm² ($p = 0.000013$) after 48 hours of recovery and 217 ± 27 nuclei/mm² ($p = 0.000024$) after 7 days of recovery.

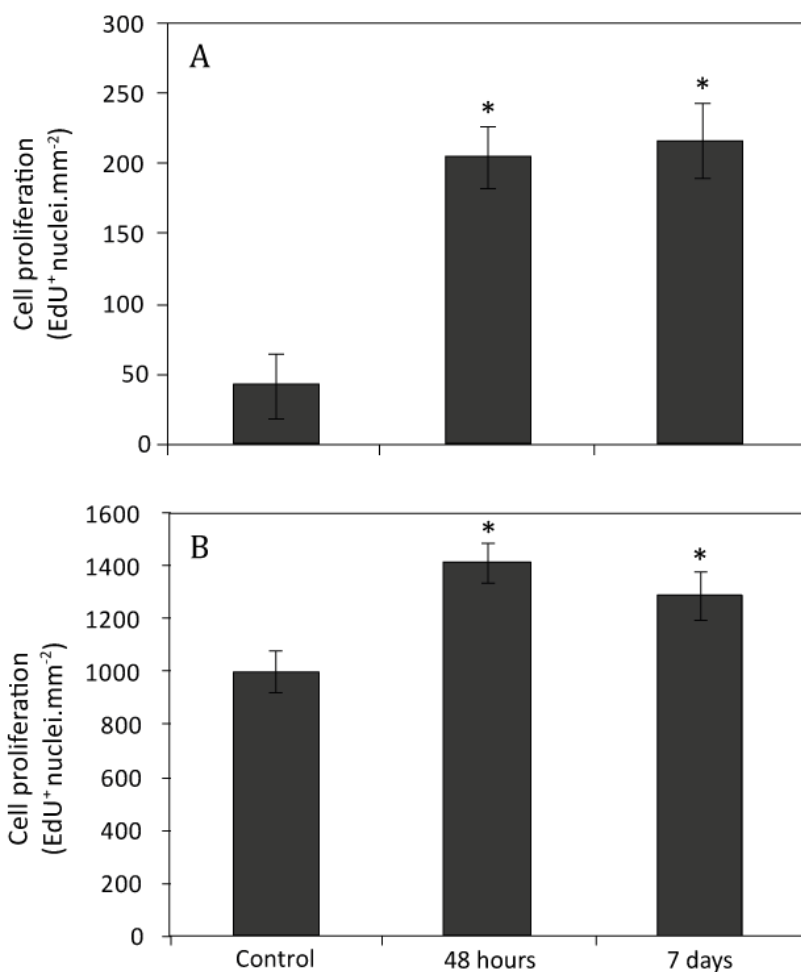


Figure 18 | Increase of cell proliferation after cold-induced bleaching. Cell proliferation (mean \pm S.E.M.) in the gastrodermis (A) and ectodermis (B) shows a rapid increase following the bleaching procedure. Asterisks represent values significantly different than controls following ANOVA and Fisher's *post hoc* test ($p < 0.001$).

Mucocytes

The cold stress had a significant effect on the number of ectodermal mucocytes found within the tentacles ($F(2,30) = 4.83$)(Fig. 19). This effect was however not significant until 7 days of recovery when mucocyte density rose substantially from 133 ± 22 cells/mm² in controls to 240 ± 26 cells/mm² ($p = 0.005$).

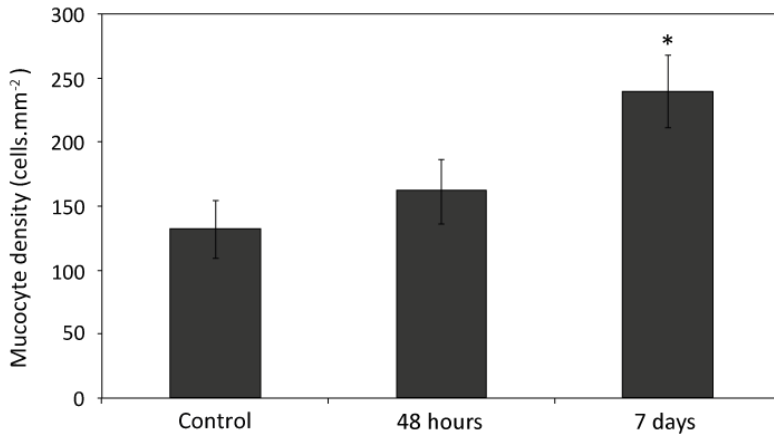


Figure 19 | Increase of mucocyte density after cold-induced bleaching. Mucocyte density (mean \pm S.E.M.) in the ectodermis shows a delayed increase following the bleaching procedure. Asterisks represent values significantly different than controls following ANOVA and Fisher's *post hoc* test ($p = 0.005$).

Discussion

Scientists extensively use the thymidine analogues BrdU and EdU. To date, such molecules have proven their usefulness in tracking cellular proliferation in many types of organisms [97, 129, 300]. Our results showed that EdU was successfully incorporated within *Aiptasia* cells and attested, for the first time, of the efficacy of this newly developed technique in studying cnidarians. Being a thymidine analogue, EdU is incorporated in cell DNA as it replicates during the S-phase of the cell cycle. Therefore, its detection, although indicating

that the labeled cell is mitotically active and proliferating, doesn't highlight the mitotic event *per se*. After DNA replication and EdU incorporation, the cell will enter the G2 phase before ultimately dividing. The duration of this G2 phase seems to be quite variable. While some organisms or cancer cells did not present an apparent G2 phase [151, 244], studies showed that this phase usually lasted only a few hours as demonstrated in cultured human cells [222]. Concerning cnidarians, information regarding the duration of the cell cycle phases are scarce and essentially focused on the *Hydra* model. Such studies demonstrated that the G2 phase could last from at least six hours for the stem cells to 12 hours for the committed precursors and even 24 hours or more for epithelial cells [70, 170]. However, the very specific cell proliferation dynamics of the *Hydra* makes it difficult to compare to *Aiptasia*. Nonetheless, considering the time necessary for the completion of the S phase, it seems appropriate to note that while short EdU incubation times would likely label cells that barely finished DNA replication, longer incubation are more likely to label cells that effectively undergo mitosis.

Our study of the circadian variations of EdU incorporation implied an incubation time of 2 hours. It is therefore very unlikely that these cells have undertaken mitosis or even finished their DNA replication. However, these results showed that DNA replication, and hence, the cycles of host cells were significantly affected by the time of the day as well as the diel light cycle. Light seemed indeed able to upregulate host cell DNA synthesis with a significant increase of this activity two hours after the beginning of light exposure. DNA synthesis stayed high during the day and progressively fell when the lights were shut off. The 2-hour latency between the beginning of the light period and the increase in EdU incorporation could be explained in two different ways. If the host cell reaction to the light is mediated by cellular mechanisms initiated in its own cytoplasm, then this lag could be

solely explained by the time needed for the progression of the cell from G1 to the S phase. Such circadian variations in host physiology have been demonstrated concerning the gene expression of the corals *Acropora millepora* [239] and *Favia fragum* [162] as well as the non-symbiotic anemone *N. vectensis* [312]. Although sharing mammalian rhythm-inducing processes like the heterodimerization of the Clock and Cycle proteins, the cellular components triggering these light-dependent, molecular clock mechanism are yet to be described. On the other hand, the detection of the light could be achieved within the symbiotic algae. The lag could then represent the time needed by light-induced mechanisms engaged by the algae as well as the response of the host cell to such mechanisms. This second hypothesis seems more likely as many studies reported influence of the diel light cycle over *Symbiodinium* algae. The influence of light seems then particularly important determining the progression of the algae through their cell cycle. A recent study demonstrated that cultured *Symbiodinium* isolated from coral undergo mitosis during the night and produce two flagellated daughter cells just before the beginning of the light period [390]. In symbiotic state however, only a small fraction of algae shows a mitotic activity. Studies performed on the anemone *Aiptasia pulchella* and the coral *Astrangia poculata* showed that 3 to 5 percent of *Symbiodinium* multiply within their host [78, 352]. Although most of the newly produced algae seem to be expelled from the host cell [78, 101], they may also have some influence over its cell cycle progression. Such link between the mitosis of the host cell and its algae has been demonstrated in the green *Hydra* [254]. The presence of two *Symbiodinium* cells within the host gastrodermal cells at the beginning of the light period may therefore be an explanation for the increased EdU incorporation detected during the following hours. Moreover, the cell cycle of host cells could be influenced by the diel fluctuation of the translocation of photosynthates from the algae. The circadian variations of *Symbiodinium* derived energetic resources,

such as lipid bodies [58], could be a factor facilitating the progression of host cells from G1 to the S phase. This may be especially true in the ectodermis where the multiplication of the algae has no direct impact.

Our bleaching experiment showed, similarly to the literature [358], that successive exposures to a water temperature of 4°C combined with incubation in darkness evoke rapid expulsion of *Symbiodinium* from *Aiptasia*. After a recovery period of 24 hours following the end of the treatment, anemones showed a striking loss of coloration accompanied by tenfold reduction of their algal density. Moreover, using EdU incorporation we also demonstrated that the rate of cell proliferation in both tissues was significantly affected by the bleaching treatment. In the gastrodermis of bleached anemones, this proliferation could be induced in order to mend the wounded tissue. Indeed, multiple studies demonstrated that, during bleaching, *Symbiodinium* loss is usually linked to the loss of its gastrodermal host cell, either through apoptosis or necrosis [6, 88, 373]. The relation between the bleaching treatment and the increased cell proliferation detected in the ectodermis is however more tricky to determine. This could be explained by the upregulation of the production of specific cell types that play a role in bleaching survival. With the breakdown of the symbiosis, the host loses most of its energy incomes and is threatened by starvation. The production of cells specialized in heterotrophic feeding and prey capture could then help the bleached host facing this menace. Our results also showing an increase in mucocyte density one week after the end of the stress support this hypothesis. Mucocytes and mucus production are indeed one of the key elements of heterotrophic feeding, accumulating particulate organic matter (POM) and carrying it to the mouth of the animal [42, 174].

The duration of this increased cell proliferation, being still significantly higher compared to control anemones after one week, casts doubt concerning the causes of such response. While the loss of algae is the main consequence of the cold stress treatment and is undoubtedly affecting the host, this treatment could also be directly affecting the host. These first results of the host histological response to bleaching are therefore promising but their interpretation is made difficult by the nature of the stress chosen for this first experiment. Further analyses using a bleaching inducing stress procedure that mimics environmental condition seem therefore essential in refining our interpretations.

Chapter 3

Increased cell proliferation and mucocyte density in the sea anemone *Aiptasia pallida* recovering from bleaching.²

Introduction

High sea surface temperature (SST) accompanied by high levels of solar irradiance are known to disrupt the symbiosis between scleractinian corals and endosymbiotic dinoflagellates of the genus *Symbiodinium* (aka coral bleaching). Studies have shown that these environmental factors can act both separately [44, 46, 125, 141, 186, 237] and in combination [131, 167, 232, 235, 394]. Coral bleaching typically involves an impairment of algal photosynthesis and eventually loss of the algae from the host tissue [179, 192, 230, 392] and therefore deprives the host of its main energy source causing a disruption of symbiosis [265]. Consequently, during the weeks following this disruption the nutritional state of the coral is compromised. Depending on the symbiont/host association and the intensity of the stress, the coral could either die [255, 256, 373] or survive [46, 80, 113] through a process of recovery that until now is still poorly characterized.

Cases of coral recovery have highlighted many cellular modifications occurring in the host tissue of the energetically

² This chapter has been published in PLOS one. (cf. [117])

compromised coral. A number of studies have come to conflicting conclusions about the role of mucocytes and mucus secretion in particular. Some hypothesize that mucus production is dependent on *Symbiodinium* for energy supply and carbon input [276, 302]. Indeed a reduction of *Symbiodinium* cell density was induced by shading, eliciting a decrease in mucus production [267, 302]. Other studies have reported increases in mucus release [6, 223, 276] or mucocyte number [132, 223] following bleaching. This variation can be partially explained by inter-specific variation. For example, Lasker et al. [223] showed that mucocyte number could increase or decrease after bleaching depending on the coral species involved.

The mucus has critical functions for coral protection and feeding [42, 138, 342] but also plays a fundamental role of energy carrier in reef ecology [52, 404]. It contains antimicrobial substances controlling the associated microbial community [321] and it stimulates planktonic or benthic microbial activity [104, 404]. Moreover, owing to its adhesive character, attached and/or secreted coral mucus acts as a particle trap and accumulates suspended inorganic and organic particles from the water column, thus supporting the retention and the recycling of essential nutrients within the reef ([404]; see Bythell and Wild [52] for review). Therefore, modifications of the mucus production caused by environmental stress factors related to climate change could have dramatic consequences in organic matter recycling and have the potential to affect the coral reef ecosystem [105, 321].

Although observations concerning mucocytes are important, post-bleaching recovery in hermatypic cnidarians is primarily characterized by the return of pigmentation due to the symbionts within the host tissue. While bleaching can be attributed to a decrease in chlorophyll concentration, it most often implies the loss of algae from host cells [156]. Many studies have focused on the mechanisms

involved in *Symbiodinium* loss [82]. Several scenarios like symbiont digestion via autophagy [85, 91], symbiont expulsion [336, 358] and host cell detachment [45, 127, 341] have been considered [394]. However, most recent findings argue for mechanisms implying the death of host cells either by necrosis or apoptosis [88, 93, 215, 299, 373]. These apoptotic pathways induced by bleaching events were first reported in the zooxanthellate sea anemone *Aiptasia pallida* [93] which is often used as a model cnidarian [63, 229, 268].

Several studies have documented the recovery of corals from bleaching [46, 80, 113], highlighting eventual modifications in the algal community of the host [17, 20, 46, 113, 156, 191]. Although understanding of re-infection mechanisms of healed host tissue is progressing [118], little is known concerning tissular mending [156] or regeneration processes that occur during this recovery. Most knowledge on tissue regeneration comes from studies on *Hydra*. In that model organism, tissue regeneration requires the cooperation of three stem cell populations: ectodermal epithelial stem cells, endodermal epithelial stem cells and interstitial stem cells. The latter provides cells committed to specific differentiation pathways leading to one class of somatic cells: neurons, nematocysts and secretory cells (of which gland cells are only detected in the body column and mucus cells in the head region), (for review see Bode [36] and Galliot and Ghila [121]). In *Hydra* mucus cells are replaced by at least two mechanisms: 1) proliferation of interstitial stem cells followed by their differentiation, and 2) transdifferentiation (no cell division) of gland cells of the body column combined with a translocation to the head region [36, 121]. Some of these pathways and mechanisms are generalized as tissue regeneration of sea anemones. However strong differences exist between these model organisms, among those is the presence of mucus cells in the tentacles of *A. pallida* but not in *Hydra* [36, 121].

