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**Étude du maintien et de la rupture de l'association
symbiotique Cnidaire-Dinoflagellés : approches
cellulaires et moléculaires chez l'anémone de mer
*Anemonia viridis***

Vincent Dani

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UNIVERSITE NICE-SOPHIA ANTIPOLIS - UFR Sciences
Ecole Doctorale n°364 Sciences Fondamentales et Appliquées (SFA)

Thèse pour obtenir le titre de Docteur en Sciences de l'Université Nice-Sophia
Antipolis

Discipline : Sciences de l'Environnement

présentée et soutenue par

Vincent DANI

**Etude du maintien et de la rupture de l'association
symbiotique Cnidaire-Dinoflagellés :
Approches cellulaires et moléculaires chez l'anémone de mer
*Anemonia viridis***

Thèse dirigée par le docteur Cécile SABOURAULT

soutenue le 3 décembre 2015 à Nice

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Résumé

L'endosymbiose trophique établie entre un hôte animal du groupe des Cnidaires et ses symbiotes Dinoflagellés photosynthétiques du genre *Symbiodinium* est à l'origine du succès évolutif des écosystèmes coralliens. Les Cnidaires hébergent leurs symbiotes dans les cellules du gastroderme, dans une vacuole qui dérive de l'internalisation par phagocytose. L'établissement et le maintien de l'interaction symbiotique dépendent d'un dialogue moléculaire intime entre les deux partenaires. Cependant, des perturbations environnementales et/ou anthropiques peuvent entraîner une rupture de l'association symbiotique (ou blanchissement), responsable du déclin mondial des récifs coralliens.

Les objectifs de mon projet de recherche étaient d'améliorer les connaissances sur les mécanismes de maintien et de rupture de la symbiose mais également de définir les acteurs moléculaires localisés à l'interface symbiotique chez l'anémone de mer, *Anemonia viridis*. Premièrement, nous avons étudié les mécanismes cellulaires impliqués dans différents types de rupture de la symbiose et nous avons suivi les modifications *in hospite* de la paroi des symbiotes. Cette étude montre que la réponse au stress dépend du type, de la durée et de l'intensité de celui-ci. Parallèlement, nous avons caractérisé les gènes *npc1* et *npc2* (impliqués dans le transport endosomal de stérols) et mis en évidence la sub-fonctionnalisation d'un gène *npc2*. Nous proposons donc que la protéine dupliquée NPC2-d est un marqueur de l'état de santé des Anthozoaires symbiotiques et que la protéine NPC1 est un marqueur de la membrane périsymbiotique. Nous avons également développé un protocole afin d'identifier les protéines associées à l'interface symbiotique entre les deux partenaires.

A terme, l'analyse fonctionnelle des gènes *npc2*, les outils développés et les cibles nouvellement identifiées permettront une meilleure compréhension des mécanismes impliqués dans la mise en place, le maintien et la rupture de la symbiose chez les Cnidaires.

Mots clés: symbiose; stress ; cnidaire ; *Anemonia viridis*; *Symbiodinium* ; blanchissement ; NPC1; NPC2; stérol; cycle cellulaire ; protéomique; apoptose ; nécrose ; autophagie

UNIVERSITY NICE-SOPHIA ANTIPOLIS - UFR Sciences
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PhD thesis presented by

Vincent DANI

**Study of the maintenance and the disruption of the
Cnidarian-Dinoflagellate symbiotic association:
Cellular and molecular approaches in the sea anemone
*Anemonia viridis***

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Abstract

The trophic endosymbiosis interaction between a cnidarian host and its symbionts, photosynthetic dinoflagellates of the genus *Symbiodinium*, form the basis of coral reef ecosystems. Cnidarians host their symbionts in gastrodermis cells, in a phagocytosis-derived vacuole. Establishment and maintenance of the symbiotic interaction depend on an intimate molecular communication between the two partners. However, environmental and/or anthropogenic disturbances can lead to the breakdown of the symbiotic association (or bleaching), which is responsible for the worldwide decline of coral reefs.

The main objectives of my research project are to improve the knowledge regarding symbiosis maintenance and disruption mechanisms, but also to define the molecular key players associated to the symbiotic interface in the sea anemone, *Anemonia viridis*. First, we have studied cellular mechanisms involved in the different types of symbiosis breakdown and the cell wall modifications of *in hospite* symbionts. Our results showed that the holobiont response depended of the type, the duration and the stress intensity. Meanwhile, the characterization of *npc1* and *npc2* genes (involved in endosomal sterol transport), showed a duplication and a sub-functionalization of the *npc2* genes. We therefore suggest that the duplicated protein NPC2-d is a biomarker of symbiosis health in symbiotic anthozoans and that NPC1 protein is a marker of the perisymbiotic membrane. We then developed a protocol to obtain the proteome of the molecular players involved in the communication interface between the two symbiotic partners.

The functional analysis of the NPC proteins, the developed tools and the newly identified targets will enable a better understanding of the mechanisms involved in the establishment, maintenance and disruption of the symbiosis among cnidarians.

Keywords: symbiosis, stress response, cnidarian, *Anemonia viridis*, *Symbiodinium*, bleaching, NPC1, NPC2, sterol, cell cycle, proteomics, apoptosis, necrosis, autophagy

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1. Johana Revel*, **Vincent Dani***, Fabrice Priouzeau, Mohamed Mehiri, Lionel Massi, Laure Capron, Maxence Wisztorski, Dounia Mouajjah, Kévin Le Brigand, Virginie Magnone, Sophie Pagnotta, Sandra Lacas-Gervais, Marjolijn Mertz, Pascal Barbry, Michel Salzet, Cécile Sabourault The symbiotic interface in the *Symbiodinium-Anemonia* interaction: Insight from proteomics and metabolomics studies. *Symbiosis* (soumise) IF=1.438
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6. **Dani, V.**, Ganot, P., Priouzeau, F., Furla, P., & Sabourault, C. (2014). Are Niemann-Pick type C proteins key players in cnidarian–dinoflagellate endosymbioses? *Molecular ecology*, 23(18), 4527-4540, IF=6.5

Liste des communications scientifiques

Communications orales

1. **V. Dani**, F. Priouzeau, C. Sabourault. "Role of NPC protein in host/symbionte communication" Journée des doctorants de l'UMR 7138. Paris, Février 2015
2. **V. Dani**, F. Priouzeau, S. Pagnotta, D. Carette, M. Mertz, C. Sabourault. "NPC proteins are key players in molecular interactions between cnidarians and their dinoflagellate endosymbionts" Journée des doctorants de l'UMR 7138. Paris, Mai 2014
3. **V. Dani**, F. Priouzeau, S. Pagnotta, D. Carette, M. Mertz, C. Sabourault. "NPC proteins are key players in molecular interactions between cnidarians and their dinoflagellate endosymbionts" 42^{ème} congrès ImmunInv. Dijon, 12-14 Mai 2014
4. **V. Dani** ; J. Revel ; L. Capron; I. Fournier ; S. Lacas-Gervais ; L. Massi ; M. Mehiri ; S. Pagnotta ; F. Priouzeau; M. Salzet ; M. Wisztorski ; **C. Sabourault** « Metabolite exchange in the cnidarian-dinoflagellate interplay" 8th International Conference on Coelenterates Biology at Eilat, Israel, 2-5 Decembre, 2013
5. **P-L. Merle**, T. Zamoum, **V. Dani**, C. Sabourault and P. Furla "Post-genomic study of carbonic anhydrase enzymes in the cnidarian-dinoflagellate symbiosis" 1er Colloque d'EcoPhysiologie Animale, Lyon, Novembre 2013
6. **V. Dani**, P. Ganot, F. Priouzeau, S. Pagnotta, P. Furla and C. Sabourault "Gene duplication and adaptation to endosymbiosis in Cnidarians : Focus on the NPC team" 17th Evolutionary Biology Meeting at Marseille, France, Septembre, 2013
7. **V. Dani**, **J. Revel**, C. Sabourault "Etude de la symbiose Cnidaire/Dinoflagellés" Journée des doctorants de l'UMR 7138. Paris, Juin 2013

Posters

1. **V. Dani**, J. Revel, F. Priouzeau, S. Pagnotta, J-P. Laugier, S. Lacas-Gervais, M. Mertz and C. Sabourault : NPC proteins are key players in the molecular interactions between cnidarians and their dinoflagellate endosymbionts Session "Establishment and maintenance of mutualism" International Symbiosis Society 12-18 Juillet 2015 Lisbonne, Portugal
2. **Sabourault C, Dani V**, Priouzeau F, Revel J, Mehiri M, Massi L, Capron L, Pagnotta S, Lacas-Gervais S, Laugier J.P, Lebrigand K, Magnone V, Barbry P, Wisztorski M, Mouajjah D, Salzet M Symbiosome membranes characterization, or how to decipher molecular interactions between cnidarians and their dinoflagellate symbionts Session "Establishment and maintenance of mutualism" International Symbiosis Society 12-18 Juillet 2015 Lisbonne, Portugal
3. **V. Dani**, J. Revel, F. Priouzeau, S. Pagnotta, D. Carette, M. Mertz, L. Massi, M. Mehiri and C. Sabourault "Are NPC proteins key players in the molecular interactions between cnidarians and

their dinoflagellate endosymbionts? ” 1st Labex SIGNALIFE Meeting Cell Signaling, Nice, France, 2014

4. **V. Dani**, P. Ganot, F. Priouzeau, S. Pagnotta, P. Furla and C. Sabourault “Gene duplication and adaptation to endosymbiosis in Cnidarians : Focus on the NPC team” 17th Evolutionary Biology Meeting at Marseilles, France, 2013
5. S. Barney-Verdier, R. Christen, **V. Dani**, D. Forcioli, P. Furla, C. Mallien, P-L. Merle, B. Ponderini, F. Priouzeau, J. Revel, C. Sabourault, P. Ventura and T. Zamoum “Anemonia viridis : A top model to study the Cnidarian-Dinoflagellate symbiosis under ecophysiological stressors” 1^{er} Colloque d’Eco-Physiology Animal, Lyon, France, 2013

Liste des abréviations

- Acc : Corps d'accumulation
- ADN : Acide désoxyribonucléique
- ANR : Agence Nationale de la Recherche
- ARN : Acide ribonucléique
- ATP : Adénosine Triphosphate
- CA : anhydrase carbonique
- CCMA : Centre Commun de Microscopie Appliqué
- CMC : Carboxyméthylcellulose
- EAO : Espèces active de l'oxygène
- EST : Express sequenced Tag
- GPCRs : G protein-coupled receptors
- GPI : Glycosyl phosphatidyl inositol
- HiCEP : High Coverage Gene Expression Profiling
- HSP : Heat shock protein
- ICN : Institut de Chimie de Nice
- inSIDE : interface symbiotique entre Cnidaire et Dinoflagellés
- ITS : Espaceurs internes transcrits
- LPS : Lipopolysaccharides
- MAA : Mycosporine-like acides aminés
- MALDI : Matrix Assisted Laser Désorption Ionisation
- MAMP : Microbe-associated molecular pattern
- MCD : Mort cellulaire programmée
- Mpb : Millions de paires de base
- n : Noyau
- NGS : Next-Generation sequencing
- Npc : Niemann-Pick Cell
- PG : Peptidoglycane
- PRISM : Protéomique-Réponse inflammatoire-Spectrométrie de masse
- PRR : pattern recognition receptor
- Pyr : Pyrenoïde
- SEM : Microscopie électronique de surface
- TEM : Microscopie électronique à transition
- TLR : Tools like receptors
- UV : Ultra-violets

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Chapitre 1: Introduction générale sur la symbiose et l'endosymbiose chez les Cnidaires

1.1. La symbiose dans le règne du vivant

1.1.1. Définition de la symbiose et théorie endosymbiotique

Avec ses travaux sur les lichens, Anton de Bary décrit alors pour la première fois en 1866 une association qu'il qualifie de spécifique et durable entre deux espèces (Bary, 1866). Ce ne sera que dix ans plus tard que le mot "symbiose" sera utilisé par l'allemand Frank (Frank, 1877) pour qualifier la coexistence d'organismes différents. Néanmoins, l'apparition de la symbiose remonte aux origines de la vie et a façonné le monde du vivant tel que nous le connaissons actuellement. Lors de l'enrichissement de l'atmosphère primitive en oxygène il y a deux milliards d'années, une cellule eucaryote primitive aurait phagocyté un procaryote apparenté aux alpha-protéobactéries, pour progressivement donner ce que l'on nomme aujourd'hui une mitochondrie. Cette bactérie ancestrale est capable de convertir l'énergie des molécules organiques issue de la digestion en énergie directement utilisable par la cellule sous forme d'adénosine-5'-triphosphate (ATP). Cette symbiose a permis un gain de productivité significatif des cellules jusqu'ici contraintes à produire de l'énergie de façon beaucoup plus lente et moins efficace, via la fermentation (Wallin, 1927). La découverte en 1962 de l'ADN des mitochondries appuie alors l'origine endosymbiotique (Nass and Nass, 1962). En effet, cette bactérie, dont le plus proche parent connu actuellement est *Rickettsia prowazekii*, est à l'origine un parasite intracellulaire obligatoire (Andersson et al., 1998). Au cours de l'évolution, l'ADN originel de la bactérie a subi plusieurs modifications par perte d'un grand nombre de gènes, parfois transférés vers le génome de la cellule hôte. Si la protéobactérie transfère de l'énergie sous forme d'ATP à la cellule hôte, cette dernière a également développé un certain nombre de mécanismes permettant le transfert de protéines vers la matrice mitochondriale (Truscott et al., 2003). De nos jours, cette alpha-protéobactérie, appelée la mitochondrie, est un organite qui a permis l'émergence de la respiration chez les cellules eucaryotes (Selosse, 2000).

Une autre endosymbiose plus tardive, apparue il y a 1,5 milliards d'années, fut déterminante dans l'émergence de la lignée des eucaryotes photosynthétiques. Une cellule eucaryote primitive possédant déjà des mitochondries a ingéré par phagocytose des procaryotes de l'embranchement

des Cyanobactéries. Ces bactéries, apparues il y a 3,8 milliards d'années, ont la capacité de transformer l'énergie solaire en énergie chimique via la fixation de dioxyde de carbone. Ces organismes sont à l'origine de l'augmentation de l'oxygène dans l'atmosphère.

Au cours de l'évolution, la cyanobactérie endosymbiotique a établi un réseau métabolique stable notamment par transferts de gènes vers le génome de l'hôte, perdant ainsi son autonomie. Il devient alors un organite à part entière de la cellule eucaryote photosynthétique, connu de nos jours sous le nom de chloroplaste (Taylor, 1970). La spécificité de l'ADN du chloroplaste et de la division indépendante de ces derniers, prouvent l'origine endosymbiotique de ces organites dans les cellules eucaryotes (Bisalputra and Bisalputra, 1967).

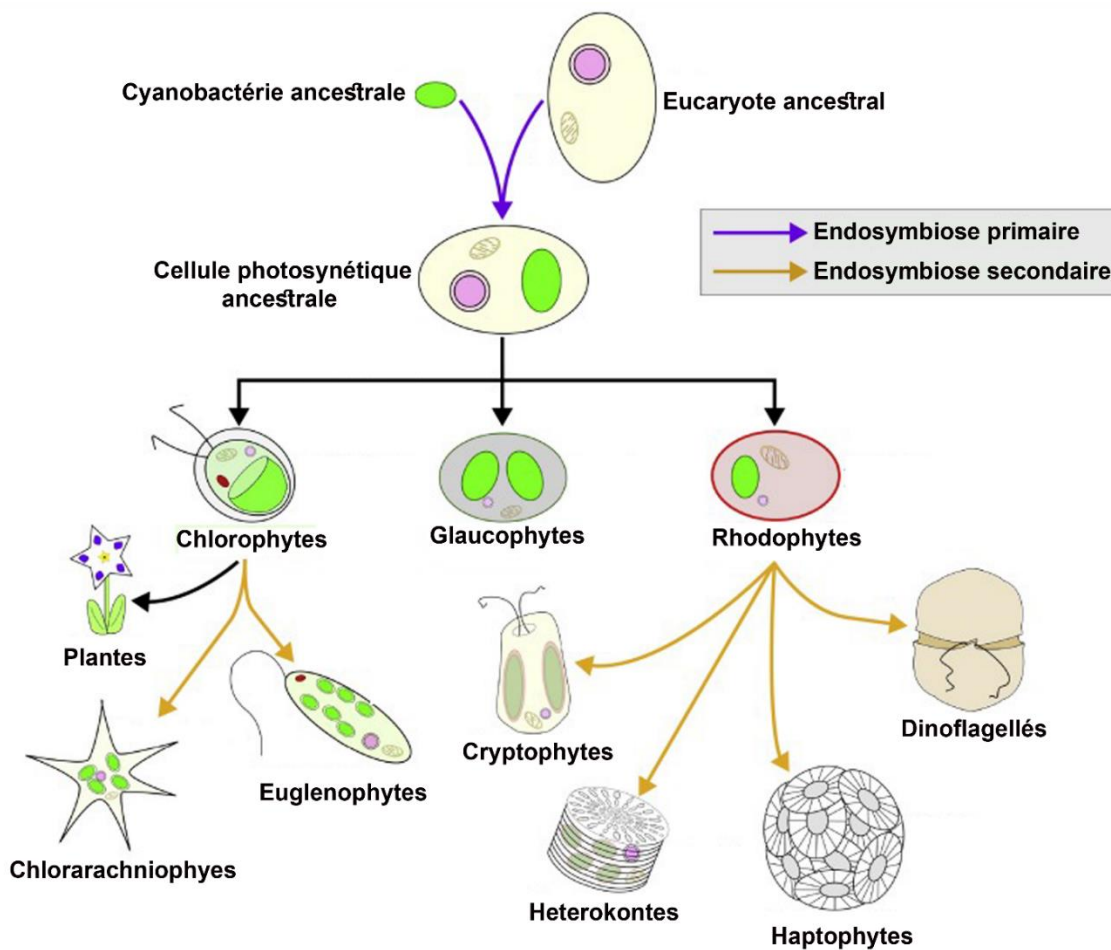


Figure 1: Phylogénie des groupes majeurs de la lignée des eucaryotes photosynthétiques et origine endosymbiotique. On note la différence entre l'endosymbiose primaire et l'endosymbiose secondaire effectuée par certains groupes chez les *Chlorophytes* et les *Rhodophytes*. Modifié d'après (Cooper and Smith, 2015)

L'acquisition de ces organites (mitochondries et/ou chloroplastes par la cellule eucaryote primaire) est définie alors comme une endosymbiose primaire (McFadden, 2001), caractérisée par un important transfert de gènes d'origine endosymbiotique vers le génome de l'hôte. Il en résulte un système à quatre membranes (membrane de l'hôte, membrane du phagosome et les doubles membranes des endosymbiotes), qui évolue vers un système à trois membranes lors de la perte de la membrane de phagocytose. Par diversification, la cellule eucaryote photosynthétique primitive a permis l'émergence des trois lignées que sont les Glaucophytes, les algues vertes et les Rhodophytes (Figure 1) (Cooper and Smith, 2015). Chez les deux dernières lignées, des événements d'endosymbiose secondaires et/ou tertiaires, qui aboutissent à une dégénération du noyau et du cytoplasme du symbiote, ont entraîné une diversification.

Plusieurs définitions de la symbiose ont vu le jour au cours de la fin du 20^{ème} siècle. En 1994, Douglas propose que le dénominateur commun de la symbiose ne serait pas forcément un bénéfice mutuel mais le résultat de l'acquisition d'une nouvelle compétence métabolique acquise par l'un des deux partenaires (Douglas, 1994). Cette définition sera nuancée plus tard par Zook en 1998 qui ajoute que la symbiose est l'acquisition par un organisme d'un ou plusieurs autres organismes lui conférant un avantage, celui-ci étant maintenu par la sélection naturelle (Zook, 1998). Dans cette association, l'hôte est défini comme le plus gros des deux partenaires. Néanmoins, cette association doit être envisagée dans son ensemble en considérant l'ensemble de la communauté microbienne associée. Cet ensemble est alors défini sous le terme d'holobionte. Globalement, nous pouvons dire que tous les organismes vivant sur la planète sont en symbiose, ne serait-ce que par leur association avec les bactéries (Gilbert et al., 2012). Ainsi, l'évolution de toutes les espèces est potentiellement influencée par les symbioses comme le décrit Paul Portier dans son ouvrage « Les symbiotes » publié en 1918 (Portier, 1918).

1.1.2. Différents types de symbiose, des mécanismes communs

Il est difficile de convenir d'une définition stricte de la symbiose. Le type d'interaction entre deux espèces est soumis aux variations des conditions environnementales et aux facteurs de temporalité et d'espace auxquels l'observateur se place. Cependant, de Bary définissait en 1866 différents termes pour distinguer les divers types de relation entre espèces (Bary, 1866) tels que le commensalisme, le parasitisme et le mutualisme, défini par Van Beneden en 1875.

Théorisé par Pierre-Joseph Van Beneden, le commensalisme est un type de symbiose dans lequel seule l'une des deux espèces tire un avantage, sans dommages pour l'autre partenaire. On dénote de nombreux exemples dans la nature dont plus de 264 sont cités dans l'ouvrage de Van Beneden (Van Beneden, 1878), tels que les plantes épiphytes vivants sur d'autres plantes afin de bénéficier d'une exposition optimale à la lumière et pour bénéficier de l'eau qui ruisselle sur les feuilles. On peut également citer le poisson-pilote qui trouve dans son association avec le requin, une protection et une source de nourriture via les débris laissés par ce dernier.

Le mutualisme, association dans laquelle les deux partenaires tirent un bénéfice réciproque, est largement réparti au sein du règne du vivant. La symbiose entre un ou plusieurs champignons hétérotrophes, appelés mycobiontes et des cyanobactéries autotrophes, représente l'association symbiotique des lichens (Yuan et al., 2005), dont l'observation du fonctionnement a donné l'origine du concept de symbiose (Bary, 1866). En effet, le champignon va assimiler de l'eau et des sels minéraux dont une partie sera transmise à l'algue, notamment sous forme de vitamine C. En échange, la production photosynthétique de l'algue sera transmise en partie sous la forme de polyols et de vitamine B aux champignons. Le mycobionte va également produire des substances lichéniques, uniquement en présence de l'algue, permettant la protection contre les herbivores et neutralisant la lumière délétère pour l'appareil photosynthétique de l'algue (Lohézic-Le Dévéhat et al., 2013). Au niveau agronomique, les symbioses fixatrices d'azote chez les plantes actinorhiziennes (bactérie actinomycète), les plantes légumineuses (bactérie *Rhizobium*) et les symbioses mycorhiziennes qui colonisent environ 80% des plantes vasculaires terrestres (Selosse, 2000), sont d'un intérêt économique primordial au niveau de la planète. Ces associations apportent des bénéfices réciproques aux deux partenaires. Par exemple, les mycorhizes augmentent les capacités d'absorption de l'eau via le mycélium très dense qui se développe au niveau des racines. Cela permet l'absorption d'éléments nutritifs présents dans le sol, via la solubilisation des minéraux contenus dans les fragments de roches, et rend disponible les matières organiques contenues dans le sol sous forme d'azote assimilable par la plante (Hayman, 1986). En échange, la plante offre un environnement stable au niveau biotique et un abri contre la prédation aux mycorhizes. Il leur transfère également une quantité importante de composés carbonés directement assimilables, que celui-ci est incapable de synthétiser lui-même (Pankow et al., 1991). Il en résulte un partage des voies de biosynthèses entre les deux partenaires de l'association. Dans la symbiose légumineuses/bactéries fixatrices d'azote, les bactéries, contenues dans un bactéroïde, transfèrent

des quantités importantes d'azote atmosphérique assimilables par l'hôte. En échange, la plante transfère, via des transporteurs situés sur les membranes du bactériocyte, une partie de ses photosynthétats sous forme de sucres, assimilables par la bactérie fixatrice d'azote (Lodwig et al., 2003; Nutman, 1976).

Le parasitisme est défini comme une association où l'une des espèces tire un avantage et l'autre un inconvénient. Dans le monde du vivant, une majorité des microeucaryotes sont des parasites et parmi eux se retrouvent les Apicomplexes (*Plasmodium*, *Toxoplasma*, *Babesia*), les Kinétoplastidés (*Trypanosoma*, *Leishmania*) et les Amibozoaires (*Entamoeba*). Ces parasites sont internalisés par un mécanisme de phagocytose (en revue dans Schwarz, 2008) et infectent les cellules de l'hôte en induisant un remodelage du réseau de microtubules (Dobrowolski and Sibley, 1996). Le parasite manipule le système immunitaire de l'hôte (Bradley and Sibley, 2007) et se maintient dans une vacuole parasitaire (Martin et al., 2007). Ce dernier recrute alors à son profit des organelles de l'hôte, telles que les mitochondries et le réticulum endoplasmique (Mercier et al., 2005), permettant l'acquisition de métabolites tels que les lipides (Gupta et al., 2005). L'étude comparée de mécanismes d'infection des parasites du groupe des Apicomplexes, groupe frère phylogénétiquement des Dinoflagellés (qui vivent en symbiose avec les Cnidaires), révèle un intérêt dans l'étude des mécanismes de reconnaissance et de maintien dans les cellules de l'hôte Cnidaire. Egalement, l'étude des mécanismes d'interaction et d'échanges métaboliques dans les symbioses fixatrices d'azote se révèle pertinente via la mise en évidence de mécanismes communs.

En effet, à l'heure actuelle, environ 440 000 études comportent le mot clé « *Plasmodium* », environ 93 000 le mot clé « *Rhizobium* » et seulement 6 300 pour le partenaire symbiotique chez les Cnidaires, « *Symbiodinium* ». Récemment, le statut mutualiste strict de la symbiose Cnidaire-Dinoflagellés a été discuté par de nombreuses études qui mettent en avant l'aspect dynamique de l'interaction. Cette symbiose peut alors être caractérisée par un continuum entre une relation de type mutualiste sous des conditions favorables, vers une relation de type parasitique dans des conditions qui s'écartent de l'optimum symbiotique (Lesser et al., 2013; Sachs and Wilcox, 2006a). Ce même type de continuum entre parasitisme et mutualisme est également observé chez les symbioses de type plante-mycorhizes, en fonction de la fertilité du sol (Neuhauser and Fargione, 2004). Ces différents types d'interactions décrits ne sont alors que des types idéaux extrêmes

(Selosse, 2000) qui définissent de façon anthropique les relations identifiées dans chaque modèle d'étude.

1.2. La symbiose Cnidaire/Dinoflagellés

1.2.1. Importance économique et écologique

Les Cnidaires symbiotiques, à la base de la formation des écosystèmes des récifs coralliens, abritent plus d'un tiers de la biodiversité marine alors qu'ils n'occupent que 0.1% de la surface totale du globe (Hoegh-Guldberg, 1999). Leur succès évolutif repose sur la capacité de ces organismes à pouvoir coloniser des milieux oligotrophes (pauvres en nutriments) tels que les océans. Cet avantage repose uniquement sur la capacité de certains organismes du groupe des Cnidaires à établir une endosymbiose mutualiste avec des Dinoflagellés photosynthétiques du genre *Symbiodinium*. En effet, seule une minorité des apports trophiques du Cnidaire est assurée via les voies hétérotrophes (par la prédation) et, suivant les espèces, jusqu'à 90% de leurs apports trophiques proviennent des photosynthétats transmis par les symbiotes (Furla et al., 2005).

L'importance de cet écosystème se mesure au niveau écologique et économique (Moberg and Folke, 1999). En effet, la bio-calcification active de ces organismes forme la structure des écosystèmes coralliens et la grande barrière de corail est la seule bio-construction visible depuis l'espace. Les récifs coralliens offrent un abri contre la prédation à de nombreuses espèces, notamment pour les stades juvéniles, et constituent également un substrat pour une grande diversité d'organismes. On peut ainsi définir les organismes coralliens comme des espèces ingénieuses des écosystèmes. L'importance des récifs coralliens se mesure également au niveau de leur rôle économique. Premièrement, ils forment des barrières naturelles protégeant de nombreux littoraux de l'érosion. Le bénéfice écosystémique est considéré à hauteur de neuf milliards de dollars par an (Cesar et al., 2003). De plus, la grande diversité trophique existant au sein des récifs coralliens permet l'exploitation par la pêche locale et l'industrie hauturière des écosystèmes associés tels que les mangroves, et génère 5,7 milliards de dollars annuels (Cesar et al., 2003). Cette grande diversité écologique est également la fondation d'une économie écotouristique qui génère chaque année plus de 10 milliards de dollars de bénéfices (Wilkinson, 1996).

1.2.2. Description générale de l'hôte : cas des Anthozoaires symbiotiques

1.2.2.1. Position phylogénétique des Cnidaires chez les Eumétazoaires

Les Cnidaires forment un phylum très ancien situé à la base de la lignée des Métazoaires, ayant divergé des Bilatériens il y a environ 650 Millions d'années à partir d'un ancêtre commun Eumétazoaire. Ce phylum comporte plus de neuf mille espèces qui vivent dans des milieux aquatiques majoritairement marins (Steele et al., 2011). Les Cnidaires ont divergé en deux groupes majeurs, les Anthozoaires (anémone de mer, coraux et pennatules) qui ont un unique stade polype, et les Méduzoaires (méduses, hydres) dont la majorité possèdent un stade méduse, en plus du stade polype (Figure 2). Cette divergence est apparue à la même période que la séparation Protostomes et Deutérostomes chez les Bilatériens (Technau and Steele, 2011). Quatre des cinq classes actuelles des Cnidaires (Anthozoa, Hydrozoa, Cubozoa, Scyphozoa and Staurozoa) ont été identifiées dans les fossiles issus du Cambrien (Daly et al., 2007). Ceci suggère une diversification considérable ayant eu lieu il y a 500 millions d'années, probablement au moment de l'explosion cambrienne (Darling et al., 2005). Comme le montre la Figure 2, les Anthozoaires sont divisés en deux sous classes, les Octocoralliaires et les Hexacoralliaires. Cette dernière sous classe comprend l'ordre des Scléractiniaires (coraux constructeurs de récifs) et l'ordre des Actiniaires (anémones de mer).

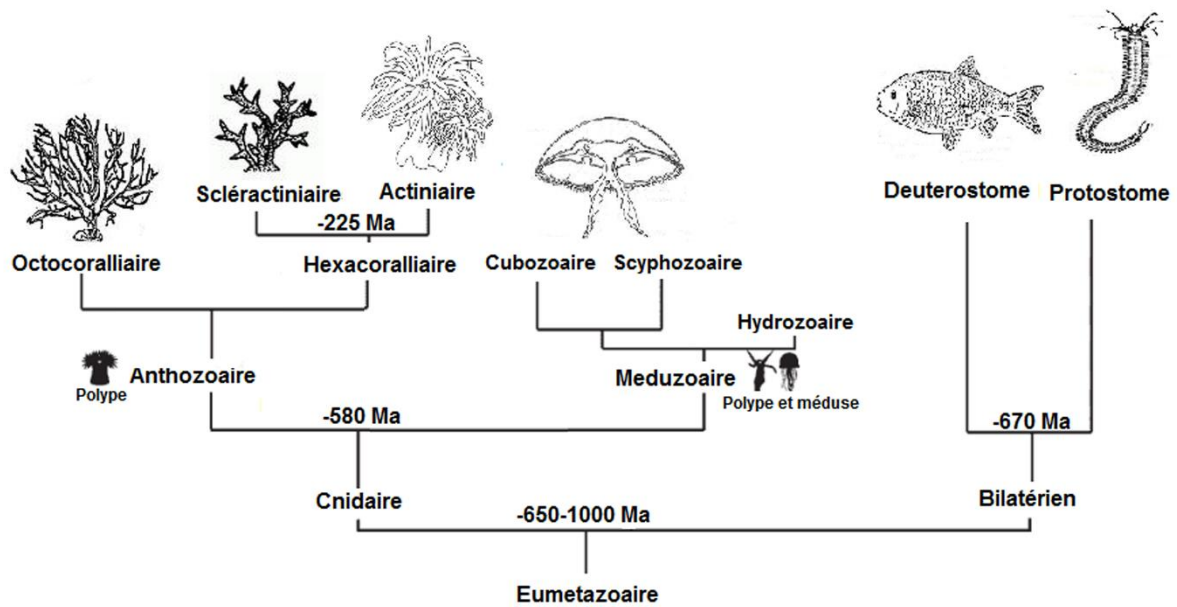


Figure 2: Evolution des Métazoaires à partir de l'ancêtre commun Eumétazoaire. Les âges indiquent le temps de divergence des nœuds. Les pictogrammes polype et méduse représentent les acquisitions à l'origine des nœuds. Modifié d'après (Darling et al. 2005).