In the present study we try to clarify some of the histological modifications induced by bleaching stress in cnidarians. We hypothesize that new host cells rapidly replace cells lost during bleaching in order to regenerate the damaged gastrodermis. To address our hypothesis, we investigate the cellular proliferation following bleaching stress (high temperature combined with high irradiance) in the zooxanthellate sea anemone *Aiptasia pallida*. Our results provide insight in changes occurring in the gastrodermis and ectodermis as well as mucocyte dynamics following thermal stress and bleaching, with important potential insights into the response of reef-building corals to similar challenges.

Material and Methods

Biological material

Aiptasia pallida specimens were collected in the public aquarium of the University of Liège. Individuals were kept in artificial seawater (Reef Crystals, Aquatic systems, France) for several weeks providing a multi-clonal population of anemones. Light was provided on a daily cycle of 12 hours/day at an intensity of 30-50 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. The temperature in the aquaria was electronically controlled with a Dupla T-Control Delta (Dohse Aquaristik, Germany) in combination with a cooling unit (Titan 150, Aqua Medic, Germany) to ensure a constant temperature of 26 ± 0.2 °C. *A. pallida* were fed weekly with frozen *Artemia* shrimps, except during experiments.

Induction of bleaching by thermal/photic stress

Twenty-four hours before the beginning of the experiment, sea anemones were placed in Petri dishes in which the water was constantly renewed by a flow-through mechanism using a peristaltic pump. Anemones were maintained at control conditions or subjected

to a stress treatment (adapted from Bhagooli and Hidaka [34]). The stress treatment consisted of a 30 hours exposure to 33°C and illumination of approximately 1900 $\mu\text{mol photons.m}^{-2}.\text{s}^{-1}$ (measured in the Petri dishes using a Submersible Spherical Micro Quantum Sensor (Walz, Germany) connected to a LI-250A Light Meter (Li-Cor, USA)) produced by led bulbs (12W, 6000K, Elix Belgium). Such light intensity has previously been detected in the field [43] and the combination of temperature and high irradiance is widely used to induce loss of symbionts or bleaching in cnidarian hosts [163, 298]. All anemones were then returned to delimited parts of the same experimental aquarium and allowed to recover under normal conditions (see Biological material). The first group of anemones (one day post-stress group) was incubated immediately after the stress in EdU-containing seawater for 24 hours, a second group (one week post-stress group) was incubated for 24 hours in EdU-containing seawater at the 6th day after the end of the stress, a third group at the 20th day (3 weeks post-stress group) and the last group at the 55th day (8 weeks post-stress group). Anemones that were not subjected to stress were sampled and incubated in EdU-containing seawater for 24 hours at the same time points as the stressed groups (1 day, 1 week, 3 weeks and 8 weeks post-stress) and served as controls (N = 10-17/time point). In addition, anemones (pre-stress group) were also sampled and incubated in EdU-containing seawater before stress conditions commenced. Sampling was performed at the same time of the day for each group.

***Symbiodinium* identification and population density**

The dominant *Symbiodinium* type from our pool of *A. pallida* was identified as a clade B1 by denaturing gradient gel electrophoresis (DGGE) and sequencing of the internal transcribed spacer region 2 of the ribosomal DNA (ITS2 rDNA). Following DNA extraction with a

DNeasy Plant Mini Kit (Qiagen, Netherlands), the ITS2 rDNA region was amplified using the forward primer 'ITSintfor 2' (5'-GAATTGCAGAACTCCGTG-3') and a reversed primer with a GC-clamp 'ITS 2 clamp' (5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCGCCCCGGGATCCATATGCTTAAGTTCAGCGGGT-3') producing a fragment size of 330-360 bp. Amplification products were screened for polymorphisms using DGGE (Biorad DCode system) and run on acrylamide gels (30-65 % gradient) following the manufacturer's instructions (Biorad Laboratories). Dominant bands were excised, re-amplified and subsequently sequenced at the Australian Genome Research Facility University of Queensland, Australia) using an ABI 3730x/ sequencer in combination with BigDye Terminator sequencing reaction kits. Sequences were then examined using Codoncode Aligner version 3.5.3. (Codoncode Corporation) and identified by BLAST comparisons in GenBank.

Bleaching was estimated in each set of experimental groups using a coral health chart, as usually done on coral reefs to grossly assess the health of coral colonies during diving. In addition, to confirm that bleaching resulted from a loss of *Symbiodinium* cells as previously described in *Aiptasia* sp. [93] we evaluated *Symbiodinium* density in tentacle sections of the pre-stress and bleached anemones during the recovery period (N = 3/time point).

Tissue histology

Histological techniques were used to evaluate the cellular proliferation and the number of mucocytes in control and bleached anemones isolated at each time point (N = 10-17/time point). Cell proliferation assays consisted of counting nuclei which incorporated thymidine analogue during DNA synthesis. To do so, each anemone was incubated for 24 hours in a solution of 1 μ M EdU (5-ethynyl-2'-deoxyuridine, thymidine analogue, Invitrogen, Eugene-Oregon-USA) in

seawater [291]. Anemones were then anesthetized for 15 minutes in a 1:1 solution of seawater and 0.37 M MgCl_2 before fixation in a solution of 4% paraformaldehyde in seawater. Fixed specimens were subsequently dehydrated, embedded in paraffin (paraplastXtra, Sigma), cut into 5 μm -thick slices and finally placed on silane-coated slides. After dissolution of the paraffin and re-hydration, the slides were washed 3 times for 5 minutes in Phosphate-Buffered Saline (PBS; 3.82 g/L $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$; 10.48 g/L Na_2HPO_4 in 0.45 M NaCl). Then the slides were incubated for 10 minutes in a blocking solution of 3% Bovine Serum albumin 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 3 PBS washes for 5 minutes 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). After 3 washes for 5 minutes in PBS the slides were incubated for 15 minutes in a 5 μM solution of WGA (wheat germ agglutinin + Alexa 594, Invitrogen, Eugene-Oregon-USA) in order to label the mucocytes [302]. Finally, the slides were washed 3 times for 5 minutes in PBS, dried and mounted for microscopy (Vectashield + DAPI, Vektor labs, Burlingame CA, USA). Slides were examined under a fluorescence microscope (Nikon TE2000-U). Omission of Click-iT solution during the revelation step and detection of fluorescence in anemones that were not incubated in EdU were used to verify the specificity of fluorescent signals. Some sections were also observed following standard hematoxylin/eosin staining procedures to visualize *Symbiodinium* cells using transmitted light microscopy.

Analyses and Statistics

Mean densities of *Symbiodinium*, EdU+ nuclei and mucocytes were calculated from 5 counts made in randomly sampled tentacle

3

sections of each anemone using Nikon NIS software v3.1. The numbers of *Symbiodinium*, EdU+ nuclei and mucocytes were reported to the tissue area [6, 138, 302]. The ectodermal area, rather than the entire tissue area, was used to standardize counts because bleaching is known to affect the gastrodermis due to algal loss and cell death [93]. Counting the total number of ectodermal nuclei was not possible because nuclei were too tightly packed to distinguish individual nuclei. At each time point, cell proliferation was obtained by dividing the density of EdU+ nuclei of stressed anemones by that of control anemones. Statistical analyses were performed using Statistica v10. *Symbiodinium* densities were analyzed at each time point using Student t-test. Analyses of variance (one-way ANOVA) followed by Dunnett's *post hoc* test were used to compare ratios of cell proliferation and mucocyte densities after stress to pre-stress ratio.

Results

Population density of *Symbiodinium*

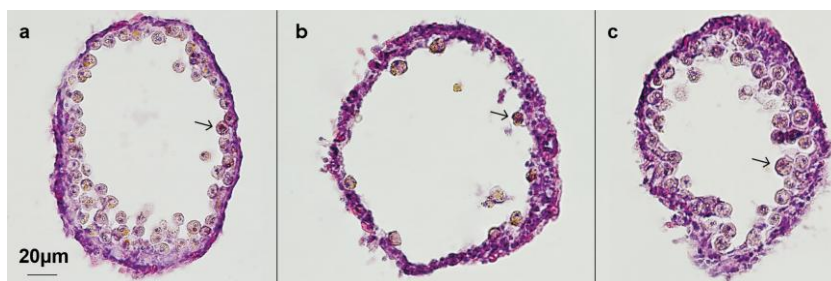


Figure 20 | Transient reduction of *Symbiodinium* density following photic/thermic stress. H&E stained transversal sections of tentacles illustrating *Symbiodinium* (arrows) density in the gastrodermis of anemones before the bleaching procedure (a) and after 1 week (b) and 8 weeks (c) of recovery. After 8 weeks, the gastrodermis has regained its normal appearance.

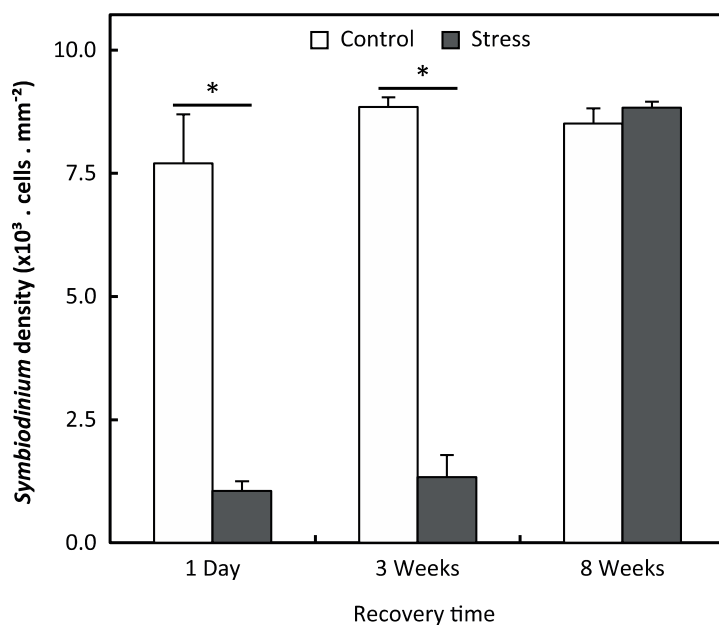


Figure 21 | Loss of *Symbiodinium* following photic/thermic stress. *Symbiodinium* density (mean \pm S.E.M.) was lower in stressed anemones than in controls 24 hours and 3 weeks after the stress. No difference between groups was detected 8 weeks after stress. Asterisks represent values significantly different than controls (Student *t* test; $p < 0.05$).

The light and temperature treatment successfully bleached anemones as observed by evaluating the color of anemones before and after the induced stress using the coral health chart (coral watch) as reference. Similarly variations of algal densities after stress were also detected in hematoxylin/eosin stained sections of tentacles (Fig. 20). A quantitative confirmation that bleaching was caused by loss of algae was obtained by measuring *Symbiodinium* density before and after stress (Fig. 21). Control anemones showed a similar density (about 8×10^3 algae per mm^2 of ectodermal area; mean \pm S.E.M.) at each time point after photic/thermic stress. Twenty four hours after the stress, anemones of the stress group showed a density ($1.1 \pm 0.2 \times 10^3$ algae. mm^{-2}) significantly lower than controls ($7.7 \pm 1.0 \times 10^3$ algae. mm^{-2} ; Student *t* Test; $p = 0.0003$). *Symbiodinium* densities were still

lower ($p = 0.00001$) in stressed anemones 3 weeks after the stress ended ($1.3 \pm 0.5 \times 10^3$ and $8.9 \pm 0.2 \times 10^3$ algae. mm^{-2} , respectively). *Symbiodinium* densities were similar in control and stress groups 8 weeks after the end of the stress (Fig. 2) with a density of $8.5 \pm 0.3 \times 10^3$ and of $8.8 \pm 0.1 \times 10^3$ algae. mm^{-2} , respectively ($p = 0.16$).

Proliferation of cells within ectoderm and gastrodermal tissues.

Histological analyses revealed that EdU was detected in tentacle tissues. Histological controls confirmed that fluorescent signals corresponded to EdU labeling in tentacle tissues and not to cellular autofluorescence or methodological artifacts due to the protocol of EdU revelation.

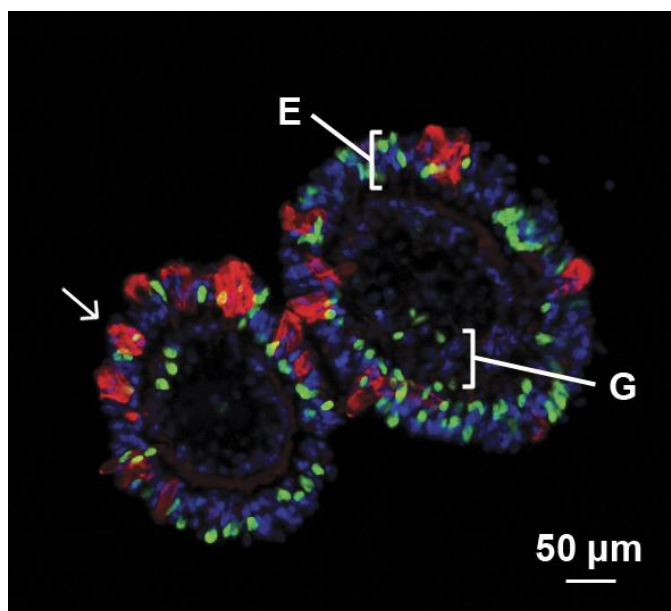


Figure 22 | EdU and WGA labeling. Transversal section of a tentacle showing histological labeling of EdU⁺ nuclei (green) and mucocytes (arrow) stained with WGA (red). DAPI staining (blue) was used to visualize nuclei. E, endodermis; G, gastrodermis.