L'étude des Cnidaires, comme organisme « basal » de la lignée des Métazoaires et groupe externe des Bilatériens, peut amener des informations clés sur l'évolution des génomes Métazoaires, depuis l'ancêtre commun Eumétazoaire.

1.2.2.2. Organisation générale des Cnidaires au niveau morphologique et histologique : cas des Anthozoaires

L'ensemble des Cnidaires a une symétrie radiale. On s'intéressera dans la suite de l'étude au stade polype, unique stade présent chez les Anthozoaires. Le polype peut être séparé en plusieurs parties que sont le pied, le corps, dont le pôle apical se termine par un renflement que l'on nomme orifice gastrovasculaire, et les tentacules distribués en couronne (Figure 3). Chez les Anthozoaires, les tentacules se répartissent selon une symétrie radiale d'ordre six chez les Hexacoralliaires et d'ordre huit chez les Octocoralliaires. Le pôle basal du pied est constitué d'un disque pédieux très musculueux qui permet la fixation au sédiment chez les organismes non

calcifiant. Chez les organismes calcifiants, l'épiderme calcicoblastique situé au niveau du pied, va sécréter la loge calcaire dans lequel se fixe le polype. Le corps contient la cavité gastrovasculaire divisée en septa et les gonades impliquées dans la reproduction.

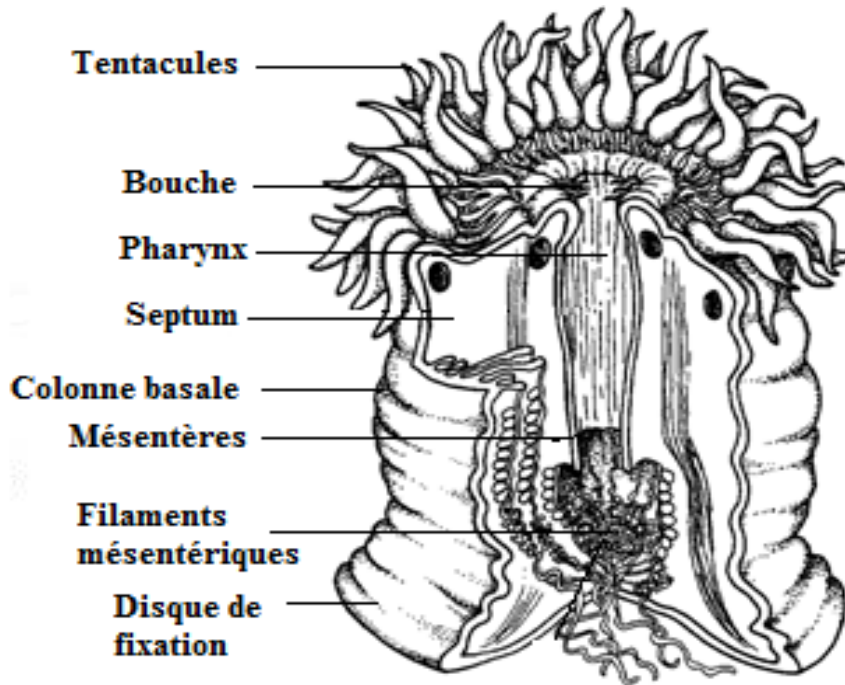


Figure 3: Schéma en coupe transversale d'un polype solitaire d'Anthozoaire. Modifié d'après Morin A. 1994

Les Anthozoaires effectuent une reproduction de type sexué et/ou asexué selon les espèces, avec parfois une alternance des deux types chez certaines espèces. Ainsi, les polypes peuvent se multiplier de façon asexuée par bourgeonnement, fission binaire et régénération, mais également par reproduction sexuée par émission de gamètes dans le milieu extérieur. La fusion des gamètes va constituer le zygote qui va se développer en une larve planula mobile. Chez les organismes coloniaux, la larve planula va constituer un organisme génétiquement différent des parents dont la colonie va se développer par division asexuée alors que d'autres espèces sont solitaires et ne forment pas de colonies.

Les Cnidaires sont des organismes diploblastiques, i.e. deux compartiments cellulaires : épiderme et gastroderme (Figure 4). Ces deux compartiments sont séparés par une couche majoritairement acellulaire appelée la mésoglyée (Collins et al., 2005).

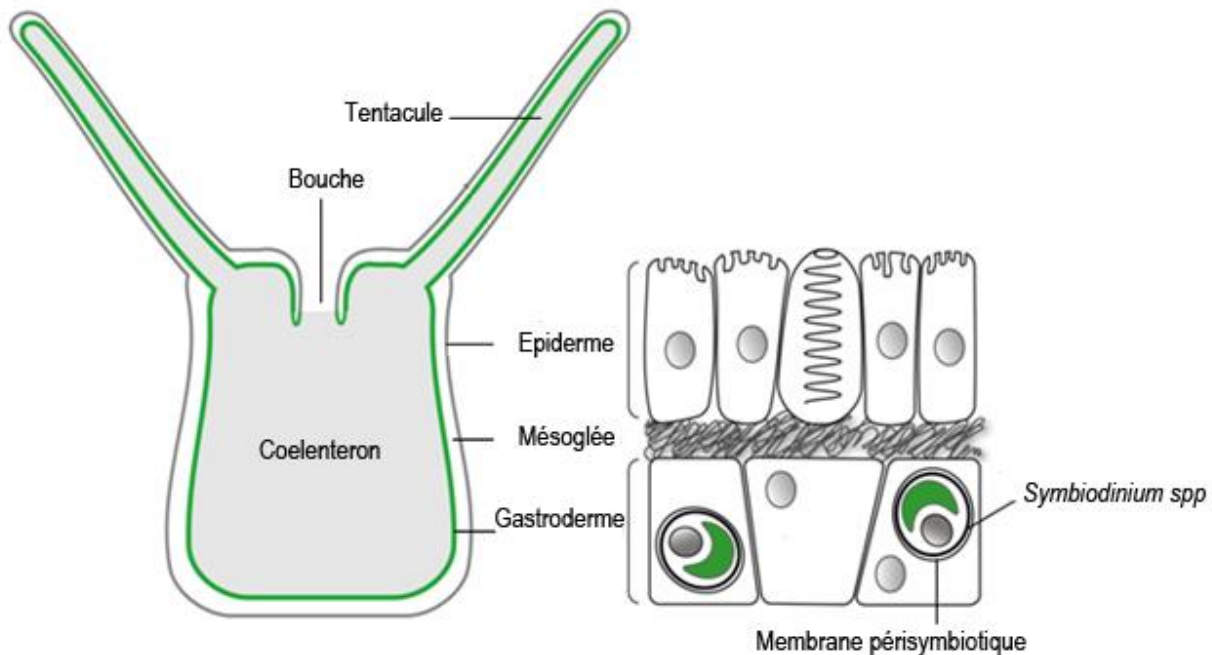


Figure 4: Schéma structurel des différents compartiments d'un polype d'Anthozoaire symbiotique non calcifiant et localisation intracellulaire des *Symbiodinium* spp (en vert sont représentés les thylakoïdes). Modifié d'après (Ganot et al., 2011)

L'épiderme pseudostratifié est le feuillet cellulaire faisant face au milieu extérieur, en contact direct avec l'eau de mer. Les Cnidaires (du grec *knide*= ortie et du latin *aria*= qui ressemble) tiennent leur nom de cellules caractéristiques retrouvées dans l'épiderme : les cnidocytes. Ces cellules sont constituées d'une vésicule nommée cnidocyte qui contient un filament urticant permettant la prédation. Chez les Anthozoaires, il existe différents types de cnidocytes. Les spirocytes, que l'on retrouve plus spécifiquement et en très grande quantité dans les tentacules spécialisés dans la prédation, et les nematocytes, organisés le plus souvent en cluster (Fautin and Mariscal, 1991). L'extrémité du cnidocyte est constituée d'un cil qui va permettre la détection d'une proie par contact, et ainsi déclencher la dévagination du filament participant à la capture de la proie. L'épiderme est également constitué de cellules à mucus qui excrètent ce dernier à la surface de l'épiderme. Ces cellules ont un rôle dans la capture de proie et un rôle protecteur via la présence d'une faune microbienne spécifique qui constitue la première ligne de défense de l'organisme (Shnit-Orland and Kushmaro, 2009). Le mucus est également un moyen de protection contre la dessiccation (Romaine et al., 1997) et est secrété dans une moindre mesure dans le gastroderme. Les cellules situées au pôle apical de l'épiderme sont également ciliées et permettent

de mettre en mouvement le mucus. Ainsi, l'épiderme joue un rôle dans la nutrition par la capture de matière organique dissoute (Schlichter, 1984). L'extrémité basale de l'épiderme est constituée d'un plexus nerveux diffus étroitement lié avec les cellules myoépithéliales, organisées en structure longitudinale et étroitement liées avec la mésoglée (Muscatine, 2012). Enfin, on note la présence de cellules interstitielles permettant la régénération du tissu (Figure 5).

La mésoglée est décrite comme généralement acellulaire (Chapman, 1953). Néanmoins, des études montrent que chez les Anthozoaires, elle est majoritairement constituée de collagène avec la présence des cellules nommées ameobocytes. Aucune étude n'a permis de définir le rôle précis de ces cellules. Le rôle de cellules sécrétrices de collagène, de cellules immunitaires, de cellules souches ou encore de cellules impliquées dans la phagoctyose a été proposé (Fautin and Mariscal, 1991; Larkman, 1984). Chez les Cnidaires calcifiants, la majorité des sclérites (éléments calcaires, unité de base du biosquelette) est contenue dans la mésoglée et le coenchyme (Dunkelberger and Watabe, 1974). Chez les Cnidaires, la densité des fibres de collagène et leur orientation dépendent des conditions environnementales, influençant sur les propriétés mécaniques de la mésoglée (Koehl, 1977).

Le gastroderme est également en contact avec l'eau de mer dans la cavité gastodermique. Ce tissu intervient majoritairement dans la digestion car il contient des nombreuses cellules digestives qui vont notamment libérer de nombreuses enzymes dans la cavité gastrovasculaire. Il est également le siège de l'endosymbiose avec les Dinoflagellés du genre *Symbiodinium*. Au niveau de l'organisation du tissu, on retrouve accolé à la mésoglée, des cellules musculaires circulaires, antagonistes aux muscles longitudinaux côté épiderme (Figure 5). Ce tissu contient également un plexus nerveux beaucoup plus réduit que dans l'épiderme, permettant la contraction des cellules musculaires préalablement décrites (Fautin and Mariscal, 1991). Le gastroderme contient également des cellules interstitielles et des cellules ciliées permettant d'induire un flux du liquide contenu dans la cavité gastrovasculaire.

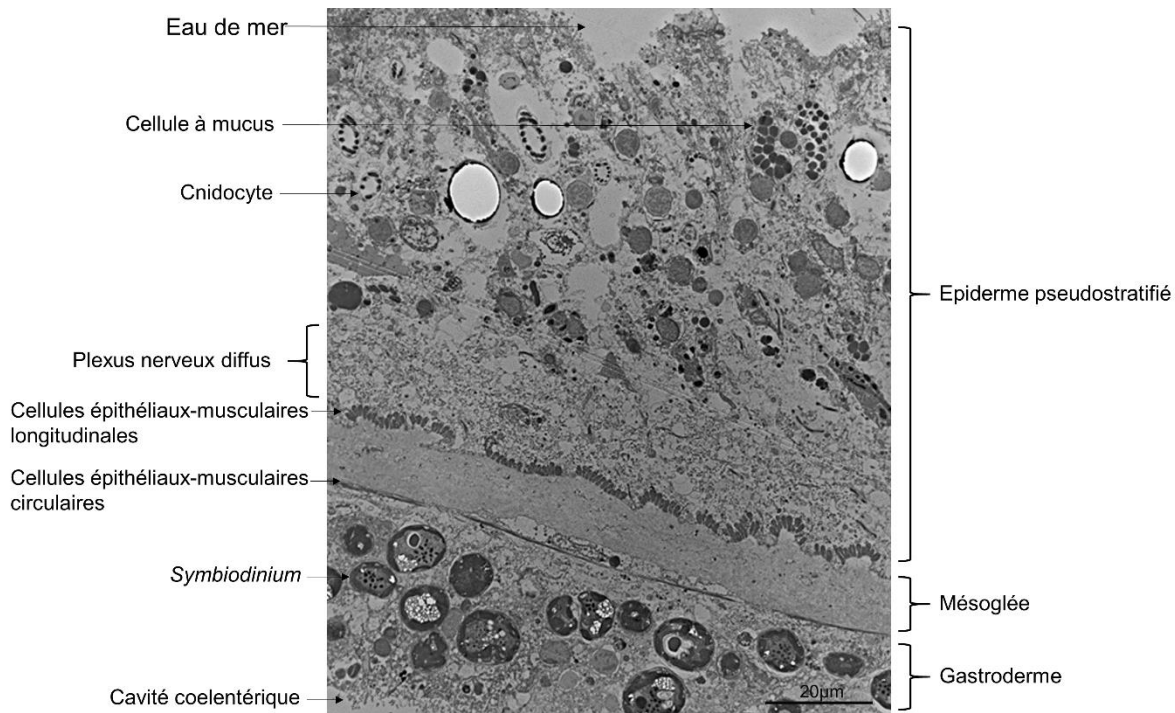


Figure 5: Coupe de tissu d'un tentacule d'*Anemonia viridis* effectuée au laboratoire, observée en microscopie électronique à transmission. Visualisation des différents types cellulaires et de l'organisation ultrastructurale du tissu.

1.2.2.3. Données génétiques disponibles : génomes et transcriptomes

En 2007 et 2010 ont été réalisés respectivement le premier séquençage d'un génome de Cnidaire : l'anémone de mer modèle *Nematostella vectensis* (Putnam et al., 2007), puis de l'hydre verte *Hydra* (Chapman et al., 2010). Récemment, le séquençage du génome du corail symbiotique *Acropora digitifera* (Shinzato et al., 2011) et de celui de l'anémone de mer symbiotique *Aiptasia* (Baumgarten et al., 2015), ont mis en évidence la présence de gènes potentiellement impliqués dans la relation symbiotique et dans le partage de voies métaboliques entre l'hôte et le symbiote. De nombreux projets de séquençage ont également permis de déterminer des cibles dans l'étude des mécanismes de la symbiose, via l'analyse du transcriptome à l'état symbiotique (Kortschak et al., 2003; Schwarz et al., 2008b) ou par comparaison entre des individus symbiotiques et aposymbiotiques (Ganot et al., 2011; Kuo et al., 2004; Kuo et al., 2010). De nombreuses données de transcriptomique ont été réalisées dans le but de suivre l'expression de gènes cibles potentiellement impliqués dans la rupture de la symbiose (voir partie 1.3.4.2).

D'une façon globale, la comparaison des génomes de Cnidaires entièrement séquencés montre que l'évolution de la diversité des Cnidaires est accompagnée de néofonctionnalisation et de pertes de gènes (Steele et al., 2011). De plus, le répertoire de gènes et la structure intron/exon des Cnidaires, sont plus proches de l'homme, que ceux des organismes modèles *Drosophila melanogaster* et *Caenorhabditis elegans* (Kortschak et al., 2003). Dans certains cas, les Cnidaires ont conservé des gènes orthologues présents chez les Deutérostomes (e.g. *Homo sapiens*) mais qui ont été perdus dans la lignée Protostomiens (e.g. *Drosophila melanogaster*, *Caenorhabditis elegans*) (Putnam et al., 2007). Ces résultats suggèrent que la plupart des gènes conservés entre Cnidaires et Bilatériens, comme par exemple ceux impliqués la mise en place de caractères clés des Bilatériens (mésoderme, symétrie bilatérale), ainsi que les mécanismes impliqués dans la régulation de ces derniers, sont apparus avant la divergence Cnidaire-Bilatériens, il y a 600 millions d'années (Technau and Schwaiger, 2015). L'étude de ces organismes en tant que groupe frère des Bilatériens est donc pertinente dans la compréhension de certains processus conservés chez l'homme.