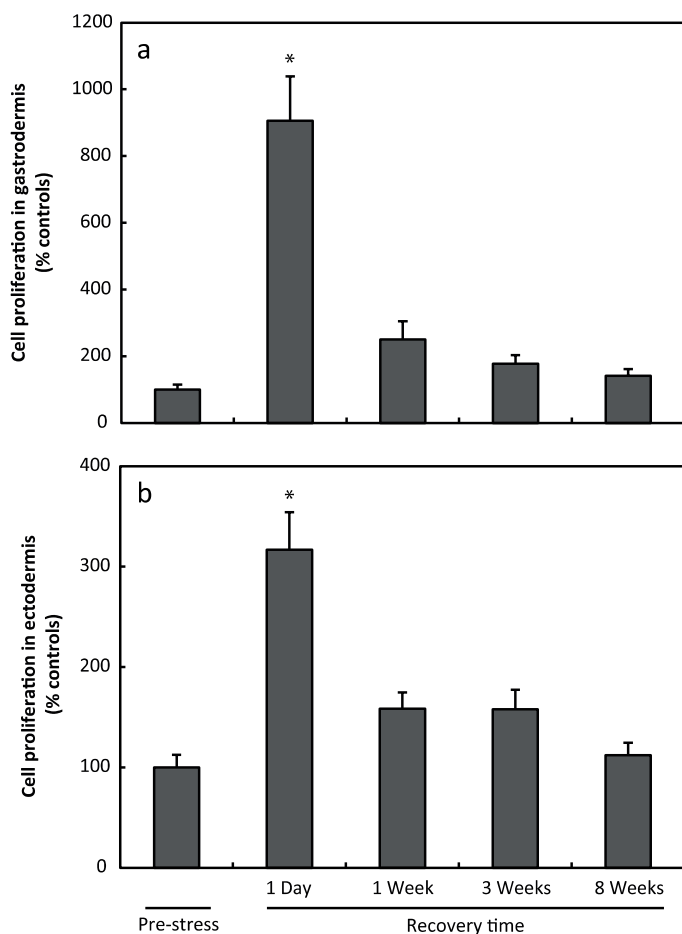


Figure 23 | Increase of cell proliferation after heat/light-induced bleaching. Cell proliferation (mean \pm S.E.M.; EdU+ cell density in treated anemones divided by EdU+ cell density in controls) in the gastrodermis (a) and ectodermis (b) shows a rapid and transient increase following the bleaching procedure (N = 10-17/time point). Asterisks represent values significantly different than pre-stress values following ANOVA and Dunnett's *post hoc* test ($p < 0.001$).

Under normal conditions (i.e., in anemones of the pre-stress group) EdU+ cells were observed in both the gastrodermis and the ectodermis. However, the number of EdU+ cells strongly differed between these tissues (Fig. 22). The number of EdU+ cells in the ectodermis of control anemones was about 16-fold higher than in

their gastrodermis. Similarly, in stressed anemones, higher numbers of EdU+ cells were also observed in the ectodermis compared to the gastrodermis.

To assess the effects of stress on cell renewal in the gastrodermis and the ectodermis, we determined the ratio of EdU+ cell densities between stressed and control tissues at each time point. In the gastrodermis of bleached anemones, a rapid increase of cell proliferation ratio was observed following the stress period (Fig. 23a). During the first day following the bleaching stress, cell proliferation increased to 905 ± 133 % of controls (mean \pm S.E.M.). After 1 week, the ratio was down to 250 ± 54 % and remained low at 3 weeks and 8 weeks after stress (177 ± 26 and 141 ± 21 % of controls, respectively). ANOVA ($F(4,62) = 21.350$) followed by Dunnett's *post hoc* test confirmed that cell proliferation ratio was significantly higher immediately after stress, at the beginning of the recovery phase than before stress ($p < 0.001$). In the ectodermis, we observed the same trend as in the gastrodermis, that is, a transient increase in cell proliferation ratios after stress (Fig. 23b). Immediately after the stress was induced the cell proliferation (317 ± 38 % of controls) was slightly higher than when measured before stress. One week, 3 weeks and 8 weeks after stress, the ratios (158 ± 16 , 158 ± 20 and 112 ± 13 % of controls, respectively) were close to those measured in anemones of the pre-stress group. Statistical analyses revealed that cell proliferation in the 1 day post-stress group was significantly higher than in the pre-stress group ($F(4,62) = 14.628$; Dunnett's *post hoc* test, $p < 0.0001$).

Mucocytes

Although the number of ectodermal mucocytes varied between batches of anemones used for each experiment (73 ± 27 to 437 ± 154 cells.mm⁻²), it remained similar in all control anemones (controls of

the pre-stress group as well as controls of 1 day, 1 week, 3 weeks and 8 weeks post stress groups) within a given experiment. The ratios of mucocyte densities were similar in the ectodermis of anemones before stress, 1 day and 1 week after stress. However the ratio was higher 3 weeks after the end of the bleaching stress (184 ± 22 % of controls; $F(4,60) = 7.822$; $p < 0.0001$). No significant difference was observed 8 weeks after stress (Fig. 24).

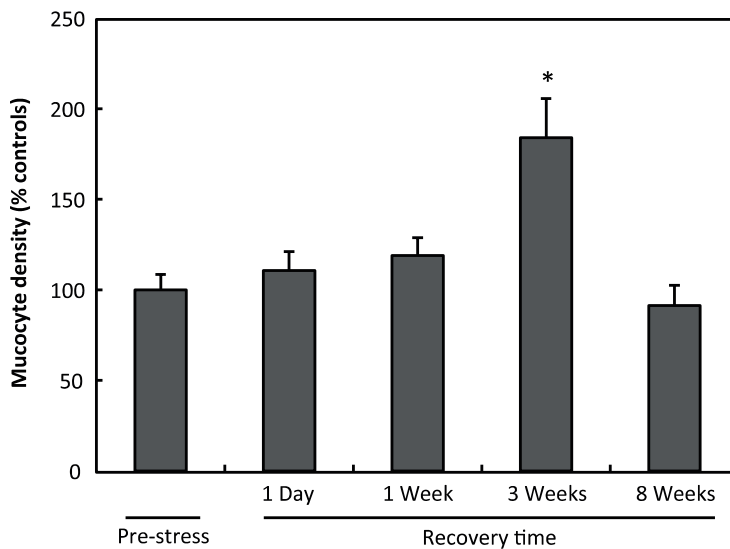


Figure 24 | Increase of mucocyte density after heat/light-induced bleaching. Mucocyte density (mean \pm S.E.M.) in the ectodermis shows a delayed and transient increase following the bleaching procedure (N = 10-17/time point). Asterisks represent values significantly different than pre-stress values following ANOVA and Dunnett's *post hoc* test ($p < 0.0001$).

Discussion

We explored an important step in tissue regeneration occurring in cnidarians following an exposure to high temperature and irradiance leading to a transient disruption of symbiosis with *Symbiodinium* algae. We focused on histological modifications taking place in *Aiptasia pallida* during this recovery period.

Recent studies on mechanisms involved in the loss of symbiotic dinoflagellates during coral bleaching highlighted that this loss is potentially related to loss of gastrodermal host cells, leaving the gastrodermis heavily damaged [127, 341]. Here, we show that cellular proliferation was significantly enhanced in the gastrodermis following bleaching in the sea anemone *Aiptasia pallida*. This suggests that a massive cellular proliferation rapidly occurs in response to stress. The exact timing of initiation of this response is uncertain. Cell proliferation could be triggered at the beginning, during or at the end of the induced stress, when conditions return to normal. In any case, cell proliferation returned to normal levels one week after the induced stress, suggesting that the gastrodermis has recovered from its stress induced cell loss. Indeed, a large part of the gastrodermis can remain healthy, despite the bleached state of anemones [93]. Therefore, a normal level of cell proliferation may suffice to rebuild the gastrodermal cell layer with time. Studies of cell turnover in the gastrodermis may clarify this suggestion.

Cell proliferation increased immediately following bleaching in stressed anemones, most likely in order to regenerate the damaged tissue and eventually regain the symbiotic state with algae. New *Symbiodinium* cells in recovering anemones were sourced from the proliferation of those that remained in the bleached hosts or were recently expelled from them as the seawater used in our experiments was artificial, regularly renewed and thus lacked any live *Symbiodinium*. These observations complement results of other studies reporting on the loss of host cells during bleaching [88, 93, 373] and suggest that gastrodermal regeneration may represent an important step in the recovery process of bleached cnidarians.

Surprisingly, our results also show an increase in cell proliferation in the ectodermis of bleached anemones. Although this increase is less

striking than that observed in the gastrodermis, with values 3 times higher in the ectodermis one day after bleaching stress than in the control experiment, this observation was unexpected because the ectodermis of *A. pallida* is reported to suffer only little damage following bleaching [93]. Cell proliferation in the ectodermis is generally relatively high, even under normal conditions, suggesting a large potential to recover from the relatively limited damage induced by stress. It is therefore unlikely that our observations are solely related to regeneration processes. A plausible additional explanation would be an augmentation in the production of cellular phenotypes that potentially improve the survival of the bleached host. Here, we focus on mucocytes, a cell type that has often been reported to be crucial for the holobiont (host animal and symbionts)[52], but whose response to bleaching is not yet completely understood and still subject to debate [132, 302].

Although an increase in mucus release has been well documented in stressed corals following various environmental stressors, including heat stress and high irradiance [291] changes in the population density of mucocytes in bleached organisms remain unclear. Lasker et al. [223] found an augmentation of epidermal mucocyte cells in bleached samples of *Favia fagrum*. Glynn et al. [132] observed divergent results in bleached corals. Bleached samples of *Pavona clavus* presented an increase in mucous secretory cells of the epidermis but samples of *P. gigantea* and *P. varians* had fewer mucous secretory cells compared to the healthy samples. More recently Piggot et al. [302] reported that the number of epidermal mucocytes in *Montastraea annularis* diminished when shading increased and, conversely, was higher in samples obtained during a seasonal increase in sea surface temperature. Both conditions resulted in a reduction of algal density. All these observations suggest

that factors, such as host species identity and the nature of the stress, may influence the number of epidermal mucocytes.

We found that mucocyte densities range between 70 to 440 cells.mm⁻² in *A. pallida*. These values seem relatively consistent with mucocyte density reported in the epidermis of coral species, ranging from 220 to 3000 cells.mm⁻² depending on the host species and light conditions [138, 302]. The coral *Mycetophyllia reesi* harbors about 3000 mucocytes.mm⁻² of epidermis [138] while *Montastraea annularis* showed seasonal variation of mucocytes densities from 220 cells.mm⁻² during spring to 1750 cells.mm⁻² during summer [302]. We found that epidermal mucocyte density was slightly low in the sea anemone *Aiptasia pallida* in absence of stress. This density may be explained by intrinsic differences between sea anemones and coral species or by the low level of light intensity used in culture (30-50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) [302].

The mucocyte density was affected by a combination of hyperthermia and increased irradiance. We did not observe a decrease in mucocyte density, which is to be expected if the host is energetically impaired immediately following stress. It is possible that such changes occurred during the induced stress and that by sampling one day after the stress such reduction in mucocyte density was overlooked if recovery was rapid. However, we observed a significant and transient augmentation of mucocyte density in the ectodermis 3 weeks after bleaching. The lag period observed between the cell proliferation peak and mucocyte density peak could account for the time needed to produce mature mucocytes, that may even have been produced outside of the tentacle as secretory cell precursors as seen in *Hydra* [349]. Another plausible and non-exclusive explanation for this delay is that differentiation of new cells into mucocytes are only engaged after a certain threshold (depletion of lipid stores [11] or

other physiological signals [406]) that was not yet reached one week after bleaching induction.

By producing and secreting mucus, mucocytes contribute to important roles in the holobiont such as: UV protection, microbial defense, sediment cleansing, energy carrying and particle trapping [11, 42, 52, 138, 241, 242, 276, 342]. In the bleached anemone, the ability of mucus to trap particles and carry them to the hosts' mouth is highly profitable. Therefore, even if the host is energetically impaired, increased production of mucocytes and mucus are in fact a helpful strategy. Heterotrophic feeding can sustain the hosts' energy incomes and compensate for a reduction of algal autotrophic contribution [42, 149, 276]. This idea is corroborated by a recently developed model in which autotrophy significantly offsets effects of bleaching principally by restoring lipid stores inside host cells [11]. Augmentation of mucus production as such, potentially reflects a strategy to limit photoinhibition in algae and subsequent production of oxidative radicals [406] or help to protect the bleached, and thus more susceptible, host against UV radiation or pathogens [42, 276]. Conversely, when stressed anemones have recovered *Symbiodinium* densities similar to controls (at 8 weeks after stress), mucocyte densities in stressed anemones were also similar to controls, arguing for a relationship between mucocyte and *Symbiodinium* densities in *Aiptasia*.

Mucocytes are not the only cell type derived from the increased proliferation in the ectodermis. Indeed, the density of EdU+ cells was several fold higher than the density of mucocytes (see Fig. 3). Among those EdU+ cells, a small number will differentiate and mature in mucocytes. Some of those newly produced ectodermal cells may differentiate into other cell types such as cnidocytes (aka nematocysts or stinging cells). Increased differentiation into cnidocytes is very likely

in bleached anemones considering their major role in heterotrophic feeding [174]. Some of the EdU+ cells may also migrate to the gastrodermis. Indeed, in *Hydra*, ectodermal cells committed to secretory cell lineages can migrate through mesoglea to the gastrodermis [36]. If similar mechanisms are present in *A. pallida* some new ectodermal cells may migrate to the gastrodermis to participate in gastrodermal regeneration after bleaching.

3 The origin of new cells in the gastrodermis and ectodermis has yet to be identified. Since a short time of incubation in EdU-containing seawater suffices to label cells in both tissue types, it seems likely that cells are produced locally by division of either precursor cells (i.e. cells committed to a lineage) or multipotent stem cells (i.e. interstitial stem cells). Such multipotent stem cells located in the mesoglea have previously been reported during regeneration of damaged tissue [207, 259, 319]. New cells could also differentiate from interstitial stem cells. These cells are located under the ectodermal surface between epithelia-muscular cells and are known to be stem cells producing gametes and other phenotypes, including secretory cells [319]. While EdU+ cells are most likely produced locally, the origin of mature mucocytes remains to be solved. Transdifferentiation and migration of precursor cells from another part of the anemone, such as the tentacle basis or the oral region, cannot be excluded. Further investigation with shorter incubation periods conducted during and directly after the stress treatment will help clarify the origin of proliferating cells as well as mucocytes.

Finally, additional studies are needed to elucidate the trigger of cell proliferation (the induced stress or a consequence of a reduction in *Symbiodinium* density) as well as the nature of the signal itself. The latter question applies specifically to the ectodermis, which is not directly affected by the effects of algal loss.

Chapter 4

Impairment of symbiont photosynthesis increases cell proliferation in the ectodermis of the sea anemone *Aiptasia pallida*.³

Introduction

The ecological success of reef-building corals throughout tropical oligotrophic waters relies on the symbiosis between cnidarians and photosynthetic dinoflagellates of the genus *Symbiodinium* (commonly referred to as zooxanthellae). This mutualistic relationship is established following a multi-step process involving both partners (see [118] for review). Once in symbiosis, the symbiont is intracellularly located within gastrodermal host cells and provides up to 95% of its photosynthetic products to the host [265], thus contributing massively to: respiration, tissue growth, calcification, gamete production and survival [348]. In return, the host ensures protection of *Symbiodinium* from planktonic grazers, provides a source of inorganic nutrients (CO_2 , NH_3 and PO_4^{3-} ; [411]) and a fixed position in the water column allowing an optimal harvesting of light [348]. This mutual relationship continuously adjusts to subtle changes in the environment to optimize the survival of the holobiont (the entity including the host and the symbionts).

Nevertheless, over the last decades, reef-building corals have faced recurrent large-scale bleaching events compromising their

³ This chapter is submitted for publication in Marine Biology.

survival [167, 177, 283]. Currently, it is widely accepted that high sea surface temperature accompanied by high levels of solar irradiance is responsible for the impairment of photosynthesis [392] and the induction of an oxidative stress that ultimately ends with the disruption of the symbiosis between cnidarians and *Symbiodinium* [394]. Among several proposed mechanisms for coral bleaching, the most supported one involves the death of the gastrodermal host cells. This could happen either by the regulated pathway of apoptosis or more abruptly by cell necrosis [93, 299, 373]. In both cases, the gastrodermis of the bleached host is left damaged and partly depleted of symbiont-containing cells.

The loss of symbionts has drastic consequences on the symbiotic cnidarian, which relies on its photosynthetic partners for energy supply. Therefore, when photosynthesis is suppressed or lowered such as during bleaching, the host relies on its own energy reserves (proteins, carbohydrates and lipids) but also on its ability to modify its metabolism and feeding habits. It has been found that in such conditions, several temperate and tropical coral species were able to increase their feeding effort when preys were available [10, 106, 107, 172]. Moreover, studies conducted on the scleractinian coral *Montipora capitata* reported that bleached and recovering specimens were able to meet their daily metabolic energy requirements by markedly increasing their feeding rates and their CHAR (per cent contribution of heterotrophically acquired carbon to daily animal respiration) from less than 20% to over 100% [148, 149]. Such increase in heterotrophy permitted *M. capitata* to maintain its energy income in absence of *Symbiodinium* and to survive during prolonged bleaching events and recovery. These changes in the nutritional balance necessarily involve significant cellular rearrangements. In a recent study, Franolet et al. [117] have demonstrated that, following high seawater temperature combined with high light irradiance, stressed

sea anemones *Aiptasia pallida* showed an increase in cell proliferation in the gastrodermis, which had to recover from the host cell depletion caused by the stress, but also in the ectodermis. These observations led the authors to hypothesize that the increase in cell proliferation observed in the ectodermis could reflect an augmentation of the holobiont heterotrophic capacity due to the loss of endosymbionts [117]. Such modifications could be achieved by increasing the number of ectodermal cells specialized in the nutritional processes, such as cnidocytes and mucocytes [117, 172, 302].

The comprehension of the cellular mechanisms induced by the host to maintain its energy balance is therefore critical to understand how the cnidarians can acclimate to lower autotrophic inputs and recover from bleaching. In this context, the present study aimed to investigate the impact of a reduction of energy supply provided by photosynthetic symbionts on cell proliferation in the tissues of *A. pallida*. To do so, *A. pallida* specimens were treated with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), an herbicide that binds to the acceptor side of photosystem II (plastoquinone Q_B) and inhibits photosynthesis by blocking the electron transport between photosystems II and I (Hill reaction). Then, *Symbiodinium* densities, cell death and cell proliferation in both the ectodermis and gastrodermis were assessed using histological techniques.

Material and Methods

Biological material

Sea anemones *Aiptasia pallida* (Verrill) were collected in the Dubuisson aquarium belonging to the University of Liège. Specimens were maintained in artificial seawater (Reef Crystals, Aquatic systems, France) for several weeks, and thus generated a multi-clonal population of *A. pallida*. Sea anemones were kept in a 12 h/12 h light

cycle at an intensity of 30-50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. The temperature in the aquaria was electronically controlled and maintained at $26 \pm 0.2^\circ\text{C}$ using a Dupla T-Control Delta (Dohse Aquaristik, Germany) in combination with a cooling unit (Titan 150, Aqua Medic, Germany). *A. pallida* were fed weekly with frozen *Artemia* shrimps, except during experimental treatments.

Experimental treatments

Twenty-four hours before the beginning of the experiment, *A. pallida* specimens were placed in individual beakers. Beakers were placed in a tank where water temperature was controlled with the same devices as in stock aquaria. Sea anemones were maintained at control conditions or exposed to seawater containing 20 mM DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea; Sigma-Aldrich) for 1 week. Control and DCMU-containing seawater was renewed on a daily basis. During this period, both control and DCMU-treated samples were exposed to a light intensity of approximately 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, provided by 12 W LED bulbs (6000 K, Elix Belgium) and measured in the bottom of the beaker using a Submersible Spherical Micro Quantum Sensor (Walz, Germany) connected to a LI-250A Light Meter (Li-Cor, USA). One week after the beginning of the experimental treatment, DCMU-treated samples were returned to control seawater and allowed to recover under normal conditions (recovery period; Fig. 25). Five control and five DCMU-treated *A. pallida* specimens were sampled two days, one, two and four weeks after the beginning of the experimental treatments (Fig. 25).

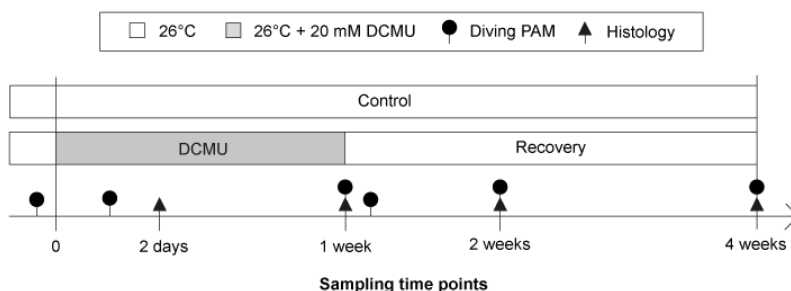


Figure 25 | Experimental treatments and sampling strategy.

Chlorophyll fluorescence measurements

Chlorophyll fluorescence parameters of *Symbiodinium* sp. in hospite of *A. pallida* were measured using a diving-PAM chlorophyll fluorometer (Walz GmbH, Germany). Measurements were always made by placing the fiber-optic of the diving-PAM 1 to 2 centimeters above the apical part of the sample placed in a 3 mL plastic cuvette. After a dark adaptation of 20 min, the initial fluorescence level (F_0) was determined by applying weak modulated pulses of red measuring light. A 1 second saturating pulse of actinic light ($>3000 \mu\text{mol photon m}^{-2} \text{s}^{-1}$) was then applied to measure the maximum fluorescence level (F_M). The maximal photochemical quantum yield was calculated as (F_V/F_M) , where $F_V = F_M - F_0$. Measures were made on samples of the DCMU-treatment groups before exposure to DCMU, 1 and 7 days after the beginning of the treatment, and 1, 7 and 21 days after the beginning of the recovery period (referred hereafter as 8 days, 2 weeks and 4 weeks, respectively; Fig. 25).

Tissue histology

Histological techniques were used to evaluate *Symbiodinium* densities, the density of cells engaged in cell cycle, the number of mucocytes and the extent of cell death in control and DCMU-treated

sea anemones sampled at each time point. Assessment of mitotic cells consisted of counting nuclei which incorporated a thymidine analogue, 5-ethynyl-2'-deoxyuridine (EdU; Invitrogen, Eugene, Oregon, USA) during DNA synthesis. To do so, 24 hours before each sampling time point, 5 control and 5 DCMU-treated samples were incubated in 10 μ M EdU-containing seawater [117, 291]. Samples were then processed for histological analyses as previously described [117]. Briefly, samples were anesthetized in a 1:1 solution of seawater and 0.37 M $MgCl_2$ before fixation in seawater containing 4% paraformaldehyde. Fixed specimens were subsequently dehydrated, embedded in paraffin (paraplast Xtra, Sigma), cut into 5 μ m-thick slices and finally mounted on silane-coated slides. After paraffin removal and re-hydration the slides were washed in Phosphate-Buffered Saline (PBS; 3.82 g/L $NaH_2PO_4 \cdot 2H_2O$; 10.48 g/L $NaHPO_4$ in 0.45 M NaCl). Then the slides were incubated for 10 minutes in 3% Bovine Serum Albumin made in PBS followed by 20 minutes in 0.5% Triton x-100 made in PBS. After PBS washes the slides were incubated 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). After 3 washes for 5 minutes in PBS, the slides were incubated for 15 minutes in PBS containing 5 μ M WGA (wheat germ agglutinin + Alexa 594, Invitrogen, Eugene, Oregon, USA) in order to label mucocytes [117, 302]. Finally, the slides were washed 3 times in PBS, dried and mounted for microscopy (Vectashield + DAPI, Vektor labs, Burlingame-California-USA). Slides were examined under a fluorescence microscope (Nikon TE2000-U). Omission of Click-iT solution during the revelation step and detection of autofluorescence in *A. pallida* that were not incubated in EdU were used to verify the specificity of the fluorescent signal.

Some sections were also observed following standard hematoxylin/eosin staining procedures to visualize *Symbiodinium* cells by transmitted light microscopy and assess their densities.

Another set of slides was used for the assessment of cell death using the TUNEL assay (ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit, Millipore, Billerica, Massachusetts-USA) as previously used on coral [6]. Following paraffin removal and re-hydration, the slides were incubated for 10 minutes in PBS containing 10 μ M Proteinase-K. They were subsequently washed 3 times in deionized water, exposed 5 minutes in a solution of 3% H_2O_2 in order to quench endogenous peroxidases and washed 2 times in PBS before 30 minutes of incubation in equilibration buffer. Slides were then incubated for 1 hour at 37°C with the TdT cocktail. Reaction was then stopped by washing the slides for 10 minutes in the stop/wash solution and 3 times in PBS. After 30 minutes of incubation in Anti-Digoxigenin Conjugate and 4 more washes in PBS, revelation was made using DAB Peroxidase Substrate. The slides were finally washed, counterstained with 0.5% methyl green and mounted for light microscopy using successive bathes of N-butanol and xylene.

Counting and statistical analysis

Mean densities of *Symbiodinium*, EdU+ nuclei and mucocytes were calculated from 5 counts made in randomly sampled tentacle sections of each anemone using Nikon NIS software v3.1. For each tentacle section, the numbers of *Symbiodinium*, EdU+ nuclei and mucocytes were reported to the tissue area according to Fransolet et al. [117]. At each time point, cell proliferation was obtained by dividing the density of EdU+ nuclei of DCMU-treated sea anemones by that of control sea anemones.

Statistical analyses of the data were performed in SigmaPlot 11.0 (Systat Software, USA). The difference between treatments within time points was investigated by using a two-way analysis of variance (two-way ANOVA) followed by Tuckey's *post hoc* test. Differences were considered statistically significant when $p < 0.05$.

Results

Treatment with DCMU inhibited the photosynthetic electron transport through PSII

In order to suppress the energy supplies coming from the *Symbiodinium*, we treated *A. pallida* specimens with 20 mM DCMU, an herbicide that inhibits photosynthesis by blocking the electron transport between photosystems II and I. After 24 hours of treatment, we observed a drastic decrease in the maximal photochemical quantum (F_V/F_M) yield from 0.674 ± 0.003 to 0.051 ± 0.007 (mean \pm S.E.M.; Fig. 26). This decrease was further more accentuated 7 days after the beginning of the exposure to DCMU and F_V/F_M values reached 0.0154 ± 0.005 . After DCMU removal, F_V/F_M values recovered progressively and were similar to pre-treatment values, 2 and 4 weeks after the beginning of the experimental treatment (0.626 ± 0.007 and 0.690 ± 0.003 , respectively; Fig. 26).

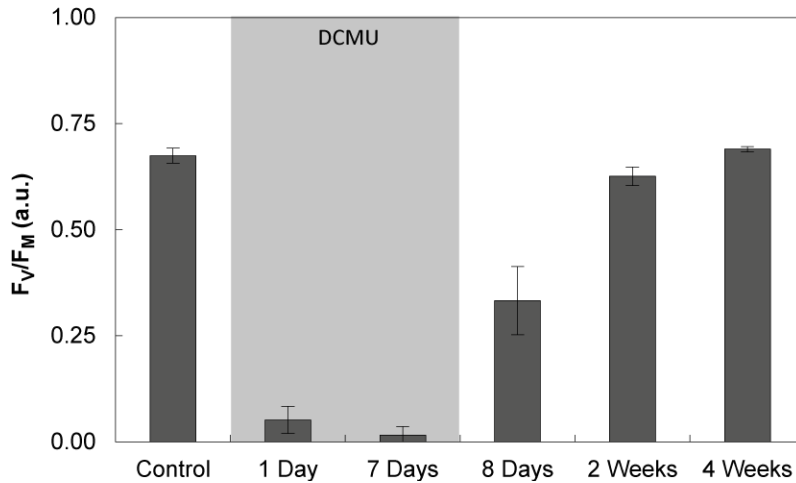


Figure 26 | The maximal photochemical quantum yield (Mean \pm S.E.M.) in *Symbiodinium* of the sea anemone *Aiptasia pallida*, before, during and after treatment with 20 mM DCMU. The maximal photochemical quantum yield was calculated as (F_v/F_m) , where $F_v = F_m - F_0$.

The inhibition of photosynthesis led to bleaching

While *Symbiodinium* density in control samples was similar throughout the experiment, and was comprised between 9.32 and 11.45×10^3 algae per mm^2 of tissue area, it drastically decreased in *A. pallida* specimens treated with DCMU (Fig. 27-28). Indeed, after 1 week of DCMU exposure, *Symbiodinium* density was 5-fold lower than controls and reached $1.9 \pm 0.6 \times 10^3$ algae mm^{-2} (two-way ANOVA and Tuckey's *post hoc* test; $p < 0.001$). Although *Symbiodinium* density partially recovered, it was still lower in DCMU-treated sea anemones 2 weeks after the beginning of the DCMU treatment than in controls ($5.1 \pm 0.5 \times 10^3$ and $9.9 \pm 0.3 \times 10^3$ algae mm^{-2} , respectively; $p < 0.001$). Then, it fully recovered to control values at the end of the experimental treatment ($10.7 \pm 0.6 \times 10^3$ algae mm^{-2} ; Fig. 27).