1.2.3. Les Dinoflagellés du genre *Symbiodinium* : le partenaire symbiotique

1.2.3.1. Position phylogénétique et diversité génétique

Les Dinoflagellés sont des organismes eucaryotes unicellulaires et appartiennent avec les Apicomplexes et les Ciliées, le règne des Alvéolés (Lecointre and Guyader, 2006). Ce sont des organismes marins ou dulçaquicoles qui possèdent une alternance de forme mobile grâce à la présence de deux flagelles et de forme coccoïde non mobile, au cours de leur cycle de vie (Freudenthal, 1962).

En 1975, McLaughlin et Zahl (Zahl and McLaughlin, 1957), isolent pour la première fois des Dinoflagellés à partir de la méduse *Cassiopeia xamachana*. La souche maintenue en culture a été décrite comme *Symbiodinium adriaticum* puis renommée *Symbiodinium microadriaticum*. Ces organismes unicellulaires symbiotiques sont classés dans l'ordre des Suessiales, dans la famille des Symbiodiniacées. Les espèces du genre *Symbiodinium* vivent à l'état libre dans l'eau de mer mais peuvent également entrer en symbiose avec une grande variété d'hôtes tels que les Foraminifères, les Mollusques, les Platyhelminthes, les Porifères et les Cnidaires (Coffroth and Santos, 2005). A

l'origine, plusieurs espèces ont été décrites, se basant sur des critères morphologiques (Freudenthal, 1962; Trench and Blank, 1987), chacune possédant des caractéristiques ultra-structurales et physiologiques distinctes (Coffroth and Santos, 2005). L'apparition de nouvelles techniques d'identification moléculaire ont révélé une forte diversité à l'intérieur du genre *Symbiodinium* (Baker, 2003; LaJeunesse et al., 2004, 2005; Santos et al., 2003). Basée sur l'étude des sous-unités 18S et 28S de l'ADN ribosomal, neuf clades ont été définis (Carlos et al., 1999; LaJeunesse, 2001; Pochon et al., 2004; Rowan and Powers, 1991; Takabayashi et al., 2004). Plus récemment, les analyses des « espaceurs internes transcrits » (ITS 1 et 2) et des sous-unités ribosomales ont révélé une diversité intra-clade importante (Baker, 2003; Coffroth and Santos, 2005; LaJeunesse, 2001). Une phylogénie non exhaustive par comparaison entre les résultats acquis via des marqueurs nucléaires et chloroplastiques a été publiée récemment (Pochon and Gates, 2010) (Figure 6). En effet, l'étude des gènes chloroplastiques et mitochondriaux a démontré la présence d'une grande diversité de souches au sein des clades précédemment identifiés (Lesser, 2013). Le développement de marqueurs spécifiques permettra à terme d'identifier la spécificité hôte/symbiote et de préciser les processus de coévolution entre les deux partenaires symbiotiques (Pochon et al., 2012). Parmi cette grande diversité, des études ont montré que certains hôtes étaient capables d'entrer en symbiose avec des *Symbiodinium* phylogénétiquement distants, alors que d'autres espèces de Cnidaires étaient à priori spécialistes d'un type donné de *Symbiodinium* (Baker, 2003). Initié par les travaux de Rowan en 1997, il a été démontré que chaque espèce de *Symbiodinium* possédait des optimums photosynthétiques et une gamme de tolérance variable en fonction des conditions environnementales (Rowan et al., 1997).

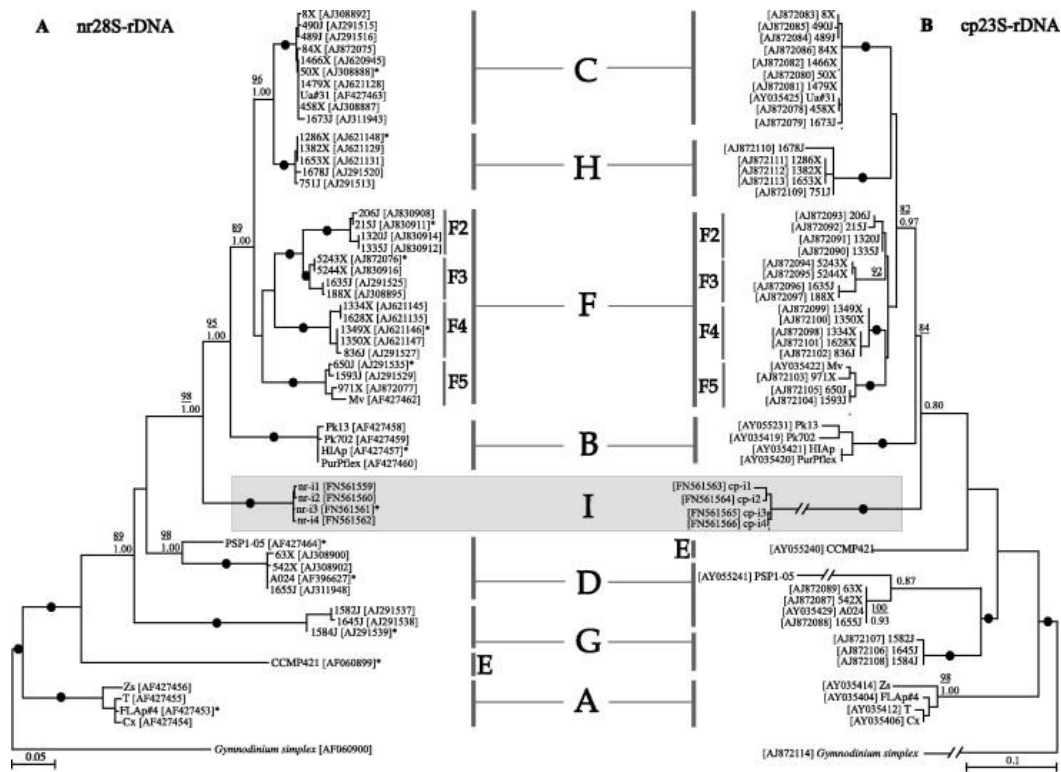


Figure 6: Distribution phylogénétique par maximum de vraisemblance des différents clades du genre *Symbiodinium* identifiés, phylogénie basée sur des données issues de la sous unité 28S du ribosome nucléaire (A) et de la sous unité 23S chloroplastique (B). (Pochon et Gates 2010)

En effet, il existe une régulation bathymétrique, saisonnière et phylo-géographique des différents clades de *Symbiodinium* dans le milieu extérieur. Ainsi, en Méditerranée, le clade dominant est le clade A tempéré (Casado-Amezúa et al., 2014; Visram et al., 2006b). La régulation de la proportion des différentes souches de *Symbiodinium* présentes au sein de l'organisme permettrait donc une adaptation aux conditions fluctuantes de l'environnement et un mécanisme d'adaptation lors d'épisodes de stress. En effet, lors d'un stress température chez *Montastrea annularis* et *Montastrea faveolata* qui possèdent les clade A, B et C, la concentration des symbiotes du type C a décliné en comparaison avec les populations de type A et B (Rowan et al., 1997). Ceci démontre d'une part les différences physiologiques existant entre les différentes espèces de *Symbiodinium* et d'autre part la capacité de l'hôte à réguler la diversité de symbiotes présents dans ses tissus. La pression sélective exercée par les conditions environnementales serait donc un mécanisme majeur dans le processus de coévolution dans la symbiose Cnidaire-Dinoflagellés (Finney et al., 2010).

1.2.3.2. Organisation morphologique et histologique des *Symbiodinium*

Les Dinoflagellés possèdent deux stades majeurs dans leur cycle de vie, que l'on nomme mastigote pour le stade motile et cocoïde pour le stade non-motile (Freudenthal, 1962). Ces deux stades correspondent à des phases du cycle cellulaire. En effet, le stade G1 correspond à la phase mobile et est caractérisé par la présence de plaques thécales composées essentiellement de cellulose et par la présence de deux flagelles permettant la motilité (Kwok and Wong, 2003) (Figure 7B). Ce stade correspond également au niveau physiologique à la phase active de croissance, avec une activité forte du métabolisme carboné et lipidique. Lorsque la taille de la cellule atteint un seuil spécifique, les Dinoflagellés perdent leurs flagelles et entrent dans la phase S. La suite du cycle cellulaire correspond à la phase G2 et M, au cours de laquelle la division cellulaire se produit (Fitt and Trench, 1983b).

Au niveau structural, les Dinoflagellés du genre *Symbiodinium* ont une taille comprise entre 6 et 13 μm en fonction des espèces (Wakefield et al., 2000a). Ils sont constitués d'une paroi cellulaire en contact avec le milieu extérieur et d'une membrane plasmique. La morphologie intracellulaire est caractérisée par un chloroplaste multilobé avec des thylakoïdes arrangés en parallèle, des carboxysomes, un pyrénoïde et des mitochondries essentiellement concentrées au centre de la cellule. Le noyau est large et possède une chromatine condensée ce qui permet de bien observer les chromosomes en microscopie électronique (Figure 7A).

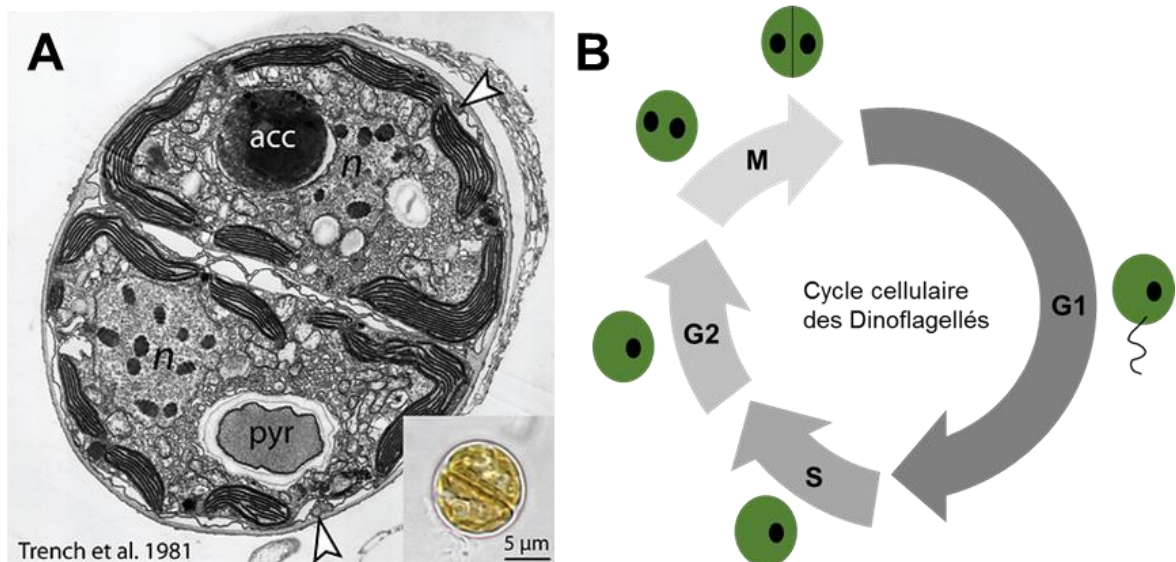


Figure 7: (A) Visualisation par microscopie électronique d'une cellule de *Symbiodinium* spp (n: noyaux condensés; pyr: pyrénoïde; acc: corps d'accumulation ; flèche du bas : paroi cellulaire ; flèche du haut : membrane plasmique) (Trench et al., 1981). (B) Cycle cellulaire d'un Dinoflagellé (d'après Kwok et al., 2003).

1.2.3.3. Génomes et transcriptomes chez le genre *Symbiodinium*

Malgré l'importance primordiale dans le succès évolutif des Cnidaires symbiotiques, peu d'études ont été menées afin d'obtenir des données sur le génome et le transcriptome des espèces du genre *Symbiodinium*, en comparaison avec les données génomiques acquises pour leur hôtes.. A l'heure actuelle, seul le génome de *Symbiodinium minutum* a été séquencé (Shoguchi et al., 2013) et assemblé en 616 Mbp, alors que la taille estimée du génome serait de 1 500 Mbp. Pour des tailles de génome estimées entre 1 500 et 245 000 Mbp chez les Dinoflagellés (Lin, 2011; Wisecaver and Hackett, 2011), *Symbiodinium minutum* a donc été choisi pour la petite taille estimée de son génome. La grande taille des génomes des Dinoflagellés explique en partie la limitation actuelle des projets de séquençage. Une autre limitation aux projets de génomique réside également dans la grande diversité de clades présents dans le genre *Symbiodinium* et capable de rentrer en symbiose avec des organismes du groupe des Cnidaires. Il est donc difficile d'établir un consortium autour de l'espèce de *Symbiodinium* modèle à étudier dans l'association Cnidaire-Dinoflagellés. Une étude de transcriptomique a récemment comparé deux espèces de *Symbiodinium* du clade A et du clade B (Bayer et al., 2012). Cette étude a permis d'identifier environ 56 000 gènes chez les deux espèces, en accord avec les résultats prédits par le séquençage du génome.

Dans le but de connaître les gènes potentiellement exprimés par *Symbiodinium* en condition symbiotique, Voolstra et ses collaborateurs ont comparé le transcriptome d'une espèce de *Symbiodinium* du clade A en culture et le transcriptome d'une espèce de *Symbiodinium* du clade C isolé à partir d'un hôte (Voolstra et al., 2009b). Parmi les transcrits identifiés, on retrouve des gènes impliqués dans le métabolisme énergétique et glucidique, dans la transduction de signaux extracellulaires et des gènes impliqués dans la communication cellule-cellule. Récemment, deux études ont analysé la réponse de *Symbiodinium* au cours de différents stress température et ont permis de mettre en évidence la régulation de gènes impliqués dans l'adhésion cellulaire, la modification du cytosquelette, des modifications post-transcriptionnelles, le métabolisme et les gènes de réponse au stress (Leggat et al., 2007; Xiang et al., 2015).

1.3. Fonctionnement, échanges trophiques

Du point de vue physiologique, l'embranchement des Cnidaires se distingue par la capacité d'une majorité d'espèces (e.g. coraux mous et durs, anémones de mer, méduses et hydrozoaires) à vivre en endosymbiose avec des eucaryotes unicellulaires photosynthétiques du genre *Symbiodinium* sp. (Freudenthal, 1962) (Dinoflagellata, Protozoa), et cela depuis au moins 65 millions d'années d'après les paléontologistes. Ces organismes Dinoflagellés sont acquis par transfert horizontal (Babcock et al., 1986) et/ou vertical (Davy and Turner, 2003) selon les espèces. Il a été démontré récemment que l'ancêtre commun des Scléactiniaires était vraisemblablement colonial et symbiotique (Barbeitos et al., 2010). Au cours de l'évolution, la capacité à vivre en symbiose aurait été perdue chez certains Scléactiniaires et l'on peut formuler l'hypothèse d'une évolution similaire chez les Actiniaires. La symbiose se définit ainsi comme une association étroite et durable entre les deux partenaires, l'hôte Cnidaire hétérotrophe et le symbiote Dinoflagellé autotrophe. Cette particularité physiologique a assuré leur succès évolutif et l'on retrouve des organismes Cnidaires symbiotiques dans les latitudes tempérées et subtropicales. Le symbiote se localise dans les cellules gastrodermiques de l'hôte, dans une vacuole ou symbiosome, formée par une membrane périsymbiotique d'origine animale, acquise lors de l'internalisation par phagocytose du symbiote (Wakefield and Kempf, 2001b).

On distingue trois grandes phases d'étude dans la relation symbiotique, qui sont la mise en place de la symbiose par des mécanismes de reconnaissance et d'internalisation, le maintien de la symbiose en conditions stables, et la rupture de la symbiose lors d'un stress anthropique ou environnemental.