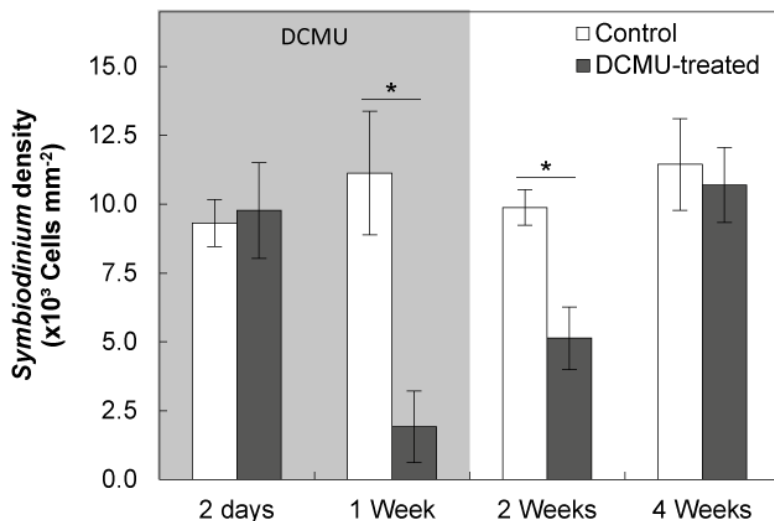


Figure 27 | *Symbiodinium* density (Mean \pm S.E.M.) in control and DCMU-treated sea anemones *Aiptasia pallida*. Asterisk indicates statistically significant differences (two-way ANOVA and Tuckey's *post hoc* test; $p < 0.001$).

TUNEL revealed the presence of a few apoptotic nuclei in both ectodermis and gastrodermis of control and DCMU-treated *A. pallida* (Fig. 28); particularly no difference in TUNEL was detected in the ectodermis 2 days and 1 week after DCMU treatment. Histological analysis also showed numerous healthy *Symbiodinium* in the gastrodermis of control *A. pallida* (Fig. 28A). After 2 days of incubation with 20 mM DCMU (Fig. 28B), most *Symbiodinium* were TUNEL-positive. After 1 week of DCMU treatment, the gastrodermis was depleted of most *Symbiodinium* cells (Fig. 28C), thinning the gastrodermis cell layer. This observation on tentacle sections confirmed the *Symbiodinium* cell density determined tentacle sections (Fig. 27). It also suggested that the drastic loss of *Symbiodinium* was likely related to apoptotic cell death (Fig. 28C).

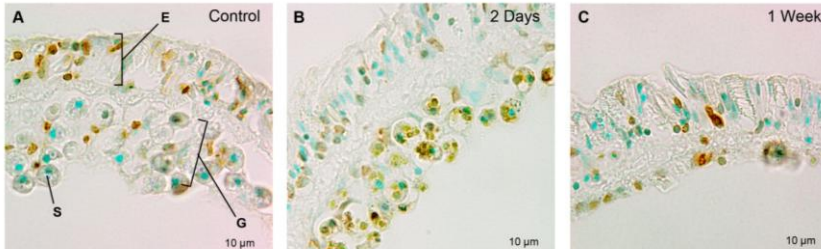


Figure 28 | Apoptotic cell death and *Symbiodinium* density in tentacles of *Aiptasia pallida*, before the treatment with 20 mM DCMU (A), after 2 days (B) and 1 week (C). Apoptotic nuclei, brown; counterstained nuclei, blue; E: epidermis, G: gastrodermis, S: *Symbiodinium*.

Cells proliferated within ectodermal and gastrodermal tissues

EdU+ cells were observed in both the gastrodermis and the ectodermis with a strong difference in their relative numbers. The number of EdU+ cells in the ectodermis of control samples was about 11-fold higher than in the gastrodermis (data not shown). Similarly, in DCMU-treated *A. pallida*, higher numbers of EdU+ cells were also observed in the ectodermis compared to the gastrodermis (data not shown). To assess the effect of exposure to DCMU on cell proliferation in both tissues we determined the ratio of EdU+ cell densities between control and DCMU-treated samples at the different sampling time points (Fig. 29).

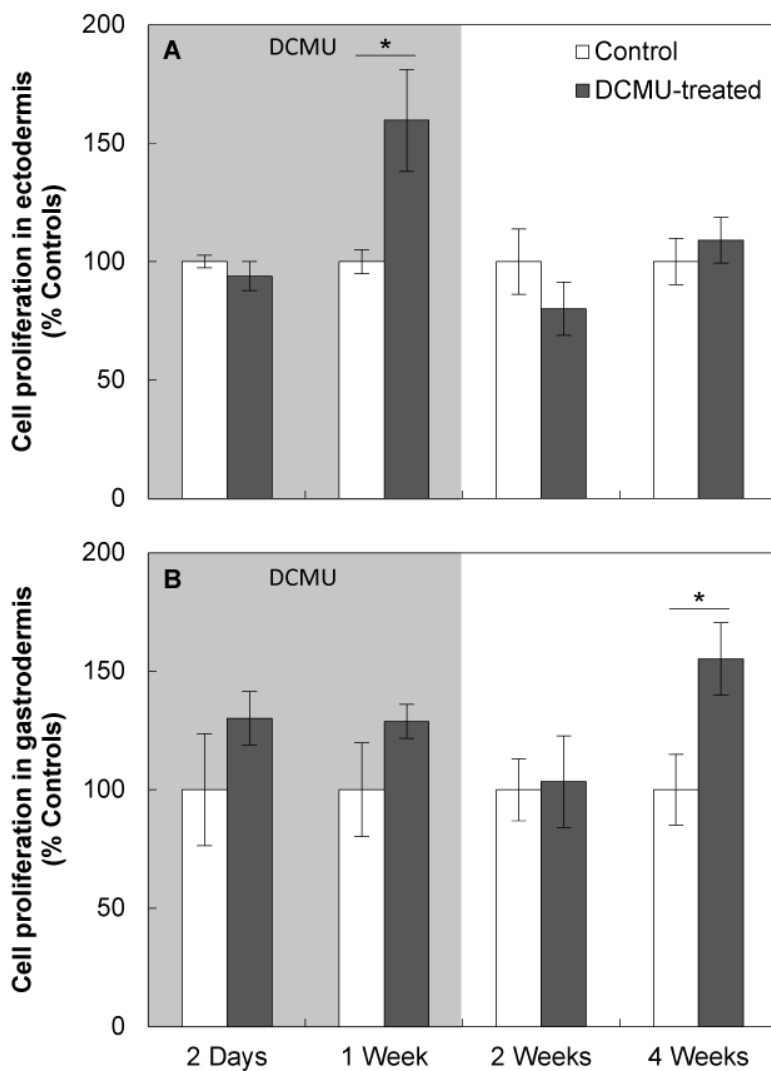


Figure 29 | Cell proliferation (Mean \pm S.E.M.) in the ectodermis (A) and the gastrodermis (B) of control and DCMU-treated sea anemones *Aiptasia pallida*. Asterisk indicates statistically significant differences (two-way ANOVA and Tuckey's *post hoc* test; $p < 0.001$).

In the ectodermis of DCMU-treated samples, EdU+ cell densities were similar to controls 2 days after the beginning of the DCMU

treatment. It increased significantly and reached $160 \pm 5\%$ of controls after 1 week of exposure (two-way ANOVA and Tuckey's *post hoc* test; $p < 0.001$). But then, EdU+ cell density rapidly decreased during the recovery period, reaching values similar to controls after 1 and 4 weeks of treatment. In the gastrodermis of *A. pallida* exposed to DCMU, EdU+ cell densities were similar to controls throughout the experimental treatment, excepted after 4 weeks where EdU+ cell density increased significantly to $155 \pm 15\%$ of controls (two-way ANOVA and Tuckey's *post hoc* test; $p < 0.05$).

Mucocyte density increased after DCMU treatment

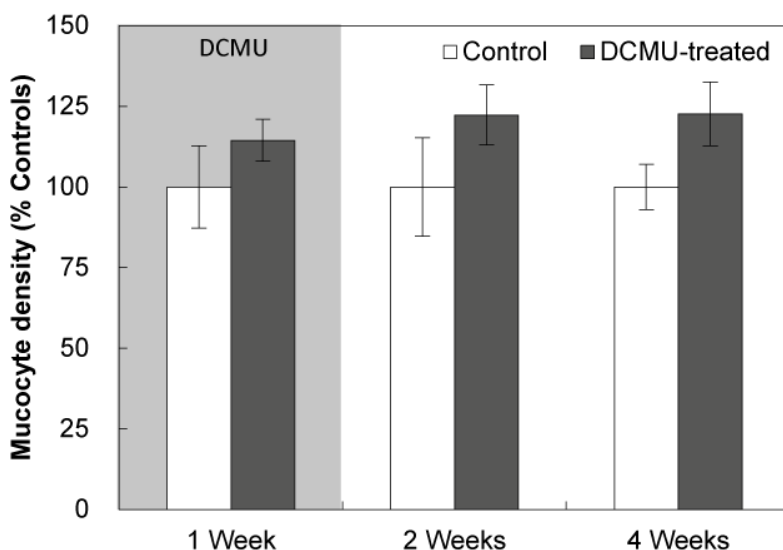


Figure 30 | Mucocyte density (Mean \pm S.E.M.) in the ectodermis in control and DCMU-treated sea anemones *Aiptasia pallida*.

Finally, we assessed the effect of DCMU exposure on the number of mucocytes in the ectodermis by determining their relative densities in control and DCMU-treated samples (Fig. 30). We observed that control values were similar at each time point (about 400 mucocytes per mm^2 of ectodermal area; data not shown) and that the mucocyte

density in the ectodermis was higher in DCMU-treated *A. pallida* than in controls. However, despite statistical analyses revealing an overall effect of treatment (two-way ANOVA and Tuckey's *post hoc* test; $p < 0.05$), at no time point a significant difference was observed.

Discussion

When photosynthesis in *Symbiodinium* cells fails, sea anemones, like other symbiotic cnidarians, likely implement alternative mechanisms to compensate for the lack of autotrophic energy income [149]. For example, sea anemones can change location to find a spot with a better light irradiance, metabolize stored energy reserves and modify their feeding strategy to favor heterotrophy. This latter mechanism takes place in the ectodermis and can involve several aspects of tissue re-organization involving cell recruitment (i.e.: migration, de-differentiation), cell maturation (i.e.: increase in mucus production, differentiation of precursor cells) and/or cell division (i.e.: stem cell division; [134]). Here, we examined how cell proliferation participated in the tissue response to a diminution of photosynthesis efficiency in the sea anemone *A. pallida*.

Photosynthesis efficiency was reduced by tenfold 1 day after the beginning of the treatment with 20 mM DCMU and such reduction persisted for the entire duration of the treatment. One day after the end of the DCMU treatment, photosynthesis efficiency had already recovered about 50% of its pretreatment values (Fig. 26). DCMU treatment also induced a significant drop of *Symbiodinium* density (Fig. 27). This loss occurred after several days of DCMU treatment as illustrated by the same algal density in control sea anemones and samples treated for 2 days. At that time, although algae were still present in the gastrodermis, impairment of photosynthesis had already affected their physiology and many *Symbiodinium* cells were

committed to cell death as illustrated by TUNEL histology (Fig. 28B). After 1 week of DCMU treatment, *Symbiodinium* loss was obvious. Such bleaching effect was not surprising as coral bleaching has previously been reported after exposures to PSII herbicides [193, 194]. Transient inhibition of photosynthesis and the subsequent loss of *Symbiodinium*, placed host cells in a deficit of algae-derived energy input, drastically reducing autotrophic energy supply. In such conditions, bleached and recovering hosts need additional means to obtain their daily metabolic energy. Among these, are the use of stored energy supplies and the enhancement of food capture [149, 172].

We observed that cell proliferation was significantly increased in the ectodermis of DCMU-treated *A. pallida* (Fig. 29A). One week after DCMU treatment, when *Symbiodinium* density and photosynthesis efficiency were at the lowest, the density of EdU+ cells was maximal in the ectodermis. Considering that DCMU treatment did not modify the level of ectodermal cell death, it seems likely that the augmentation of EdU+ cells reflects a response to the reduction of energy supply rather than to a direct damage to the ectodermal tissue. We observed a 150% increase of EdU incorporation within the ectodermis at the end of the stress treatment. This observation contrasts with a previous study where proliferation of ectodermal cells was detected as early as 24 h after exposure to a combination of high temperature and light irradiance [117]. However, in that study, one cannot exclude a direct effect of seawater temperature on the ectodermis such as modifications of cell metabolism (i.e., [87]). However, in the study of Fransolet et al. [117], as in the present study, ectodermal cell proliferation was increased when autotrophic energy supplied by photosynthesis was at the lowest. Grottoli et al. [149] reported that when autotrophic energy supply is drastically reduced the coral *Montipora capitata* could sufficiently increase the rate of zooplankton

capture and the heterotrophic energy income to prevent depletion of energy reserves. Conversely, other coral species such as *Porites compressa* or *Porites lobata* were unable to modify the contribution of heterotrophically acquired carbon [149]. An increase in prey capture was also observed in *Cladocora caespitosa* when cultured in darkness [172]. Surprisingly, changes in heterotrophy efficiency could also occur in *C. caespitosa* placed in high light intensity [172]. In that case, feeding increase mostly served to augment supplies of nitrogen and phosphorus (to allow tissue growth) rather than carbon. To achieve these heterotrophic shifts, ectodermal tissue needs to change its heterotrophic ability through the production of cnidocytes to capture large preys and/or mucocytes to trap particulate food [42, 126, 174, 321]. Our results support this hypothesis, showing that mucocyte density is significantly higher in stressed *A. pallida* following diminution of the photosynthesis efficiency (Fig. 30). Further analyses and study of cell population dynamics are still needed to determine the relative proportion of new cells committed to mucocytes and to cnidocytes as well as their lifespan.

Cell proliferation also increased in the gastrodermis (Fig. 29B). Four weeks after DCMU treatment, EdU incorporation within the gastrodermis was increased by 150%. In this tissue, cell proliferation likely contributed to tissue regeneration following host cell damage due to bleaching. Indeed, many nuclei were positively stained for apoptosis 48 h and especially 1 week after the onset of the DCMU treatment. Such gastrodermal cell death has been observed after/during bleaching episodes in corals and other symbiotic cnidarians [93, 299, 373]. In the present study, *Symbiodinium* density partially recovered at 2 weeks, even before gastrodermal cell proliferation was increased. This suggested that some gastrodermal cells were able to host new algae at this time. Several possibilities exist: (1) some cells, although competent, did not host *Symbiodinium*

cells at the time of the stress and then remained healthy and available for *Symbiodinium* symbiosis ; (2) some cells might have expelled *Symbiodinium* cells before any injury [394] and remained competent to host new *Symbiodinium*; (3) or some cells, like nutritive cells, which are the main cell phenotype of Anthozoans gastrodermis, were able to undergo dedifferentiation and transformation into symbiotic host-cell when phagocytosing competent *Symbiodinium* [71, 134]. Proliferation of these putative host cells would then depend on their encounter with new algae, whose presence within the water could only rely on the release and proliferation of the few that survived the stress. Further studies should examine the cellular origin of cells able to host new *Symbiodinium* cells after stress as well as the fate of new gastrodermal cells.