1.3.1. Reconnaissance et spécificité d'interaction

La mise en place de la symbiose requiert d'une part la reconnaissance par l'hôte de la cellule de *Symbiodinium* présente à l'état libre dans le milieu extérieur, ainsi que la mise en place des mécanismes permettant la spécificité d'interaction. La reconnaissance intervient par des mécanismes moléculaires de reconnaissance extracellulaire entre les motifs exprimés à la surface de *Symbiodinium* ; les motifs moléculaires associés aux pathogènes (MAMP) et les récepteurs de reconnaissance de motifs moléculaires (PRR) exprimés par l'hôte (en revue dans Davy et al., 2012;

Toledo-Hernández and Ruiz-Diaz, 2014). Les études *in silico* ont permis de répertorier un ensemble de gènes présents chez l'hôte qui pourraient avoir un rôle dans le mécanisme de reconnaissance MAMP/PRR. En effet, 92 lectines de type C ont été identifiées dans le génome de *Nematostella vectensis* (Wood-Charlson and Weis, 2009), mais également chez des Cnidaires symbiotiques tels que *Montastrea faveolata* (Schwarz et al., 2008a), chez l'octocorail *Sinularia lochomodes* (Jimbo et al., 2000; Jimbo et al., 2005; Jimbo et al., 2013), le corail *Ctenactis echinata* (Jimbo et al., 2010), chez *Acropora millepora* (Kvennefors et al., 2008), *Pocillopora damicornis* (Vidal-Dupiol et al., 2009) et chez *Anthopleura elegantissima* (Schwarz and Weis, 2003, 32).

Chez *Symbiodinium*, des études *in vivo* ont montré la sécrétion de composés glycoconjugués en culture et en condition symbiotique (Lin et al., 2000; Markell and Trench, 1993a; Markell and Wood-Charlson, 2010; Markell et al., 1992). Une caractérisation par cytométrie de flux de motifs exprimés à la surface des *Symbiodinium* a également mis en évidence la grande diversité des motifs exprimés (Logan 2010). De plus, ces études montrent qu'il existe une spécificité des motifs exprimés et une variabilité du glycome dans chaque espèce (Markell and Trench, 1993a; Markell and Wood-Charlson, 2010). Les premières études fonctionnelles relatives aux mécanismes de reconnaissance ont été initiées par les travaux chez l'hydre, en symbiose avec les chlorelles. En effet, ils ont montré qu'une inhibition des motifs exprimés à la surface des symbiotes réduisait de façon importante la mise en place de la symbiose (Pool, 1979). De la même façon, le blocage des récepteurs impliqués dans les mécanismes de reconnaissance chez l'hôte bloquerait l'initiation de la mise en place de la symbiose (Meints and Pardy, 1980). En effet, le blocage du ligand lectine associé au récepteur mannose/glycane, a permis de mettre en évidence le rôle de l'interaction lectine/récepteur glycane dans la mise en place de la symbiose. De la même façon, chez les Anthozoaires, le masquage ou la suppression des motifs glycanes à la surface des *Symbiodinium* réduisent significativement le succès de la mise en place de l'association symbiotique (Lin et al., 2000; Wood-Charlson et al., 2006).

Néanmoins, au niveau moléculaire, peu d'études ont permis *in hospite* la colocalisation directe au niveau de l'interface hôte/symbiote de protéines potentiellement impliquées dans la reconnaissance des symbiotes. La protéine Sym32 (adhésion cellulaire) a été démontrée comme étant associée avec la membrane périsymbiotique, et pourrait donc activement participer à la reconnaissance des *Symbiodinium* (Schwarz and Weis, 2003). De la même façon, la protéine D-

galactose-binding lectine, (SLL-2) purifiée de l'octocorail *Sinularia lochomodes*, a été retrouvée autour des symbiotes dans le gastroderme (Jimbo et al., 2000). Chez le corail *Acropora millepora* et *Pocillopora damicornis*, deux lectines ont également été découvertes associées autour des symbiotes dans le gastroderme (Kvennefors et al., 2008; Vidal-Dupirol et al., 2009). Ainsi, l'ensemble des motifs impliqués dans la reconnaissance hôte/symbiote a fait l'objet d'une revue dont est tiré le schéma ci-dessous (Weis et al., 2008a)(Figure 8).

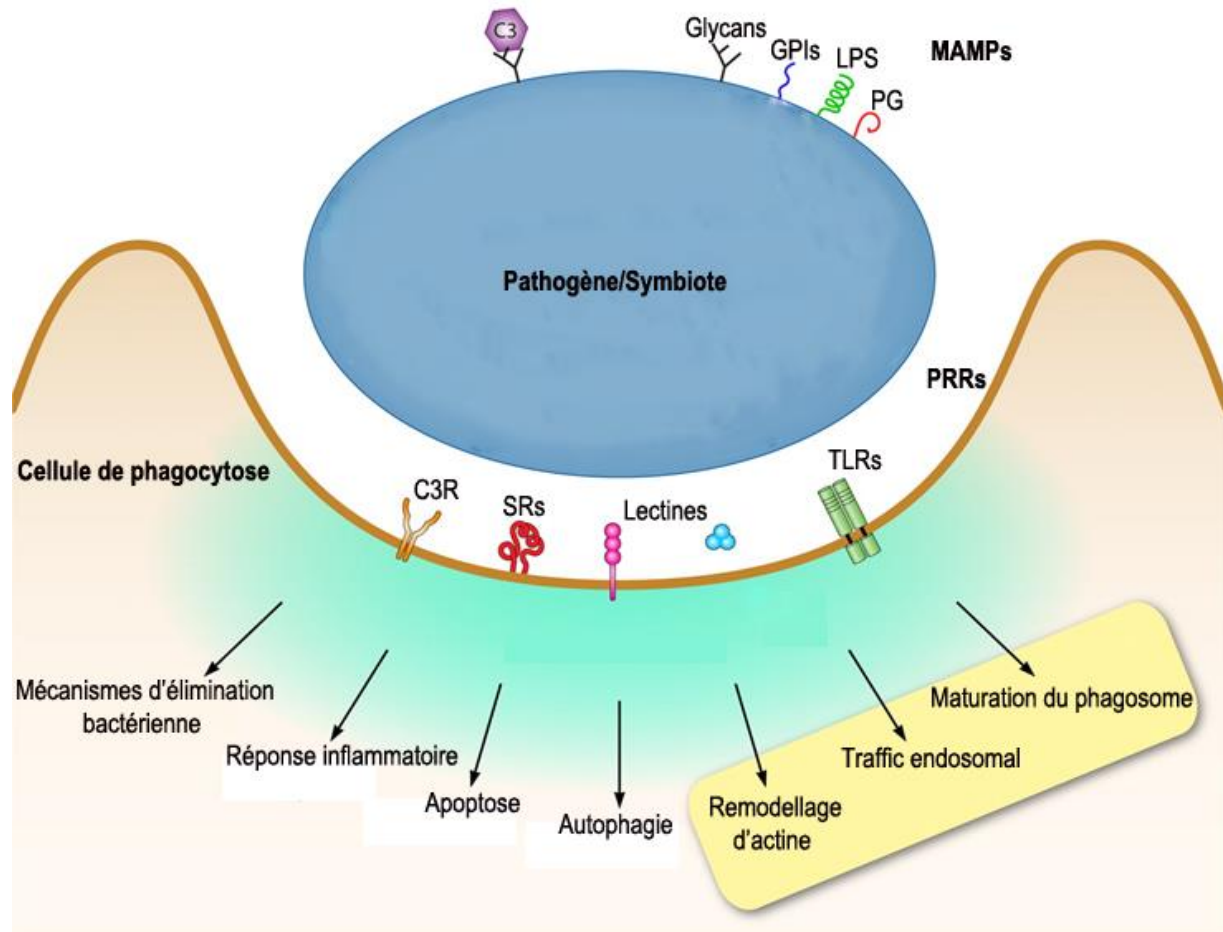


Figure 8: Signalisation hôte/pathogène lors de l'internalisation d'un pathogène ou d'un symbiote chez les Cnidaires. Le pathogène ou le symbiote exprime à sa surface cellulaire des motifs moléculaires associés aux pathogènes (MAMPs) qui vont être reconnus par les récepteurs de reconnaissance de motifs moléculaires (PRRs). récepteur au Complément C3 (C3R), récepteurs scavengers (SRs), Toll-like récepteurs (TLRs), ancrage glycosylphosphatidylinositol (GPIs), lipopolysaccharide (LPS), peptidoglycan (PG)(Modifié d'après Davy et al., 2012))

Plus récemment, l'étude du génome d'*Aiptasia* a souligné la présence de gènes codant des Cnidarian ficolin-like proteins (CniFLs), absents du génome de l'anémone non symbiotique *N.vectensis*. De plus, il apparaît que ces gènes sont surexprimés dans la condition aposymbiotique

et que leur expression diminue après la mise en place de la symbiose (Baumgarten et al., 2015). Ces protéines possèdent un domaine immunoglobuline et seraient alors impliquées dans la reconnaissance et la mise en place de la symbiose avec une souche compatible de *Symbiodinium*.

1.3.2. Processus d'internalisation

Le site d'internalisation des symbiotes n'est pas clairement défini. Lors de l'acquisition par transmission verticale (lors de la reproduction sexuée), des études ont décrit la présence de symbiotes au niveau de la planula (Davy and Turner, 2003). Cette population pourrait servir de stock initial. Néanmoins, l'acquisition de symbiotes par transfert horizontal (acquisition de l'environnement) se ferait initialement par ingestion dans la cavité gastro-vasculaire (Hirose et al., 2008), suivi d'un processus d'ingestion cytoplasmique dans les cellules du gastroderme par un processus de phagocytose. Plusieurs études sur l'hydre (McNeil, 1981; McNeil et al., 1981; Muscatine et al., 1974) et les Anthozoaires (Weis et al., 2001) ont montré que la phagocytose était le mécanisme d'internalisation des *Symbiodinium* dans les cellules de l'hôte (Muscatine et al., 1975; Rinkevich, 2004). Un plus grand intérêt a été porté par la communauté scientifique sur la capacité du symbiote à persister dans les cellules du gastroderme de l'hôte. Définie comme un mécanisme de l'immunité innée, la phagocytose permet l'élimination de pathogènes (Aderem and Underhill, 1999). La maturation du phagosome entraîne la fusion de ces derniers avec les lysosomes, responsables de l'élimination de l'organisme phagocyté par déversement d'enzymes lytiques. Il paraît alors essentiel de déterminer les mécanismes permettant l'arrêt de la maturation du phagosome, entraînant la persistance des symbiotes dans les cellules du gastroderme, dans des conditions symbiotiques stables.

Un premier élément de réponse a été fourni par des études dans le modèle d'infection des *Paramecia* par *Chlorella*, mettant en évidence une inhibition de la fusion phagosome-lysosome (en revue dans Kodama and Fujishima, 2010). Chez l'hydre, l'inoculation de symbiotes tués à la chaleur est suivie d'une dégradation lysosomale de ces symbiotes, alors que l'inoculation de symbiotes viables entraîne un arrêt de la maturation des phagosomes (Hohman et al., 1982). Des résultats similaires ont été démontrés chez Cassiopée (Fitt and Trench, 1983a). Ces résultats mettent en évidence que les motifs exprimés à la surface des symbiotes ne sont pas suffisants à la persistance de la relation symbiotique. L'arrêt de la maturation des phagosomes est donc un

mécanisme actif mis en place lors de l'internalisation via la régulation du trafic vésiculaire. Les études menées sur la famille des protéines Rab ont mis en évidence une expression spécifique de ces protéines au cours de l'internalisation et du maintien de la symbiose chez *Aiptasia pulchella*. Les protéines Rab, activées chez les vertébrés par liaison d'une molécule de GTP, sont impliquées dans la régulation du trafic vésiculaire intracellulaire et la dynamique des membranes (Novick and Zerial, 1997). Les études de Chen et ses collaborateurs ont montré lors de la mise en place de la symbiose la co-localisation des protéines Rab 4 et Rab5, uniquement au niveau des *Symbiodinium* viables (Chen et al., 2004, 5; Hong et al., 2009b, 4). La présence de ces protéines bloque l'ancrage de Rab7, retrouvé uniquement sur des symbiotes inactivés par choc température ou par traitement chimique (Chen et al., 2003). Cette étude souligne également que Rab11, impliqué dans les processus de recyclage des endosomes (Novick and Zerial, 1997), est présent sur les symbiotes endommagés et absent des symbiotes viables (Chen et al., 2005). Des mécanismes similaires, impliquant la manipulation du trafic vésiculaire de l'hôte par des bactéries pathogènes, ont montré l'importance de l'inhibition de l'expression des Rab GTPases dans les mécanismes d'arrêt de la dégradation lysosomale, du transport intracellulaire, de croissance et de différenciation (Stein et al., 2012). L'arrêt de la maturation des phagosomes entraîne donc la persistance des symbiotes dans les cellules du gastroderme de l'hôte. La membrane du phagosome devient alors la membrane pérисymbiotique et donne la structure fonctionnelle nommée symbiosome, composée de la membrane pérисymbiotique et du symbiote viable. Entre la membrane pérисymbiotique d'origine animale (Rands et al., 1993a; Wakefield and Kempf, 2001b) et le symbiote, se trouve un espace pérисymbiotique dont le pH est légèrement plus acide que le cytoplasme animal (Venn et al., 2009). Des études de microscopie électronique ont montré que le symbiote est entouré d'une membrane complexe résultant de la succession des cycles cellulaires du symbiote, secrétant une nouvelle membrane à chaque bouclage du cycle cellulaire (Wakefield et al., 2000a). La membrane pérисymbiotique est donc considérée comme l'interface de communication cellulaire entre les deux partenaires.

1.3.3. Fonctionnement de la symbiose à l'état stable

En condition symbiotique stable, l'hôte offre aux symbiotes un abri contre la prédation et une exposition optimale à la lumière nécessaire à la photosynthèse. Il leur transfère également des acides aminés, des éléments minéraux tels que l'azote, le phosphore, le soufre, et le principal composé nécessaire à la photosynthèse, le carbone inorganique (Yellowlees et al., 2008a). En contrepartie, le symbiote transfère vers son hôte du carbone organique issu de la photosynthèse, sous différentes formes telles que du glycérol, du glucose, de l'acide glutamique et des lipides (Figure 9)

Ces éléments sont utilisés comme des constituants structurels, de réserve, ou des composants de la matrice organique comme le mucus. L'apport trophique du symbiote à l'hôte Cnidaire peut aller jusqu'à 90% du carbone fixé par les Dinoflagellés lors de la photosynthèse (Falkowski et al., 1984; Furla et al., 2005). Parmi les éléments transférés par le symbiote, on retrouve des molécules de faible poids moléculaire, tels que le glycérol, élément majoritairement transféré (Burriesci et al., 2012), le glucose, des acides aminés et des acides organiques (Muscatine and Cernichiaro, 1969; Trench, 1971). En effet, l'étude des voies de biosynthèse des acides aminés dans le génome d'*Aiptasia* montre que les Anthozoaires n'ont pas la capacité de synthétiser 12 des 20 acides aminés (Baumgarten et al., 2015; Lehnert et al., 2014). Des molécules plus complexes sont également transférées comme des acides gras libres (Papina et al., 2007), des glycoprotéines et des glycolipides (Markell and Trench, 1993b). Des études ont également rapporté le transfert bidirectionnel de composés via des gouttelettes lipidiques (Kellogg and Patton, 1983; Patton et al., 1977; Peng et al., 2011). Ces échanges métaboliques entre les deux partenaires, ainsi que les nombreuses adaptations génétiques et moléculaires nécessaires à la communication entre les deux partenaires, expliquent en partie le succès évolutif de l'association Cnidaire/Dinoflagellés (Davy et al., 2012a; Weis and Allemand, 2009).

Le carbone inorganique, élément indispensable à la photosynthèse et à la biosynthèse du squelette (CaCO_3), est transporté majoritairement sous la forme de bicarbonate (HCO_3^- à 2.2 mM) par rapport au dioxyde de carbone (30 μM) dans l'eau de mer. Les travaux pionniers de Goreau (Goreau, 1959) ont permis la découverte chez les Anthozoaires de l'anhydrase carbonique (CA), enzyme capable de convertir le bicarbonate en CO_2 , et la mise en évidence du transport de carbone

inorganique du milieu extérieur vers les cellules du gastroderme de l'hôte. L'augmentation de la concentration intracellulaire de carbone inorganique dissous, permet sa fixation par la rubisco (ribulose-1,5-bisphosphate carboxylase / oxygenase) dans les cellules du symbiote. Ces enzymes ont été très étudiées dans le contexte de la mise en place de la biocalcification et des processus physiologiques impliqués dans la symbiose, notamment depuis le développement des techniques de biologie moléculaire depuis les années 2000 (en revue dans (Bertucci et al., 2013)). L'activité des CA est augmentée d'un facteur dix en condition symbiotique par rapport à la condition aposymbiotique (Bertucci et al., 2013; Weis and Reynolds, 1999; Weis et al., 1989). Ces protéines ont été retrouvées par immunofluorescence indirecte au niveau des symbiotes (Moghrabi et al., 1996; Venn et al., 2009) et de l'épiderme calicoblastique chez *Stylophora pistillata* (Moya et al., 2008). Ainsi, l'activité des CA est sous le contrôle de l'état symbiotique (Ganot et al., 2011). En effet, chez *Anemonia viridis*, on retrouve deux CA surexprimées en présence de symbiotes. De façon plus intéressante, l'une, CA2-c, est cytoplasmique et exprimée dans l'épiderme et le gastroderme alors que l'autre, CA2-m est membranaire et exprimée préférentiellement dans le compartiment symbiotique, à priori au niveau de la membrane périsymbiotique. Cette adaptation moléculaire permet la mise en place d'une photosynthèse active par les symbiotes au sein des cellules de l'hôte.

En conditions stables, les photosynthétats sont produits et retransmis vers les cellules de l'hôte sous forme de carbone organique (Muscatine and Hand, 1958). Dépendant des organismes, les apports autotrophes issus des symbiotes représentent de 60 à 90% des apports trophiques totaux de l'organisme. Une série d'études menées par Trench en 1971 a montré que la quantité de métabolites transférés par le symbiote était conditionnée par la présence de l'hôte et par le clade de *Symbiodinium*. Ces résultats démontrent d'une part que l'hôte contrôle le type et la quantité des métabolites excrétés par le symbiote mais également que les différents clades de *Symbiodinium* diffèrent biochimiquement (Trench, 1971). Les composés transférés par le symbiote sont majoritairement des composés sous forme de glycérol, mais également des acides aminés essentiels, du glucose, de succinate, fumarate, des glycoprotéines et une grande variété de lipides (Lewis and Smith, 1971; Yellowlees et al., 2008a).

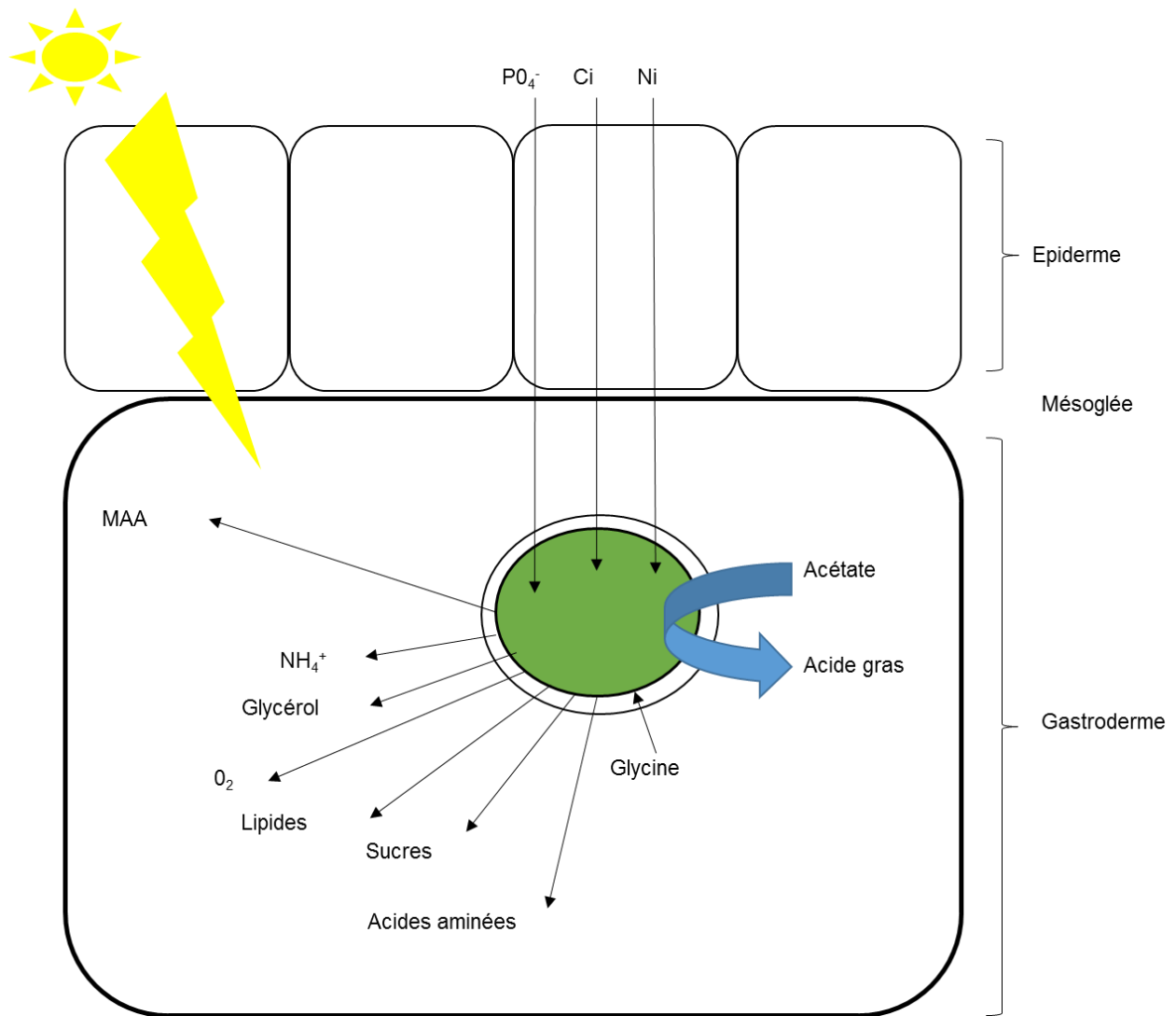


Figure 9: Echanges métaboliques dans la symbiose Cnidaire/Dinoflagellés (Modifié d'après Gordon et Leggat 2010). Les flèches noires pleines indiquent des transferts actifs

Parmi les éléments transférés par le symbiote, les lipides représentent une grande majorité des apports photosynthétiques (Imbs, 2013; Venn et al., 2008). Ce transfert est primordial pour l'hôte, celui-ci ne possédant pas toutes les enzymes nécessaires à la synthèse *de novo* de stérols (Baumgarten et al., 2015; Tarrant et al., 2009). Ainsi, de nombreuses voies de biosynthèse des dérivés des stérols partagés entre l'hôte et le symbiote ont été mises en évidence (Baumgarten et al., 2015; Harland et al., 1991a; Sarma et al., 2009). Cependant, les acteurs moléculaires qui régulent ce transport bidirectionnel à travers la membrane pérисymbiotique restent à définir (Davy et al., 2012a; Imbs et al., 2010; Peng et al., 2010a). Un autre exemple de voie de biosynthèse

partagée entre l'hôte et le symbiote a déjà été largement décrit. Il s'agit des mycosporine-like amino acids (MAA), des métabolites secondaires permettant d'atténuer les effets des UV. Certains précurseurs de MAA sont synthétisés par les symbiotes puis transférés à l'hôte. Celui-ci les transforme en une grande variété de MAA, assurant ainsi une protection contre l'exposition à la lumière, nécessaire à l'activité photosynthétique des symbiotes (Ferrier-Pages et al., 2007).

Ainsi, le transport du symbiote vers l'hôte permet le maintien d'une relation mutualiste stable. Réciproquement, le transport actif de l'hôte vers le symbiote participe à la régulation de la population de symbiotes intracellulaires via la maîtrise du cycle cellulaire du symbiote. Des études ont montré que le cycle cellulaire du symbiote était réduit de dix fois *in hospite* en comparaison avec les *Symbiodinium* à l'état libre (Jones and Yellowlees, 1997; Wilkerson et al., 1983; Wilkerson et al., 1988). Plusieurs mécanismes de régulation par l'hôte ont été proposés, parmi lesquels le contrôle de la disponibilité en azote (McAuley, 1987), la limitation en carbone inorganique (Blank and Muscatine, 1987; Neckelmann and Muscatine, 1983) et la synthèse d'un facteur d'inhibition de croissance (HFR) (McAuley and Muscatine, 1986). Il apparaît également que les symbiotes pourraient contrôler leur propre population *in hospite* (McAuley, 1982a; Muscatine and Pool, 1979; Rodriguez-Lanetty et al., 2006). Cependant, il existerait en fait une multitude de facteurs de contrôle de la production photosynthétique encore non décrite (revue (Davy et al., 2012a).

Le maintien de l'endosymbiose et les échanges métaboliques entre les deux partenaires de la symbiose nécessite de nombreuses adaptations fonctionnelles. Dans les conditions permettant la symbiose, l'hôte exprime préférentiellement des gènes permettant les échanges trophiques avec le symbiote, favorisant le fonctionnement autotrophe (Ganot et al., 2011). Néanmoins, très peu d'acteurs moléculaires impliqués dans le transport sélectif de métabolites de part et d'autre de la membrane ont été identifiés. Une première raison provient de la difficulté technique de purifier la membrane périsymbiotique. Actuellement, une seule étude a permis l'identification de 17 protéines situées à la membrane perisymbiotique (Peng et al., 2010a) à partir d'un protocole de purification de la membrane (Kazandjian et al., 2008). Parmi les cibles identifiées, on retrouve des « GPCR-like receptors » impliqués dans la reconnaissance cellulaire, des transporteurs ABC suggérant la présence de transports actifs et des ATP synthases permettant l'acidification de l'espace périsymbiotique. Cette étude a également identifié des protéines impliquées dans le remodelage du

cytosquelette, comme l'actine, ainsi que des protéines de réponse au stress (HSP). Une caractérisation plus complète de l'interface symbiotique sera déterminante pour une meilleure compréhension de la régulation de la symbiose et des échanges entre les deux partenaires.

1.3.4. Les différents mécanismes de rupture de la symbiose

Toutefois, l'association peut être rompue entre les deux partenaires lorsque l'holobionte est soumis à divers stress anthropiques et environnementaux, tels que l'augmentation de la température, des rayonnements ultra-violets et la présence de contaminants (Coles and Brown, 2003). Ces stress entraînent un dysfonctionnement de la photosynthèse, une augmentation du stress oxydant dans les tissus animaux, une diminution de la quantité de photosynthétats transférés des *Symbiodinium* jusqu'à l'hôte, puis à terme une expulsion des symbiotes (Leggat et al., 2003). Ce phénomène est communément décrit comme le blanchissement des coraux ou « bleaching » et résulte d'un dysfonctionnement de la symbiose (Douglas, 2003), caractérisé par une perte des *Symbiodinium* et de leurs pigments photosynthétiques (Weis, 2008) (Figure 10).

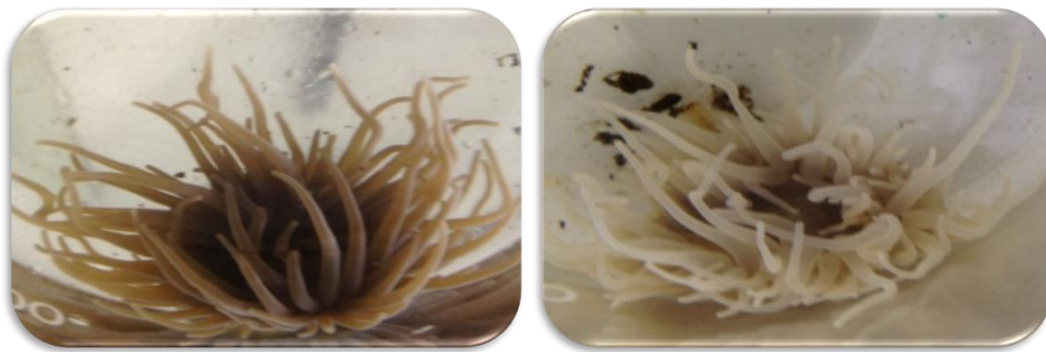


Figure 10: Individus d'*Anemonia viridis* en condition symbiotique (gauche) et aposymbiotique (droite)

Les organismes blanchis ont alors un taux de survie et une fitness drastiquement réduits ainsi qu'une augmentation de la sensibilité aux attaques de pathogènes (Hoegh-Guldberg et al., 2007). Ce phénomène est responsable d'un taux élevé de mortalité dans les écosystèmes coralliens (Hoegh-Guldberg et al., 2007). Actuellement, le réchauffement climatique global entraînant une augmentation de la température de surface des océans semble la cause principale responsable du

blanchissement des récifs coralliens, ce phénomène étant amplifié par l'augmentation des radiations UV et de l'acidification des océans.

L'un des processus majeurs impliqués dans la rupture de la symbiose en réponse au changement climatique est l'augmentation significative de la production d'espèces actives de l'oxygène (EAO). En condition symbiotique stable, l'activité photosynthétique du symbiote entraîne une hyperoxie locale dans la cellule du symbiote mais également dans les cellules de l'hôte (Richier et al., 2003). Pourtant, l'hôte a développé des mécanismes d'adaptation liés à la production intracellulaire d'EAO via l'expression d'une large palette d'enzymes antioxydantes (Catalase, ascorbate peroxydases et superoxide dismutases) (Furla et al., 2005), permettant la détoxification des EAO. Dans des conditions environnementales qui vont s'écarter de l'optimum symbiotique telle qu'une augmentation de la température, l'emballement de la machinerie photosynthétique et de la respiration des symbiotes vont entraîner une augmentation significative de la production d'EAO (Venn et al., 2008). Si la capacité de détoxification des EAO par le métabolisme anti-oxydant de l'hôte est insuffisante, cette production augmentée d'EAO va induire une oxydation des membranes, une dénaturation des protéines, des dommages sur les acides nucléiques (Lesser and Farrell, 2004) et à terme une rupture de l'association symbiotique (Weis, 2008).

Les mécanismes impliqués dans la rupture de l'association peuvent être étudiés à plusieurs niveaux, indépendamment chez l'un ou l'autre des partenaires. Il s'agira donc dans une première partie de définir les différents mécanismes cellulaires observés lors de rupture de symbiose, puis d'appréhender les mécanismes moléculaires qui interviennent en réponse à un stress.

1.3.4.1. Au niveau cellulaire

Plusieurs mécanismes responsables de la diminution des symbiotes ont été détaillés (Gates et al., 1992) et étudiés au niveau cellulaire chez plusieurs organismes lors de rupture de symbiose (en revue dans (Weis, 2008)). En effet, suivant le type de stress, son intensité et l'organisme étudié, de nombreux mécanismes ont été décrits au niveau de l'hôte et du symbiote (Figure 11).

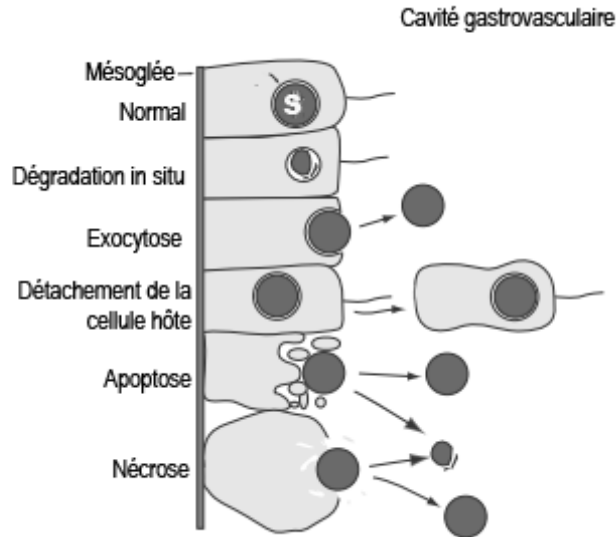


Figure 11: Schéma des différents mécanismes impliqués dans la rupture de la symbiose au niveau du gastroderme (Modifié d'après Weis et al., 2008).

Observée dans l'environnement (Brown et al., 1995) et en laboratoire sur des organismes soumis à des stress hyperthermiques (Dunn et al., 2004, 2004; Strychar et al., 2004a), la **dégradation *in situ*** des symbiotes présente un profil caractéristique de mort cellulaire programmée (MCP). Ces mécanismes ont été décrits chez des organismes unicellulaires et pluricellulaires (Ameisen, 2002) et plusieurs études ont montré la mise en place de MCP lors de la rupture de la symbiose via les mécanismes d'apoptose et d'autophagie décrits ci-dessus (Figure 11).