In conclusion, sustained decrease in photosynthesis efficiency leading to bleaching induces tissue remodeling such as cell proliferation in the ectodermis and gastrodermis of *A. pallida*. Ectodermal cell increase may contribute to a heterotrophic shift to sustain energy demands, which are drastically reduced following the loss of autotrophic carbon source. Such shift could, in part, rely on an increased production of cells, such as mucocytes, that play a role in heterotrophic feeding.

Chapter 5

Trans-tissular migration and mucocyte differentiation of bleaching-induced proliferating cells.

Introduction

Bleaching in hermatypic cnidarians does not simply consist in the expulsion of *Symbiodinium* from its host. Multiple elements, related either to the stress causing the bleaching, the mechanisms involved in the loss of the symbiotic algae or the starvation resulting from the cessation of autotrophic incomes, profoundly alter and transform the host. Scientists demonstrated that bleached cnidarians are indeed heavily affected by the phenomenon, showing multiples signs of cellular death [93, 299, 373], as well as important modifications of their feeding habits [10, 149, 172]. In the previous chapters, we confirmed some of these observations and showed in *A. pallida* that the loss of *Symbiodinium* triggers an increase of cell proliferation in both tissues as well as an increase of ectodermal mucocyte density [117]. The origin and the fate of these newly produced mucocytes are however still to be clearly determined.

Although being extensively studied in cnidarians, cell proliferation, either during growth or regenerative processes, is poorly understood in anthozoans. The production of new cells in cnidarians has, in fact, been essentially studied in hydrozoans and in particular in *Hydra* due its spectacular regenerative capacity [36, 39, 349]. These studies showed the existence of specific stem cells, or I-cells, which

are able to multiply in order to produce most of, if not every, other kinds of cells [134, 209, 263]. Anthozoans lack such I-cells but harbor similar wandering cells called amoebocytes. While amoebocytes have been identified to play a role in wound healing processes in the anemone *Nematostella vectensis* [380], the coral *Porites cylindrica* [288] or the gorgonians *Plexaurella fusifera* [259], this role is likely related to the innate immune system rather than tissue regeneration [287]. The capacity of amoebocytes to generate differentiated cell types is therefore very unlikely. Amoebocytes are most probably an artificial cellular type regrouping amorphous wandering cells with differing degrees of specialization [134]. Similarly to *Hydra*, whose epithelial cells are self-renewing, production of new cells in anthozoans seems, in fact, to be related to dedifferentiation of epithelio-muscular or nutritive-muscle cells [134]. Indeed, a recent experiment demonstrated in *N. vectensis* planulae that both ectodermal and gastrodermal epithelial cells are able to dedifferentiate and produce neurons [271]. Epithelial cells would therefore also have a stem-like function, assuming the development of tissues as well as their renewal and repair through a succession of mechanisms implying dedifferentiation, multiplication, migration and specialization [134]. Recent results showed in *N. vectensis* that this ability is not restricted to specific zones and is present throughout the body in both tissues [291].

Following its dedifferentiation into a pluripotent progenitor, the epithelial cell will most likely undergo mitosis. Regeneration without mitosis, or morphallaxis, is also possible but, within cnidarians, has only been described in hydrozoans [121, 134]. Such division of stem cells is therefore usually organized in two categories depending on the nature of the daughter cells produced. A symmetrical division produces two similar cells that will both further differentiate into specific phenotypes while an asymmetrical division produces one

committed cell and one stem cell daughter, allowing self-renewal [121]. Although largely described in *Hydra* [69], this categorization may not be suited for anthozoans. Indeed, the notion of asymmetrical division and self-renewal of stem cell pool seem linked to “classical” stem cells such as *Hydra*’s I-cells, which are always undifferentiated and pluripotent, and not newly dedifferentiated cells. Self-renewal in the case of anthozoans could therefore be seen as a symmetrical division followed by the differentiation of one of the daughter cells back to the original epithelial phenotype. Although likely, this is however uncertain, just as the belonging of this phenomenon to such classification.

Migration of these newly produced cells is well described in cnidarians. However, most observations are once again carried out in hydrozoans. The long-distance cell migration observed in hydrozoans is probably linked to the existence of specific proliferative sites [206, 209, 263]. The detection of proliferating cells throughout the body of some anthozoans [291] suggests that these cells may migrate over shorter distances. The mesoglea seems to play a crucial role in such cell migration [343]. Some experiments showed that anthozoan cells dissociated from sea anemones (including *Aiptasia*), scleractinians or alcyonaceans and exposed to media of various compositions would only migrate in contact with pieces of mesoglea [320]. Further studies identified many mesogleal components, such as integrin [310], fibronectin [359] and laminin [340], to be necessary for this migration. Once at destination, the new cells will finish their differentiation. While they seem able to adopt many cell phenotypes [209], the majority of the studies addressing this matter documented the transformations taking place during the maturation of cnidocytes, ephemeral cells that present an intensive turnover [74, 197, 206].

As we outlined here, most of the actual knowledge concerning cell proliferation, migration and differentiation in cnidarians derives from studies conducted in hydrozoans. Although providing important information, its extension to the results gathered on *A. pallida* presented in previous chapters is however hypothetical. We therefore decided to conduct some experiments in order to assess the origin of the bleaching-induced proliferating cells as well as their possible differentiation into mucocytes. First of all, we evaluated the cell proliferation in the whole individual (*in toto*) to confirm its homogeneous repartition. We then focused on the possible migration of cells from the ectodermis to the gastrodermis following heat and light-induced bleaching. Being previously described in *Hydra* [350], this phenomenon could accelerate the regeneration of the wounded gastrodermis and could also explain, in part, the increased proliferation in the undamaged ectodermis. To do so, we used an indirect technique: we counted EdU+ nuclei at different time intervals following incubation with hydroxyurea (HU), a molecule inhibiting the synthesis of nucleotides and therefore hindering mitosis [291]. We then estimated the stability of populations in the ectodermis and the gastrodermis. The mitosis being inhibited, changes in EdU+ cell densities within one tissue would therefore reflect a contribution from the other tissue (a cellular migration). Additionally, we evaluated the contiguity of mucocytes and proliferating cells 5 and 7 days following bleaching (Fig. 31). This allowed us to further confirm that the ectodermal newly produced cells participate in the increased number of mucocytes that we previously observed following bleaching-inducing stresses.

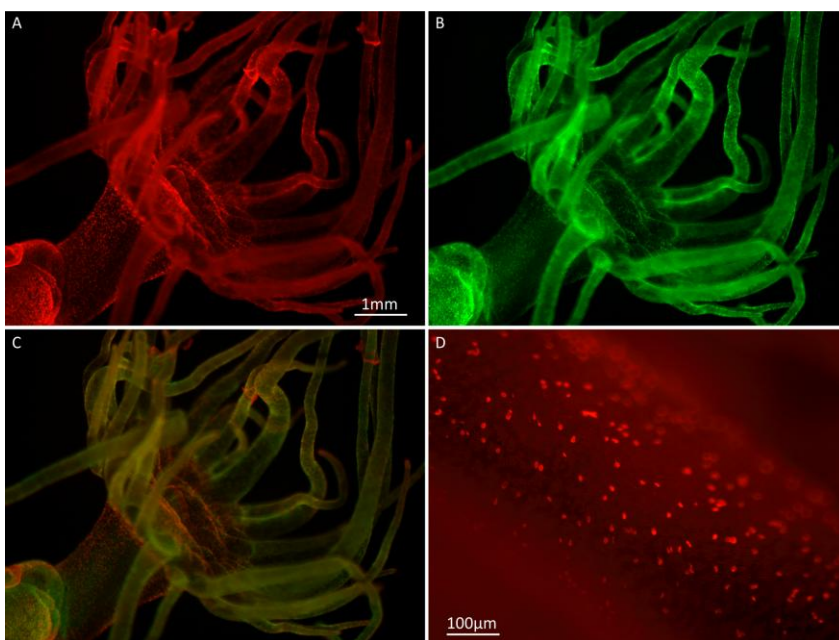


Figure 31 | *In toto* labeling of mucocytes and proliferating cells. WGA (A), EdU (B) and merged (C) staining. (D) Close-up on mucocytes in a tentacle.

Material and Methods

Biological material

Sea anemones *Aiptasia pallida* (Verrill), originating from the aquarium of the University of Liège, were maintained in our laboratory where they rapidly produced a multi-clonal population. Our aquariums were filled with artificial seawater (Reef Crystals, Aquatic systems, France) maintained at $26 \pm 0.2^{\circ}\text{C}$. A Dupla T-Control Delta (Dohse Aquaristik, Germany) in combination with a cooling unit (Titan 150, Aqua Medic, Germany) assured the stability of this temperature. Illumination following a 12 h/12 h light cycle was provided by 12 W LED bulbs (6000 K, Elix Belgium) producing $30\text{--}50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (measured on the bottom of the aquarium using a Submersible Spherical Micro Quantum Sensor (Walz, Germany)). Anemones were

fed every week with frozen *Artemia* shrimps, except during experimental treatments.

***In toto* cell proliferation**

Healthy anemones were incubated for 24 hours in a 10 μ M solution of 5-ethynyl-2'-deoxyuridine (EdU; Invitrogen, Eugene, Oregon, USA) in filtered seawater [117, 291]. They were subsequently anesthetized for 15 minutes in a 1:1 solution of 0,37 M MgCl_2 and filtered seawater before fixation in a 4% solution of paraformaldehyde in filtered seawater. Specimens were then washed 3 times for 5 minutes in Phosphate-Buffered Saline (PBS; 3.82 g/L $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$; 10.48 g/L NaHPO_4 in 0.45 M NaCl) and then incubated during 20 minutes in a 0.5 % solution of Triton x-100 made in PBS. Following 3 washes of 5 minutes in a 3 % solution of Bovine Serum Albumin made in PBS, specimens were incubated 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). Finally, anemones were washed 3 times in PBS and kept in darkness in PBS before microscopic examination.

Trans-tissular cell migration

We used a thermal/photic stress treatment to bleach our anemones during this experiment (see chapter 3, [117]). Eighteen anemones of approximately 2 centimeters in height were isolated from the stock aquarium and transferred into 100 mL beakers. They were then exposed during 30 hours to a water temperature of 33°C and an illumination of approximately 1900 $\mu\text{mol photos.m}^{-2}.\text{s}^{-1}$ (measured in the bottom of the beaker). They were then returned to normal conditions and allowed to recover for 2 hours before being incubated for 4 hours in a 10 μ M solution of EdU in filtered seawater. Following this incubation, anemones were divided into 3 groups of 6

individuals: T_0 , T_1 +HU and T_1 -Ctrl. Anemones of the T_0 group were immediately anesthetized for 15 minutes in a 1:1 solution of 0.37 M $MgCl_2$ and seawater and fixed in a 4% solution of paraformaldehyde in seawater. Anemones of the T_1 +HU group were allowed to recover during 24 more hours in a 20 mM solution of HU (Sigma) in filtered seawater, preventing any further cell division, while anemones of the T_1 -Ctrl group recovered in normal seawater. During this period, anemones of the 2 groups were kept under normal conditions of light and temperature. Another anemone was incubated at the same time in both EdU and HU in order to attest the efficacy of HU in blocking mitosis. HU blocks mitosis by inhibiting the ribonucleotid reductase and therefore the synthesis of deoxyribonucleotides. HU efficiency decreasing over time, the solutions were renewed 3 times at 8-hour intervals. After the 24 hours, the anemones were also anesthetized in $MgCl_2$ and fixed in paraformaldehyde. Following fixation, specimens were processed for histological analysis. They were dehydrated in a succession of alcohol solutions and imbedded in paraffin (paraplast Xtra, Sigma). Specimens were then cut into 5 μ m-thick slices and mounted on silane-coated slides. Following paraffin removal and rehydration, the slides were washed and treated in order to label the EdU+ nuclei. To do so, we proceeded in the exact same manner as for the *in toto* revelation described above. After the last PBS washes, the slides were dried and mounted for microscopic observation (Vectashield + DAPI, Vektor labs, Burlingame-California-USA).

WGA and EdU co-labeled cells

For this experiment, 12 anemones were bleached using the same protocol of photic/thermic stress as described above. They were then pooled together and incubated for 24 hours in a 10 μ M solution of EdU in filtered seawater. At the same time, 12 anemones isolated from the stock aquarium were incubated in the same way and served

as control group. Following the EdU incubation, the anemones were washed multiple times in seawater in order to clear out the EdU. On the fifth day following the stress treatment, 6 anemones of each group were anesthetized and fixed. The rest of the anemones were anesthetized and fixed on the seventh day. They were then all treated for paraffin embedding, cut into 2 μm -thick slices and processed for EdU revelation following the protocol described above. The slides were additionally incubated for 15 minutes in a 5 μM solution of WGA (wheat germ agglutinin + Alexa 594, Invitrogen, Eugene-Orgeon-USA) in order to label mucocytes and washed 3 times in PBS before being mounted for microscopy.

Counting and statistics

In toto anemones were observed under a fluorescence binocular microscope (Olympus SZX-16) and slides were examined under a fluorescence microscope (Nikon TE2000-U and Confocal Leica SP5). Mean densities of EdU+ nuclei were evaluated in transversal sections of tentacles randomly chosen for each anemone using Nikon NIS software v3.1. Each value was calculated from 5 counts that were reported to the ectodermal tissue area according to Fransolet et al. [117]. A cell was considered labeled by WGA and EdU when both stainings were at least partially overlapping. While this does not ensure that the new cells indeed matured into mucocytes, the thickness of the tissue slices and the criteria of analysis make it very likely. The numbers of WGA+EdU+ cells were reported to the total number of mucocytes.

Statistical analyses of the data were performed using Statistica v10. Concerning cell migration, the difference between the three treatments was investigated by using a one-way analysis of variance (one-way ANOVA) followed by Fisher's *post hoc* test. As to evaluation of the co-labeling, differences between control and stressed

anemones were assessed using Student t-test. Differences were considered statistically significant when $p < 0.05$.

Results

In toto cell proliferation

Observations of EdU labeling in the whole anemone revealed that cell proliferation occurs throughout the body, from the pedal disk to the extremities of the tentacles. Density of EdU+ nuclei seems however to be a bit higher in the basal part of the column (Fig. 32).



Figure 32 | *In toto* EdU labeling. Cell proliferation occurs throughout the whole body of the *A. pallida* with a higher density of dividing cells in the lower part of the column.