D'autre part, il a également été démontré que lors de stress sévères un autre type de mécanisme, prenant la forme de mort cellulaire non-contrôlée par nécrose, était également impliqué dans la rupture de la symbiose.

L'apoptose est un mécanisme de mort cellulaire programmée impliqué dans l'élimination des cellules infectées ou endommagées (Lasi et al., 2010a), initié par une cascade de signaux intrinsèques et extrinsèques (Rubinstein and Kimchi, 2012). Ce mécanisme déclenché préférentiellement en réponse à l'augmentation de la production d'EAO (Simon et al., 2000) est très conservé au sein des métazoaires (Raff, 1998). Cette augmentation d'EAO étant le mécanisme principal induit par l'augmentation de la température dans la symbiose Cnidaire-Dinoflagellés, ce mécanisme a été observé dans de nombreuses études (Dunn et al., 2002; Dunn et al., 2004; Dunn et al., 2007; Richier et al., 2006; Tchernov et al., 2011). Chez l'hôte, l'apoptose est caractérisée par

la condensation de la chromatine à la périphérie de l'enveloppe nucléaire et par la proéminence de la membrane plasmique, provoquée par le découplage localisé du cytosquelette (Tchernov et al., 2011). Au niveau du symbiote, les signes caractéristiques sont la condensation du cytoplasme et des organelles, ainsi que la formation de corps apoptotiques denses aux électrons (Dunn et al., 2004; Strychar et al., 2004b). Le processus moléculaire majeur permettant d'identifier la présence d'apoptose est l'augmentation significative de l'expression des enzymes caspases (Richier et al., 2006) et l'augmentation du facteur de transcription pro-apoptotic p53, mis en évidence lors d'un stress hyperthermique chez *Montastrea faveolata* (Lesser and Farrell, 2004). L'induction de la dégradation des symbiotes par l'hôte permettrait ainsi de réduire la production de d'EAO par les symbiotes (Dunn et al., 2004). Des études de transcriptomique menées chez des Cnidaires symbiotiques soumis à divers stress ont montré l'expression de gènes impliqués dans les mécanismes d'apoptose (Bellantuono et al., 2012; Rodriguez-Lanetty et al., 2006; Sunagawa et al., 2009a; Voolstra et al., 2009c).

Le processus **d'autophagie** permet la dégradation des composés intracellulaires endommagés, par l'action des lysosomes (Maria Cuervo, 2004). L'autophagie est aussi un mécanisme d'immunité innée, qui participe à l'élimination de pathogènes intracellulaires. Ainsi, la reconnaissance par la cellule de matériel organique étranger induit la fusion des phagosomes avec les lysosomes, qui détruisent la cible par déversement d'enzymes hydrolytiques dans le phagosome. De plus, les cascades moléculaires menant à l'autophagie ont fait l'objet de nombreuses études et sont retrouvées dans de nombreux systèmes biologiques (Klionsky et al., 2012a). Chez les Cnidaires, ce mécanisme de mort cellulaire programmée a été décrit aussi bien chez l'hôte (Chera et al., 2009; Dunn et al., 2007) que chez le symbiote, pour lequel il a été défini par le terme de symbiophagie par Downs, lors d'un stress hyperthermique et UV chez *Pocillopora damicornis* (Downs et al., 2009). Il est caractérisé chez l'hôte par l'apparition de structures actives d'autophagie correspondant à des accumulations de matériel cytoplasmique (Dunn et al., 2007; Hanes and Kempf, 2013) et de structures membranaires sphériques (Downs et al., 2009; Hohman et al., 1982). Chez le symbiote, la symbiophagie est caractérisée par une dissolution du noyau, une désorganisation des thylakoïdes et la transformation de la membrane symbiotique en une vacuole digestive (Downs et al., 2009; Downs et al., 2013). Dans cette dernière étude, l'augmentation des niveaux d'expression des enzymes phosphatase et des protéines Rab7 indique également une augmentation des activités autophagiques. La surexpression de gènes potentiellement impliqués

dans l'autophagie a également été décrite dans des études de transcriptomique chez l'anémone *Aiptasia pallida* (Sunagawa et al., 2009a) et le corail *Acropora palmata* (Polato et al., 2013).

Cependant, ces deux mécanismes de mort cellulaire programmée (apoptose et autophagie), sont inter-régulés et possèdent des voies moléculaires communes (Lalaoui et al., 2015). Chez les Cnidaires, Dunn et ses collaborateurs ont montré que l'inhibition des deux mécanismes simultanément était nécessaire pour prévenir le blanchissement (Dunn et al., 2007). Ce phénomène précédemment décrit chez les vertébrés (Boya et al., 2005) montre en effet que l'inhibition de l'autophagie est compensée par l'induction de l'apoptose et vice versa (Rubinstein and Kimchi, 2012). Ce processus ultra-conservé au cours de l'évolution, est un mécanisme primordial dans la dégradation d'organites endommagés (Chera et al., 2009), mais intervient également dans la dégradation de pathogènes (Koul et al., 2004; Wileman, 2013).

Un autre type de dégradation *in situ* des symbiotes a été décrit et caractérisé comme étant de la **nécrose**, de la cellule hôte et/ou du symbiote (Dunn et al., 2004). Ce mécanisme est reconnaissable par une rupture des membranes et un relargage du contenu cytoplasmique (Fink and Cookson, 2005). S'il apparait que les deux précédents mécanismes décrits sont utilisés de façon modérée par l'hôte pour éliminer les symbiotes indésirables, la nécrose a été décrite comme un mécanisme incontrôlé, entraînant une dégradation totale de la cellule animale (Dunn et al., 2002; Dunn et al., 2004) et/ou du symbiote (Strychar and Sammarco, 2009; Strychar et al., 2004b). La nécrose est alors caractérisée chez l'hôte par une vacuolisation du cytoplasme, la présence de corps d'agrégations denses aux électrons, et la rupture des membranes des organelles (Dunn et al., 2002; Dunn et al., 2004; Hanes and Kempf, 2013), ces deux dernières étant également observées chez le symbiote (Strychar and Sammarco, 2009; Strychar et al., 2004b). Ces travaux montrent dans un premier temps une dégradation conjointe du contenu cytoplasmique de la cellule gastrodermique et du symbiote. On observe ensuite une sévère vacuolisation du symbiosome ainsi qu'une forte dégradation des thylakoïdes et des membranes du symbiote. Au stade final, on observe uniquement des corps nécrotiques et une absence de la cellule de l'hôte. Ce phénomène serait donc induit lors d'un stress sévère conduisant à l'élimination de la cellule symbiotique dans son ensemble. Récemment, des mécanismes de type nécrose programmée impliquant des voies de signalisation indépendantes de l'activation de caspases ont été mis en évidence (Fink and Cookson, 2005; Leist and Jäättelä, 2001; Proskuryakov et al., 2003). Ceci suggère donc que la distinction entre mort

cellulaire programmée et non programmée se situe dans un continuum dépendant du modèle d'observation, de la thématique (cancer, infection, stress) et de l'intensité de la réponse observée.

Toutefois, il a été décrit deux autres mécanismes d'élimination des symbiotes des cellules hôtes qui n'entraînent pas une dégradation des symbiotes. Il s'agit des mécanismes d'exocytose et de détachement de la cellule symbiotique (cellule du gastroderme et du symbiote). En effet, de nombreuses études ont montré que l'expulsion de symbiotes inadaptés aux nouvelles conditions environnementales pouvait constituer un mécanisme d'adaptation. L'hôte induit donc une expulsion par **exocytose** d'une partie de ses symbiotes, permettant de changer transitoirement pour acquérir une population de *Symbiodinium* ayant des optimums de photosynthèse plus adaptés aux nouvelles conditions (Baker, 2001; Baker et al., 2004; Brown, 1997; Buddemeier and Fautin, 1993; Douglas, 2003). Ce mécanisme a été observé pour la première fois chez *Aiptasia pulchella* (Steen and Muscatine, 1987) lors d'une réduction brutale de la température et chez une variété de corail lors d'une augmentation de la température (Brown et al., 1995). Les symbiotes sont ainsi expulsés de la cellule gastrodermique dans la cavité coelentérique, puis rejetés dans le milieu extérieur sous forme de pelotes fécales ou agrégats par la bouche. Ce mécanisme est provoqué par un désassemblage des microtubules de la cellule animale et est accompagné d'une augmentation de la concentration des ions calciques cytoplasmique (Steen and Muscatine, 1987). Chez le corail *Acropora grandis*, l'expulsion par exocytose de symbiotes a été corrélée à une augmentation de la concentration calcique suite à un choc température (Fang et al., 1997). Parmi les symbiotes expulsés, on retrouve aussi bien des symbiotes encore viables (Steen and Muscatine, 1987; Wang et al., 2012) que des symbiotes totalement dégradés (Fujise et al., 2014b; Sammarco and Strychar, 2013). Une étude montre également que certains Cnidaires symbiotiques expulsent préférentiellement des symbiotes en phase de division, les proportions différant en fonction du stress (Baghdasarian and Muscatine, 2000). Ainsi, en fonction du type de stress et de l'intensité de celui-ci, les Cnidaires expulsent par exocytose des symbiotes, dont une partie pourrait infecter d'autres hôtes. Pour l'hôte, ce mécanisme permettrait également d'obtenir une nouvelle population de symbiotes mieux adaptés aux nouvelles conditions environnementales. Egalement décrit lors de stress par diminution ou augmentation de la température, le **détachement de la cellule** symbiotique correspond au relargage de la cellule gastrodermique viable, contenant encore le symbiote, dans le milieu extérieur (Brown et al., 1995). Même si la cellule gastrodermique semble rapidement se

dégrader, le symbiote est ainsi retrouvé dans le milieu extérieur à l'état viable (Gates et al., 1992). En effet, le choc température entraîne une augmentation de la concentration intracellulaire des ions calciums (Ca^{2+}) lors d'une augmentation de la température (Sawyer and Muscatine, 2001) ou lors d'un choc froid (Wang et al., 2012). En effet, une augmentation de la concentration intracellulaire du calcium entraîne une perturbation du cytosquelette et la liaison avec les molécules d'adhésion cellulaires (Geiger et al., 1992). Ainsi, un choc thermique impliquerait une modification du cytosquelette et une perturbation des propriétés adhésives de la cellule. Néanmoins, les mécanismes impliqués dans l'expulsion de cellules du gastroderme restent à définir (Weis, 2008).

1.3.4.2. Au niveau de l'expression des gènes

Ces deux dernières décennies ont vu une accélération accrue des techniques de biologie moléculaire et de séquençage. Ces avancées technologiques ont permis d'accroître les données acquises sur les régulations génétiques impliquées dans les mécanismes de la symbiose. Une liste non exhaustive des principales études en transcriptomique et protéomique a été dressée dans le Tableau 1, depuis les premières études en 1996 et la découverte de la régulation des anhydrases carboniques par Weis et Levine (Weis and Levine, 1996) jusqu'à nos jours. Elles ont porté tout d'abord sur la comparaison d'individus symbiotiques et aposymbiotiques, puis vers l'analyse de la régulation transcriptomique lors des différents stades de développement et lors de la rupture de symbiose. Il s'agit également de noter la diversité des espèces étudiées, notamment au niveau de l'hôte, permettant ainsi de comparer les résultats obtenus sur plusieurs modèles d'études dont la plasticité symbiotique diffère. Nous remarquerons l'apparition des puces à ADN à partir de 2006, permettant de comparer l'expression des gènes dans des conditions diverses, puis des méthodes de séquençages à haut débit à partir de 2011. La multiplication des banques « expressed sequenced tag » (EST) et des projets de séquençage ont également permis une annotation plus précise des gènes impliqués dans la relation symbiotique.

La synthèse de l'ensemble des résultats obtenus ces 20 dernières années souligne l'importance des gènes impliqués dans la reconnaissance des partenaires, tels que les lectines (Baumgarten et al., 2015; Ganot et al., 2011; Grasso et al., 2008; Lehnert et al., 2014; Reynolds et al., 2000; Sunagawa et al., 2009), la dynamique du cytosquelette (deBoer et al., 2007; Lehnert et

al., 2012; Rodriguez-Lanetty et al., 2006; Sabourault et al., 2009a; Voolstra et al., 2009a), et l'implication du trafic vésiculaire dans la mise en place de la symbiose (Ganot et al., 2011; Grasso et al., 2008; Yuyama et al., 2011). Ces études soulignent également l'importance du métabolisme lipidique (Baumgarten et al., 2015; Ganot et al., 2011; Rodriguez-Lanetty et al., 2006), des gènes impliqués dans la régulation du stress oxydant (Ganot et al., 2011; Lehnert et al., 2014; Rodriguez-Lanetty et al., 2006; Weis and Levine, 1996), dans l'homéostasie du calcium (Kuo et al., 2010), le transport du carbone inorganique (Yuyama et al., 2011), la communication cellulaire (Ganot et al., 2011; Lehnert et al., 2014; Reynolds et al., 2000) et moléculaire (Ganot et al., 2011; Kuo et al., 2010; Lehnert et al., 2014; Yuyama et al., 2011) dans le processus de maintien de la symbiose.

Lors de la rupture de la symbiose, ces études ont révélé l'importance des protéines chaperonnes (HSP) (Bellantuono et al., 2012; Desalvo et al., 2008; Leggat et al., 2007; Meyer et al., 2011; Polato et al., 2013; Rodriguez-Lanetty et al., 2009), du stress oxydant (Bellantuono et al., 2012; Desalvo et al., 2008; Lehnert et al., 2014; Rodriguez-Lanetty et al., 2009; Sunagawa et al., 2009a; Voolstra et al., 2009a) et du métabolisme calcique et lipidique (Desalvo et al., 2008; Lehnert et al., 2012; Lehnert et al., 2014; Meyer et al., 2011; Moya et al., 2012; Voolstra et al., 2009a; Voolstra et al., 2009a; Yuyama et al., 2011). Les gènes impliqués dans les mécanismes d'apoptose (Bellantuono et al., 2012; Richier et al., 2008; Rodriguez-Lanetty et al., 2006; Sunagawa et al., 2009a; Voolstra et al., 2009c), d'autophagie (Polato et al., 2013; Sunagawa et al., 2009a), et de phagocytose (DeSalvo et al., 2010; Sunagawa et al., 2009a) ont également été mis en évidence par ces études. Elles mettent en avant l'importance des mécanismes impliqués dans la reconnaissance, la lutte contre le stress oxydant, ainsi que le métabolisme calcique et lipidique qui jouent un rôle dans le maintien, mais également lors de la rupture de la symbiose. Il apparaît alors que lors d'un stress entraînant la rupture de la symbiose, les gènes d'interactions symbiotiques sont réprimés au profit de gènes régulant le transport vésiculaire. Ceci constituerait une réponse adaptative via l'activation des voies hétérotrophiques aux dépens des voies autotrophiques (Ganot et al., 2011).

Cette dernière étude, conduite au laboratoire sur *Anemonia viridis*, est la première à étudier de façon séparée le compartiment non symbiotique représenté par l'épiderme, et le gastroderme contenant les symbiotes. Ces résultats ont mis en évidence une réponse compartiment spécifique. En effet, 71% des gènes surexprimés en condition symbiotique l'étaient dans le compartiment

gastrodermique. La présence des symbiotes est donc corrélée avec l'expression de gènes potentiellement impliqués dans la relation symbiotique. Parmi ces gènes, on retrouve les anhydrases carboniques *ca2-c* cytosolique et *ca2-m* membranaire, le gène *npc2* impliqué dans le métabolisme lipidique, la calumenin impliquée dans le métabolisme calcique et *sym32*, impliqué dans la reconnaissance des symbiotes. De façon intéressante, tous ces gènes possèdent des duplications spécifiques au groupe des Cnidaires. Ceci implique une adaptation au niveau génétique de la mise en place et de la régulation de la symbiose chez les Cnidaires. Cette étude suggère également que les produits de ces gènes sont en interaction avec le symbiote *in hospite* au niveau de la membrane pérисymbiotique. La caractérisation de cette interface perisymbiotique paraît ainsi primordiale dans la compréhension des mécanismes impliqués dans la relation symbiotique.