Trans-tissular cell migration

The efficacy of hydroxyurea in blocking mitosis was confirmed by the absence of EdU incorporation in the anemone incubated in both

solutions (data not shown). Measures performed at T_0 revealed that, although incubation was limited to 4 hours during this experiment, a large amount of cells incorporated EdU. The number of EdU+ nuclei was significantly higher in the ectodermis (6010 ± 598 nuclei per mm^2 , Mean \pm S.E.M.), with 30 times the density observed in the gastrodermis (184 ± 65 nuclei per mm^2).

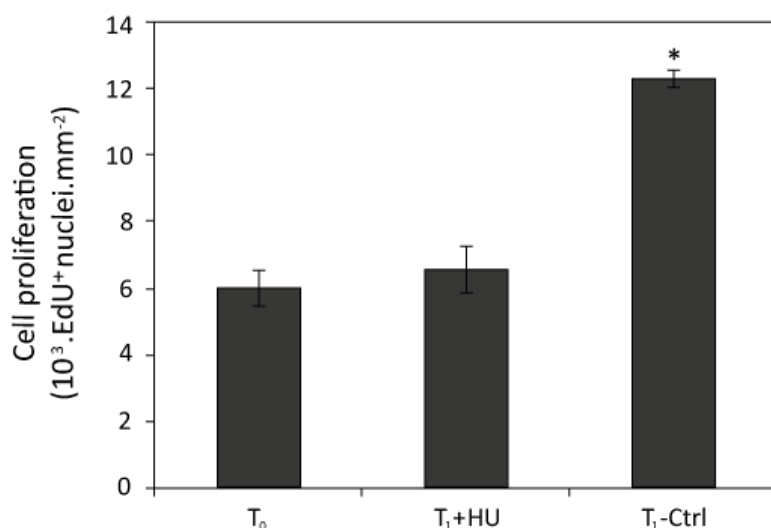


Figure 33 | Cell proliferation (Mean \pm S.E.M.) following photic/thermic stress and HU incubation in the ectodermis of *A. pallida*. The density of EdU+ nuclei does not differ from T_0 values after 24 hours of incubation in HU. This value doubles in anemones not incubated with HU ($T_1 - \text{Ctrl}$). (ANOVA and Tuckey's *post hoc* test; $p < 0.001$).

After 24h of recovery, anemones treated with HU ($T_1 + \text{HU}$) showed no variation in the ectodermal density of EdU+ nuclei compared to T_0 . In the untreated group, however, ectodermal density of EdU+ nuclei increased dramatically to 12303 ± 305 nuclei per mm^2 and thus doubling compared to T_0 and $T_1 + \text{HU}$ (Fig. 33). ANOVA ($F(2, 15) = 32.621$) followed by Tuckey's *post hoc* test confirmed the significant difference between $T_1 + \text{HU}$ and the other groups ($p = 0.0002$).

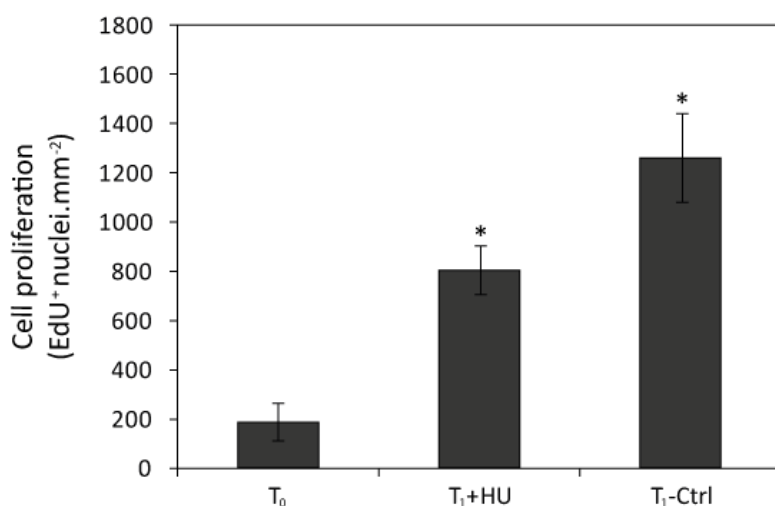


Figure 34 | Cell proliferation (Mean \pm S.E.M.) following photic/thermic stress and HU incubation in the gastrodermis of *A. pallida*. Densities of EdU+ nuclei were 4 and 6-fold higher in the T₁+HU and T₁-Ctrl groups, respectively, compared to the T₀ group (ANOVA and Tuckey's *post hoc* test; $p < 0.001$).

In the gastrodermis, the density of EdU+ nuclei increased at T₁ in both groups compared to T₀ (Fig. 34). This density reached 800 ± 92 nuclei per mm² in anemones exposed to HU and 1263 ± 163 nuclei per mm² in untreated ones (increases of 400% and 600% respectively). ANOVA ($F(2, 15) = 22.465$) followed by Tuckey's *post hoc* test confirmed that the T₁+HU group was significantly different from the T₀ group ($p = 0.005$) and that the T₁-Ctrl group was significantly different from the others ($p = 0.0002$ compared to T₀ and $p = 0.03$ compared to T₁+HU).

EdU localization in WGA+ cells

No significant difference in the number of WGA+EdU+ cells between control and stressed anemones could be observed five days following the bleaching treatment. At this time, approximately 40% of the mucocytes were EdU+ in both groups. A significant increase of WGA+EdU+ cells appeared in stressed anemones on the seventh day

following the bleaching treatment (Fig. 35). At this time, while only 38% of the mucocytes in control anemones possessed EdU+ nuclei (Fig. 36), this value reached 52% in the stressed ones (Student t-test; $p = 0.00004$).

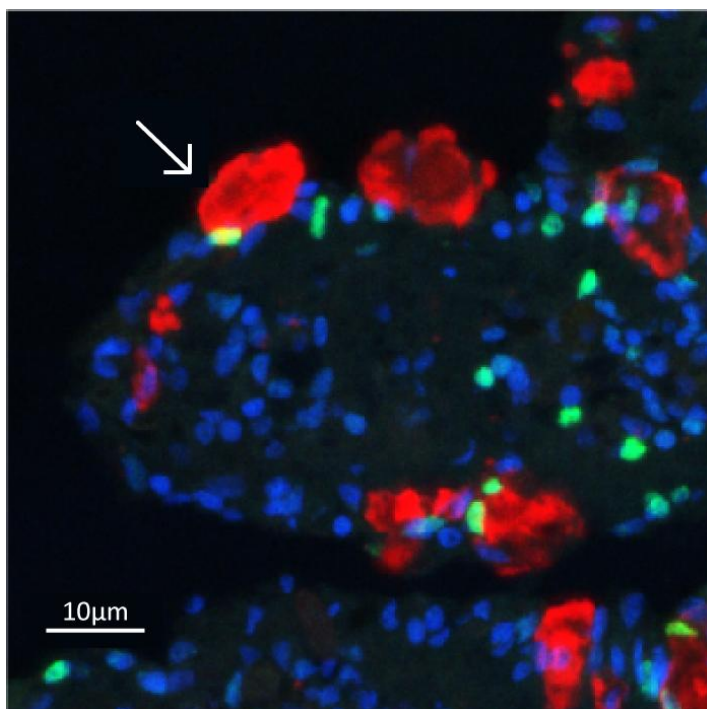


Figure 35 | Confocal picture of a WGA+EdU+ cell (arrow) in the ectodermis of *A. pallida* 7 days after bleaching treatment.

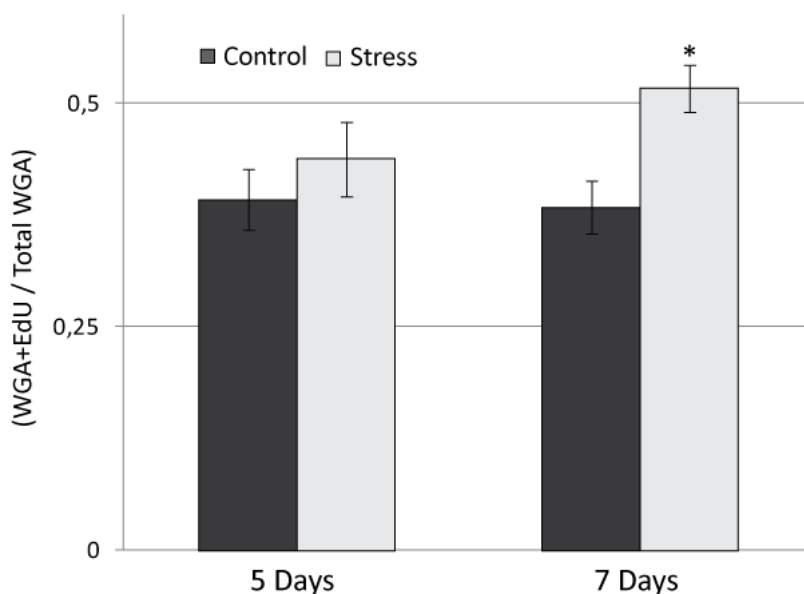


Figure 36 | Ratio of EdU+ mucocytes (Mean ± S.E.M.) 5 and 7 days following photic/thermic stress in *A. pallida*. While no difference can be observed between control and stressed anemones after 5 days, the ratio of WGA+ cells possessing EdU+ nuclei in stressed anemones rises to approximately 50% after 7 days.

Discussion

In previous chapters, we demonstrated that, following different stress treatments, bleached cnidarians show an increase of their cell proliferation. This increased production in the gastrodermis corroborates previous studies showing that algal loss is, in part, operated through the death of the host cells [93, 299, 373]. In those chapters, we also highlighted an increased density of ectodermal mucocytes following bleaching and proposed that it could be part of a strategy engaged by the host to operate a heterotrophic shift. This shift was described in the literature as a viable option for the host to compensate the loss of autotrophic incomes [10, 149, 172]. The origin of the newly produced cells and their differentiation into mucocytes were, however, still to be explored.

In toto observations of EdU labeling confirmed that cell proliferation occurs throughout the whole body of *A. pallida*. Unlike hydrozoans, where cell proliferation takes place in specific regions of the animal and cell migration occurs over long distances [206, 209, 263], new cells produced in *A. pallida* are likely to migrate over short distances to reach their final site. Moreover, cell mitosis seems more active in the basal region of the anemone. This is probably linked to the asexual mode of reproduction of *A. pallida*. This phenomenon, called pedolaceration, consists in the detachment of little fragments of tissues from the pedal disc and therefore requires active cell proliferation.

While these observations argue for a limited migration of newly produced cells, trans-tissular migration was still a possible phenomenon that had to be investigated. To test this hypothesis, we used bleached *A. pallida* that we incubated in EdU and divided into three groups. Anemones of the first group were directly sacrificed while those of the second group were incubated for 24 hours in hydroxyurea, a mitosis inhibitor. Any change in the relative numbers of EdU+ nuclei in each tissue of these anemones compared to those sacrificed directly after the EdU incubation would then reflect trans-tissular migratory events. The third group of anemones was sacrificed after 24 hours of incubation in regular seawater and served as control, showing normal increase of EdU+ nuclei.

No change of EdU+ nucleus density was reported in the ectodermis of the HU-treated anemones. This indicates that, if some cells migrated from the ectodermis to the gastrodermis, they do not represent a significant number compared to the total amount of ectodermal proliferating cells. In the ectodermis of anemones incubated for 24 hours in seawater, the density of EdU+ nuclei doubled compared to the T_0 group. Compared to the T_1 -HU group, this

observation confirms the efficacy of the HU in inhibiting the mitosis. This massive increase in EdU+ nucleus density is very unlikely related to a second mitotic event, which would take much more time. It is most probably caused by the division of the cells that incorporated EdU during the 4 hours incubation and had not yet time to undergo mitosis at T_0 . On the other hand, EdU+ nuclei located in the gastrodermis of the HU-treated anemones showed a 400% increase compared to T_0 . Since no cell could have divided following HU incubation, this observation has to be related to the migration of cells from the ectodermis. Even though this density augmentation is relatively important, the quantity of new cells only represents a tenth of the cells initially present in the ectodermis at T_0 . This would therefore explain why no diminution of EdU+ nucleus density could be measured in the ectodermis of HU-treated anemones. Finally, a 600% increase of EdU+ nucleus density was measured in the gastrodermis of anemones incubated for 24 hours in seawater. This most probably results from the division of the cells that were EdU+ at T_0 combined with the migration of EdU+ cells from the ectodermis.

These results highlight the existence of trans-tissular migratory events in bleached *A. pallida*. Already reported in *Hydra* [350], this phenomenon could bring an important amount of new cells to the bleached and wounded gastrodermis, which is largely depleted of its symbiotic host-cells and presents a reduced proliferative capacity due to the sustained stress. Moreover, the much larger quantity of cells in the ectodermis makes it a very effective production site for new cells. This mechanism could therefore accelerate the regeneration of the gastrodermis, allowing a faster replenishment of competent host-cells and a quick recovery of symbiotic algae.

The experiment assessing the ratio of EdU+ cells in WGA+ cells brought new elements that complement the results gathered in

previous chapters. In this experiment, we highlighted a significant increase in WGA+EdU+ cells 7 days after the thermic/photic stress treatment. This suggests that, at least, some of the bleaching-induced proliferating cells transform into mucocytes. These newly produced mucocytes must have fully matured quite shortly before being observed since no increase in EdU+ mucocytes was observed 2 days earlier. So far, most of the studies concerning migration and differentiation of cells in cnidarians focused on cnidocytes [74, 197, 206, 393]. This experiment is the first to date to assess the differentiation of cnidarian cells into mucocytes. These observations further strengthen our previous results showing increase in ectodermal cellular proliferation and linking it to the higher number of mucocytes following bleaching in *A. pallida*.

Altogether, these results provide new elements concerning the fate of the new ectodermal cells produced following bleaching in *A. pallida*. Some of these cells migrate to the gastrodermis where they most probably participate in the regeneration of the damaged tissue in order to re-establish symbiosis. On the other hand, another fraction of the new cells also seems to differentiate into mucocytes. The increased mucus production that follows is believed to help the bleached host to survive, via protective properties as well as functions in prey capture and heterotrophy.

Chapter 6

General Discussion

In this work, we investigated the histological modifications occurring in cnidarians following a bleaching event. While today's research continues to primarily focus on the causes of bleaching, our knowledge about the events that follow the loss of algae is still very lacunar. Two major and recent discoveries led us to approach this overlooked subject from a histological point of view:

Firstly, the mechanisms involved in *Symbiodinium* expulsion from the host's gastrodermal tissue. Many publications demonstrated that this process implies the loss of the host cell either through cell death mechanisms [92, 93, 154, 215, 223, 299] or cell detachment [45, 127, 341]. The regeneration of this wounded tissue, although being essential for the re-establishment of symbiosis, has so far, never been the subject of studies. We therefore decided to address this matter using some newly developed methods allowing detection of cell proliferation.

Secondly, the ability of some coral species to cope with the drastic reduction of their autotrophic energy incomes. Multiple studies linked this aptitude to variations of their heterotrophic capacity, either following bleaching [149] or reduction of photosynthesis [10, 366]. However, none of them investigated the mechanisms implied in such a modification. The mucus layer covering corals playing a key role in heterotrophic feeding [42], we decided to evaluate the variations of mucocyte density following bleaching. We chose to perform all our experiments in the well-documented sea anemone model *Aiptasia pallida* [396].

Post-bleaching tissular modifications

In all our experiments, whether bleaching was induced by cold and darkness, elevated temperature and light or a photosynthesis inhibitor, histological analyses revealed similar results. In every case, the bleached anemones presented an increase in cellular proliferation in both their gastrodermis and ectodermis. The respective delays of these responses varied depending on the stress protocol, which always occurred during the following days or weeks. Both these observations seem linked to the loss of *Symbiodinium* since the treatment with a photosynthesis inhibitor eluded the possible effect of the stress on the host.

The higher cell proliferation rates observed in the gastrodermis of bleached anemones are most probably induced to regenerate the wounded tissue. As said earlier, the gastrodermis is depleted of most of its algae-hosting cells during the bleaching and has to recover in order to host new symbionts. Moreover, damages could have spread to non-symbiotic gastrodermal cells if the leakage of ROS from damaged host cells was not stanching in time [338]. The function of the increased cell proliferation measured in the ectodermis of the bleached anemones is however less evident. A small fraction of these new cells could, in part, contribute to the restoration of the gastrodermis. Indeed, our experiment highlighting the migration of newly produced ectodermal cells to the gastrodermis suggests that ectodermal cells contribute to the regeneration of the gastrodermal tissue. Another fraction of these new ectodermal cells could also be linked to the higher densities of ectodermal mucocytes that we measured in bleached anemones, regardless of the stress. Further experiments revealed that, following bleaching, a higher fraction of mucocytes incorporated the thymidine analogue EdU. This reinforces the hypothesis that some of the bleaching-induced ectodermal

proliferating cells differentiate into mucocytes. The higher quantity of mucus produced, in addition to providing protection against UV and pathogens, would enhance the heterotrophic feeding capacity of the host. This modification would last until the regeneration of the gastrodermis and the reestablishment of the symbiosis allow the return of autotrophic incomes back to a normal level.

Although having been conducted in the sea anemone *Aiptasia pallida*, our results very likely reflect the events occurring during the bleaching of most hermatypic anthozoans. The value of *A. pallida* as a model for the study of coral bleaching has indeed been unanimously recognized [396]. Moreover, the use of such models is even recommended since progress moves faster when scientists focus their work on the same organism. This is due to the fact that model organisms allow easier comparisons and reproductions of results as well as development of new tools and genomic databases [396]. This kind of benefit is particularly relevant for studies, such as those deciphering the mechanisms of symbiosis, which are conducted on the cellular-scale. Altogether, the synergic efforts gravitating around model organisms ultimately enable quicker field applications, an advantage that reinforces even more their utility for the urgent study of coral bleaching.

Once extrapolated to corals, the results we gathered with *A. pallida* will hopefully help us clarify some aspects of the pandemic bleaching phenomenon that threatens them [167]. These results especially invite us to try to understand the cellular events that follow bleaching and that, along with environmental factors [146], explain at least in part, the variable surviving rates observed between different coral species.

Cell proliferation and tissue regeneration

So far, bleaching-related regenerative processes in corals have never been studied. The only information available comes from experiments evaluating re-epithelization of the gastrodermis following damages induced by ultraviolet-C radiations in acroporids [25] or wound-healing processes engaged after scrape injuries in bleached *Montastraea annularis* [252] and lacerating injuries in *Porites cylindrica* [288]. These studies suggest that anthozoans share, at least, some of the tissular plasticity and regenerative capacity of their close and extensively studied relative *Hydra* [38]. The first two studies bring however only little information aside from delays of regeneration and are not very relevant to the understanding of post-bleaching tissular modifications. The third study is more pertinent and, although inflicted wounds are very different from damage induced by bleaching, provides detailed observations of the healing process. The successive phases described by the authors are similar to those observed in other invertebrates and give a first insight on how regeneration could take place in the bleached gastrodermis. Except for the plug formation and the inflammation process, which are likely due to the open nature of the lesion, they also raise the existence of stem cells that would be stimulated by the injury. These newly produced cells would then infiltrate the lesion and further multiply to operate its regeneration [288].

Our results confirm the existence of similar processes during the regeneration that follows bleaching events. Moreover, we demonstrated that, contrary to *Hydra*, the new cells do not originate from specific niches but, instead, can be observed throughout the whole body of the anthozoan in both tissues. We also highlighted the existence of similar migratory events that are, in our case, likely to be operated on rather short distances. However, the identity of the stem

cells and thus the origin of the new cells still remain to be identified. In a recently published review, Gold and Jacobs proposed that production of new cells in anthozoans relies on the dedifferentiation of ectodermal epitheliomuscular cells and gastrodermal nutritive-muscle cells [134]. Each of these two types of dedifferentiated cells could then adopt a variety of phenotypes depending on its origin. Differentiation into cnidocytes, for example, being restricted to cells dedifferentiated from epitheliomuscular cells. Our observations suggest the revision of this hypothesis. Newly produced cells seem indeed able to migrate, at least, from the ectodermis to the gastrodermis, and this, most likely to produce new symbiotic host cells. The dedifferentiated and mitotically active cells found in anthozoans are then believed to have a wider differentiation capacity, with less dependency on their original phenotype. This further raises the question of the differentiation potential of these cells (pluripotency vs. totipotency) and encourages for a deeper investigation of this matter in other specific mechanisms such as pedolaceration.

In the gastrodermis, we observed that the delays between the peak of cellular proliferation and the complete replenishment of *Symbiodinium* density vary between the stress treatments. Comparison of these delays to other laboratory or environmental studies is very difficult considering the multitude of varying parameters such as the species utilized or observed, the type or duration of the stress and the amount of new *Symbiodinium* available for reinfection. Nonetheless, while some laboratory studies actively reinfected their bleached specimens with new algae [32], we here showed that replenishment of *Symbiodinium* density could solely rely on the few that escaped expulsion from their host. While not being one of the major results of this work, this observation, although never put forward in the literature, has important field consequences. Most

of the studies focused on the exogenous origin of new *Symbiodinium* following bleaching. They highlighted their presence in multiple habitats such as the water column and the sediments [4, 53, 59, 142, 159, 246, 248, 305, 368, 409] or the surface of macroalgae [306], and underlined the similarities between the free-living populations and the symbiotic ones [248, 368, 409]. While there is no doubt that free-living *Symbiodinium* are able to infect a bleached host [240], our results suggest that the reestablishment of the symbiosis can be operated without any external input. Furthermore, this corroborates the hypothesis that post-bleaching changes in *Symbiodinium* clade composition may be operated through a shuffle mechanism. This implies that some clades, initially present in little density within the host gastrodermis, could become dominant following bleaching. They owe this to their competitive advantage provided by their relative better fitness when exposed to stressful conditions [31, 280].

Having demonstrated that, during bleaching, the new gastrodermal host cells have two different origins (ectodermal and gastrodermal), the question of their equality before the newly infecting algae seems legitimate. Considering the multitude and complexity of the cellular components involved in *Symbiodinium* recognition and selection [71, 118], dissimilarities may exist between the new cells derived from the ectodermis and the gastrodermis. Although both type of cells being naïve to symbiosis, they could share epigenetic characteristics of their lineage [35, 413]. The cells originating from the ectodermis could therefore have a different algal affinity, especially if their trans-tissular migration is a bleaching restricted process. With today's increasing number of observations depicting *Symbiodinium* population shifts within corals and the rising interest they generate [16, 20, 189, 375], this hypothesis seems worth being investigated.

Cell proliferation and mucocyte density

The second major modification that occurs within corals following bleaching is to be found on the metabolic and energetic level [11]. Once deprived of their autotrophic energy incomes, some coral species seem able to cope with the situation by increasing their heterotrophic capacity. Contrary to other species, which only rely on their energy reserves, this strategy spares these supplies and allows for maintained sexual reproduction as well as a better survival rate in case of prolonged or successive stresses [149]. However, the mechanisms underlying this heterotrophic shift, whether it happens during sediment-related shading [10, 366] or bleaching [149] events, are not yet entirely clarified.

In our work, we propose that an increase in ectodermal mucocyte density could contribute to this acclimatization. This corroborates previous studies showing a similar increase in mucus production [276, 406] or mucocyte number [223] following bleaching events in corals. Our results further expand our limited knowledge about the development of anthozoan mucocytes [42] and show that their maturation following cell division takes at least between six and seven days. Following this period, a higher number of mucocytes would not only result in a larger amount of mucus in a thicker layer, it would also, along with increased ciliary movements, enhance the transport and therefore the quantity of food particles brought to the mouth of the coral polyps [42]. These particles include detrital POM (particulate organic matter) as well as live POM, ranging in size from the pico, nano and microplankton to the larger invertebrates belonging to the mesoplankton [174].

A major contribution of the carbon participating in mucine synthesis being initially derived from *Symbiodinium* photosynthates

[42, 276, 379, 404], increase in its production following bleaching has to imply some significant metabolic modifications. These may involve different pathways and could also result in subtle variations of mucine composition such as a reduction in the amount of oligosaccharide residues. The C:N ratio in the mucus, a highly variable characteristic as shown by multiple studies made of healthy corals [42], could therefore be strongly influenced by the loss of *Symbiodinium*. Overall, while investment of its remaining carbon resources in mucus production by the coral seem to be a gamble, it can ultimately lead to a net gain as illustrated by the salvaged lipid reserves observed in heterotrophy-shifting corals [149].

Along with food-particles capture, the thicker mucus layer that covers the bleached coral could also provide some other significant benefits. Thanks to the MAA's it contains, the mucus may confer an increased protection against UV radiations [42]. This is not negligible since the damages they would cause to the weakened coral could be potentially fatal. In a same way, the mucus layer could also protect the bleached coral against pollutants or other toxics [42]. Moreover, the mucus having some antibacterial proprieties, it may strengthen the coral defense against pathogens [42]. This could be critical since many studies linked the presence of bacteria, such as *Vibrio shiloi* or *Vibrio coralliilyticus*, to bleaching [27, 211, 212]. A reinforced protective mucus layer would therefore limit the infection probabilities and increase the chances of coral survival until algae recolonisation. Having showed that an increase in mucocyte density could be induced solely by photosynthesis dysfunction and algal expulsion from the host, heterotrophy is therefore more likely the main purpose of this increase. The mucus benefits exposed above come thus handy after bleaching but have however to be considered as collateral advantages.

Implications of the increased mucus production go beyond the functions it plays for the bleached coral. The fraction that dissolves upon release in the water (up to 80% for some acroporids) as well as the particles and threads that detach from the surface of the coral play a major role in the coral reef ecosystems [404]. These carbon-rich molecules feed bacteria and enter the trophic chain where they generate a significant nutrient release. Mucus aggregates alone can represent as much as 20% of the organic carbon metabolized by the community living in the sediments [404]. Relatively speaking, this means that an increase of mucus production by the bleached corals populating a reef could significantly increase nutrient input to the water column. While this could be a benefit for the benthic and pelagic communities, it could also enhance the growth of macroalgae. Such phenomenon could therefore be a major drawback of increased mucus production since macroalgae can be dangerous competitors for corals, especially after bleaching events [77, 98, 167, 203, 221].

Perspectives

Altogether, the results gathered by this work bring some new light to the still obscure events that take place in corals after bleaching. Although bringing conclusions that could be key elements to the comprehension of those events, they also open new leads for further research.

Clear identification of the proliferating cells and confirmation of their epithelial phenotype seem particularly important to complete the picture that we have just sketched. This could be achieved for example by using *in situ* hybridization and co-localizing cell proliferation staining with mRNA sequences specific of epithelial cells. As we said above, it could also be interesting to verify if cellular migration from the ectodermis to the gastrodermis is a constant

phenomenon or if it is only triggered during bleaching. Interesting results could also come from the analysis of the cellular proliferation in the column of bleached *A. pallida*. In this part of the animal, the gastrodermis harbors very few *Symbiodinium* but is composed of numerous secretory cells that operate digestion and are therefore important in heterotrophic feeding. Increase in gastrodermal proliferation in the column could therefore be interpreted as another element corroborating a heterotrophic shift. Multiple other experiments could also be considered in order to confirm this hypothesis. The quantity of mucus released by the bleached anemones could be measured and compared to the healthy ones. Such comparisons could also be made with the speed of ciliary movements bringing food to the mouth. Likewise, a major improvement to the heterotrophic shift hypothesis could come from the assessment of the respective cnidocyte densities between healthy and bleached *A. pallida*. The main function of these cells residing in prey capture, their increased production would, with no doubt, indicate a rise in the feeding capacity of the bleached host. However, analysis of cnidocyte density involves overcoming some difficulties. *In toto* analyses seem essential since cnidocytes are often grouped in batteries and counts made in tentacle sections would therefore not represent an accurate estimation of their density. This gets even more complicated considering that the only immunoglobins engineered so far have been developed to detect *Hydra*'s cnidocytes and do not work properly with *A. pallida*. Other detection methods will therefore have to be elaborated in order to conduct such an experiment. Genetic analyses could then be particularly useful, as to further highlight bleaching influence on cell proliferation and migration. Contrary to most transcriptomic studies focusing on symbiosis or ROS-related genes, they could be used to evaluate modifications in the expression of genes characteristic of heterotrophically active cells or cell-migration processes. Finally, even acknowledging the efficacy of

A. pallida as a model species, the extension of these experiments to some coral models would be of great interest. It would then be noteworthy to see if a similar increase in cellular proliferation can be observed in the tissues of the coenosarc, in particular in the calicoblastic epithelium, a tissue lacking in the anemone.

Conclusion

To conclude, even though this thesis brings only preliminary answers and barely lifts the veil on the events occurring during the days and weeks that follow bleaching in cnidarians, it reveals a promising field for future research. Today, as massive coral bleaching events become more and more frequent and as their intimate causes seem largely unraveled, it highlights the importance of post-bleaching mechanisms for the survival and recovery of reefs. It modestly bears optimism and lets us hope that, one day, a better comprehension of the regenerative processes and the modifications engaged to resist starvation will maybe help us to determine the key to bleaching survival and hence to the perpetuation of coral reef ecosystem.

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