Hôte	Stade d'étude	Technique utilisée	Type d'étude	Résultats majeurs et cible(s) identifiée(s)	Références
<i>Anthopleura elegantissima</i>	Adulte	Protéomique, électrophorèse 2D	Comparaison profil protéine individus symbiotiques versus aposymbiotiques	Anhydrase carbonique	(Weis and Levine, 1996)
<i>Anthopleura elegantissima</i>	Adulte	PCR semi-quantitative	Protéine majeure exprimé dans Weis et Levine, 1996	<i>Sym32</i>	(Reynolds et al., 2000)
<i>Aiptasia pulchella</i>	Adulte	Banque EST	Comparaison profil transcrits individus symbiotiques versus aposymbiotiques	147 gènes identifiés (45 spécifiques symbiotique et 46 spécifiques aposymbiotiques)	(Kuo et al., 2004)
<i>Aropora tenius</i>	Juvénile	Banque soustractive ADNc	Comparaison profil transcrits individus symbiotiques versus aposymbiotiques	2 gènes surexprimés en conditions symbiotique, non identifiés	(Yuyama et al., 2005)
<i>Heteroxenia fuscescens</i>	Juvénile	Protéomique, électrophorèse 2D	Comparaison profil protéine individus symbiotiques versus aposymbiotiques	Différences en fonction du stade de développement	(Barneah et al., 2006)
<i>Anthopleura elegantissima</i>	Adulte	Microarrays cDNA	Comparaison profil transcrits individus symbiotiques versus aposymbiotiques	Métabolisme lipidique, adhésion cellulaire, prolifération cellulaire, apoptose, stress oxydant	(Rodriguez-Lanetty et al., 2006)
<i>Fungia scutaria</i>	Larve	Protéome, Microarrays cDNA, q-PCR	Comparaison au cours de la mise en place de la symbiose	Détermination de séquences EST différentiellement exprimée	(deBoer et al., 2007)
<i>Symbiodinium</i>	Clade C3	Banque EST	Stress température	561 EST identifiés codants pour des protéines impliquées dans des modifications post-transcriptionnelles, turnover de protéines, protéines chaperonnes, production d'énergie	(Leggat et al., 2007)
<i>Montastrea faveolata</i>	Adulte	Microarrays cDNA	Stress température	Stress oxydant, homéostasie du Ca ²⁺ , cytosquelette, mort cellulaire, calcification, métabolisme, synthèse de protéine, activité chaperonnes, transposons	(Desalvo et al., 2008)
<i>Acropora palmata</i> , <i>Montastrea faveolata</i>	Embryons, larve, adulte	Banque EST	Expression lors des différents stades de développement	Récepteurs de l'immunité innée	(Schwarz et al., 2008a)

<i>Anthopleura elegantissima</i>	Adulte	Microarrays cDNA	Stress température et UV	27 gènes différentiellement exprimés : cytosquelette, prolifération cellulaire, synthèse de protéines, apoptose, transport	(Richier et al., 2008)
<i>Acropora millepora</i>	Embryon, larve, adulte	Microarrays cDNA	Expression lors des différents stades de développement	Lectines, anyhydrases carboniques	(Grasso et al., 2008)
<i>Acropora millepora</i>	Larve	Microarrays cDNA	Stress température	29 gènes différentiellement exprimés HSP, protéine fluorescente homologue, lectine type-C, stress oxydatif, anhydrase carbonique	(Rodriguez-Lanetty et al., 2009)
<i>Anemonia viridis</i>	Adulte	Banque EST	Comparaison standard et stress	14 504 gènes uniques identifiés	(Sabourault et al., 2009a)
<i>Aiptasia pallida et Symbiodinium</i>	Adulte	Banque EST	-	Stress oxydant, apoptose, autophagie, phagocytose, système immunitaire, lectine	Sunagawa et al., 2009
<i>Montastrea faveolata</i>	Embryon	Microarrays cDNA	Effet du stress température	Stress oxydant, apoptose, modification du cytosquelette et diminution du métabolisme	(Voolstra et al., 2009a)
<i>Symbiodinium</i>	Clade C3 et clade A	Banque EST	Comparaison des deux espèces	Respectivement 3336 et 1484 séquences uniques impliquées dans le métabolisme énergétique et carbohydrate, signal extracellulaire, communication cellulaire, photosynthèse	(Voolstra et al., 2009b)
<i>Fungia scutaria</i>	Larve	Microarrays cDNA	Comparaison au cours de la mise en place de la symbiose	Caractérisation des transcrits	(Schnitzler, 2010)
<i>Aiptasia pulchella</i>	Adulte	Banque soustractive ADNc	Comparaison profil transcrits individus symbiotiques versus aposymbiotiques	11 gènes différentiellement exprimés dont <i>npc2</i> , Ca^{2+} binding, protéine EFhand	(Kuo et al., 2010)
<i>Montastrea faveolata</i>	Adulte	Microarrays cDNA	Comparaison standard et stress	HSP, Catalase, Histone, cytosquelette, Thioredoxine, ATPase, facteur d'élongation	(DeSalvo et al., 2010)
<i>Acropora palmata</i>	Adulte	Microarrays cDNA	Stress température	Régulation cycle cellulaire, activité chaperonne, stabilisation des acides nucléiques, phagocytose, métabolisme, cytosquelette, perception sensorielle	(DeSALVO et al., 2010)
<i>Acropora millepora</i>	Larve	RNA-seq	Stress température	HSP, métabolisme du calcium et carbohydrate	(Meyer et al., 2011)
<i>Acropora tenuis</i>	Juvenile	Méthode HiCEP	Comparaison profil transcrits individus symbiotiques versus aposymbiotiques	33 gènes différentiellement exprimés impliqués dans le métabolisme des carbohydrates et lipidique, transduction de signal intracellulaire, transport membranaire d'ions	(Yuyama et al., 2011)

<i>Anemonia viridis</i>	Adulte	Microarrays cDNA, q-PCR	Comparaison profil transcrits individus symbiotique versus aposymbiotique par compartiment	39 gènes caractéristiques d'un stade : <i>Calumenin</i> , <i>npc2</i> , <i>npc1</i> , <i>carbonique anhydrase</i> , <i>sym32</i> ; Mise en évidence de duplication de gènes symbiose spécifique	(Ganot et al., 2011)
<i>Acropora digitifera</i>	Sperme	Séquençage NGS	Génome	Apoptose, autophagie, voie de biosynthèse des MAAs, récepteur Toll, immunité innée	(Shinzato et al., 2011)
<i>Aiptasia pallida</i>	Adulte	Transcriptome	Transcrits d'individus aposymbiotiques	Enzymes du métabolisme, cytosquelette, neuropeptide	(Lehnert et al., 2012)
<i>Stylophora pistillata</i>	Adulte	Protéomique	Stress température, métal limitation	26 protéines différemment exprimés chez le symbiote : photosystème, réparation ADN, métabolisme antioxydant, HSP, hémoprotéines, métabolisme du nitrogène, protéines virales, apoptose, transport vésiculaire, endo/exocytose	(Weston et al., 2012)
<i>Symbiodinium</i>	Clade A et B	Séquençage 454	Séquençage des transcrits	56 000 séquences obtenues Nombreux transcrits impliqué dans le stress oxydant	(Bayer et al., 2012)
<i>Anemonia viridis</i>	Adulte	Transcriptome	Stress température et UV	Mécanisme de mort cellulaire, homéostasie du calcium (<i>Calumenin</i>), métabolisme des lipides (<i>npc2</i>), reconnaissance cellulaire (<i>sym32</i>)	(Moya et al., 2012)
<i>Acropora millepora</i>	Adulte	Microarrays cDNA	Stress thermique sur des individus préconditionnés versus non conditionnés	Différence de l'expression au niveau quantitatif et pas qualitatif ; Mannose-binding lectine, calumenine, cytosquelette	(Bellantuono et al., 2012)
<i>Acropora palmata</i>	Larve	Microarrays cDNA	Stress température	<i>Ferritine</i> , HSP, cytosquelette, adhésion cellulaire, autophagie, métabolisme du calcium, <i>npc1</i>	(Polato et al., 2013)
<i>Aiptasia pallida</i>	Adulte	Transcriptome	Comparaison profil transcrits individus symbiotiques versus aposymbiotiques	900 gènes différemment exprimés: transports de nutriments, reconnaissance et tolérance des symbiotes, métabolisme lipidique, stress oxydant	(Lehnert et al., 2014)
<i>Symbiodonium</i>	Souche SSB01	RNA-seq	Comparaison de différentes conditions de luminosité	Réponse au stress, métabolisme carbohydrate, lipidique et calcium, organisation du cytosquelette, adhésion cellulaire, communication cellulaire, mort cellulaire, cycle cellulaire	(Xiang et al., 2015)
<i>Acropora microphthalma</i>	Adulte	Protéomique	Condition de stress UV et température	Anhydrase carbonique, métabolisme du calcium, Rab5, actine, HSP70, calmoduline	(Weston et al., 2015, 205)
<i>Aiptasia</i>	Souche CC7, H2	Génome	Génome Illumina MiSeq	Métabolisme lipidique (<i>npc2</i>), reconnaissance cellulaire (CniFLs), transfert de gènes horizontaux	(Baumgarten et al., 2015)

Tableau 1 : Liste non exhaustive des différentes études en génomique, transcriptomique et protéomique conduite sur la symbiose Cnidaire/Dinoflagellés, de 1996 à 2015

1.4. Contexte du projet doctoral et problématique de l'étude

1.4.1. Position du projet au sein de l'équipe symbiose marine

Les résultats de transcriptomique obtenus ces vingt dernières années et ceux obtenus au laboratoire par la comparaison de l'expression des gènes entre des individus symbiotiques et aposymbiotiques d'*A. viridis* (Ganot et al., 2011; Sabourault et al., 2009a), ainsi que lors d'un stress température et/ou UV (Moya et al., 2012), suggèrent un rôle primordial de certaines protéines membranaires animales dans les mécanismes de reconnaissance et de communication moléculaire entre les deux partenaires de la symbiose. Plus particulièrement, la membrane périsymbiotique d'origine animale, acquise lors de la phagocytose du symbiote, apparait comme l'interface de communication lors i) de la reconnaissance et l'internalisation des symbiotes, ii) des mécanismes impliqués dans la communication et iii) des échanges moléculaires mis en place pour le fonctionnement de l'interaction symbiotique.

Les travaux menés au laboratoire, en accord avec les résultats précédemment obtenus par la communauté scientifique (Tableau 1), ont permis d'identifier des cibles préférentiellement exprimées en conditions symbiotiques et dont la fonction participerait activement aux mécanismes symbiotiques chez *A. viridis*. Afin de permettre une meilleure caractérisation de l'interface symbiotique, le laboratoire a obtenu en 2012 un financement sur projet ANR porté par le Dr Cécile Sabourault. Le projet inSIDE (Symbiotic Interface of a cnidarian-Dinoflagellate Endosymbiosis) a pour but d'étudier l'interface symbiotique chez l'anémone de mer *A. viridis* via i) la localisation et la régulation au niveau transcriptomique et protéomique des gènes *Avnpc1* et *Avnpc2*, ii) la caractérisation du protéome de la membrane périsymbiotique et iii) une étude du lipidome et du métabolome d'*A. viridis*. Ce projet est réalisé en collaboration avec l'Institut de Chimie de Nice pour les analyses métabolomiques, notamment par le recrutement de Johana Revel, doctorante en charge de la partie lipidomique et métabolomique, et avec le laboratoire PRISM de l'Université de Lille1, pour les analyses de protéomique et l'imagerie moléculaire. Dans ce projet, j'ai été recruté en tant que doctorant contractuel chargé d'enseignement sur bourse ministérielle, pour travailler sur la caractérisation des gènes *npc1* et *npc2*, ainsi que la caractérisation du protéome à l'interface symbiotique.

1.4.2. *Anemonia viridis*, un modèle d'étude aux nombreux avantages

Pour ce projet, le modèle d'étude utilisé au laboratoire est l'anémone de mer symbiotique *Anemonia viridis* (Forsk., 1775) (Anthozoa, Cnidaria). Ce modèle comporte plusieurs avantages dans l'étude de la symbiose Cnidaire-Dinoflagellés. Premièrement, cette espèce est commune des paysages sous-marins en Méditerranée et très abondante tout au long de l'année. Elle se répartit entre 0 et 25m de profondeur sur les fonds rocheux de zones bien éclairées à ombragées. Elle vit en endosymbiose avec le Clade A tempéré (ou A') de *Symbiodinium* sp. (Freudenthal, 1962) (Dinoflagellata, Protozoa), clade largement dominant en Méditerranée (Savage et al., 2002; Visram et al., 2006b). Comme le décrit la Figure 12, *A. viridis* possède un très large polype solitaire fixé au substrat, pouvant atteindre jusqu'à 20 cm de diamètre et jusqu'à 200 tentacules. Ces tentacules ont également une grande capacité de régénération. Comme chez les autres Cnidaires symbiotiques, les symbiotes sont hébergés dans les cellules du gastroderme, en contact avec la cavité coelomique, séparée de l'épiderme par la mésogée (Figure 12). La grande taille des individus d'*A. viridis* permet d'une part d'obtenir de grandes quantités de matériel biologique, et d'autre part de séparer facilement par incision à la base du tentacule, le gastroderme (compartiment symbiotique) de l'épiderme (compartiment non symbiotique). La possibilité de séparer ces deux compartiments cellulaires avec un minimum de contamination est quasi unique chez les Cnidaires symbiotiques et représente un avantage déterminant de ce modèle d'étude. Il est également possible d'obtenir des *Symbiodinium* fraîchement isolés à partir des cellules du gastroderme. Ces techniques de séparation des différents compartiments ont déjà été validées dans plusieurs études au laboratoire (Ganot et al., 2011; Merle et al., 2007; Moya et al., 2012; Richier et al., 2006). De plus, *A. viridis* est un organisme solitaire non calcifiant, ce qui permet d'étudier le mécanisme de la symbiose en s'affranchissant d'une part des processus liés à la calcification et d'autre part de ceux liés à la colonialité chez les Scléactiniaires. Cet organisme permet aussi de disposer d'individus blanchis (perte des symbiotes) qui peuvent être maintenus en aquarium pendant plusieurs années, offrant ainsi la possibilité de comparer l'état symbiotique et aposymbiotique d'un même individu au cours du temps. Durant les dernières années, le laboratoire a acquis un bon nombre de données physiologiques et de transcriptomique lors de différents projets et études publiés. Ce laboratoire est également un des rares laboratoires à posséder des *Symbiodinium* clade A tempéré en culture pour lequel un projet de transcriptomique a été initié.

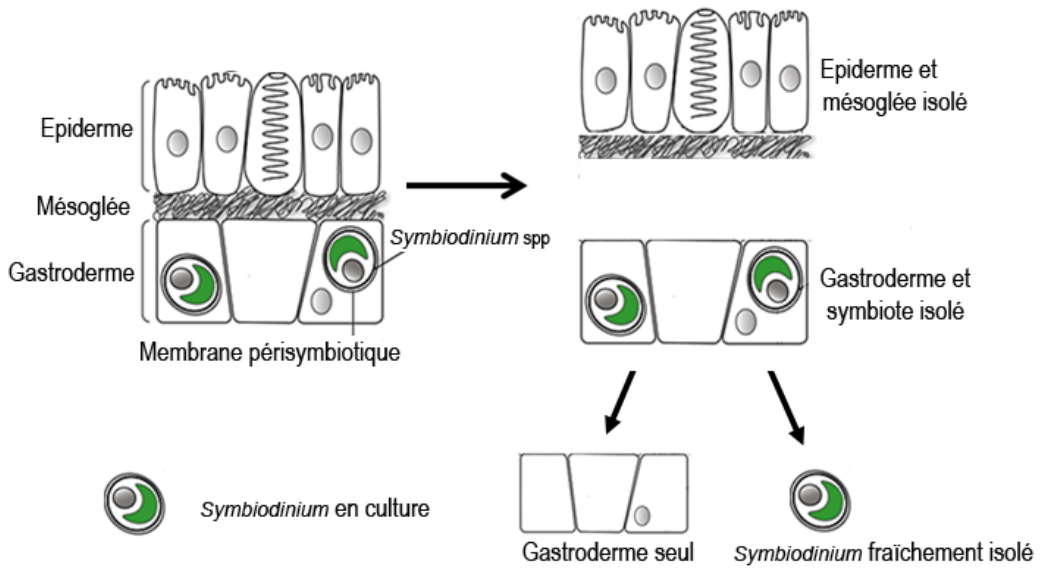
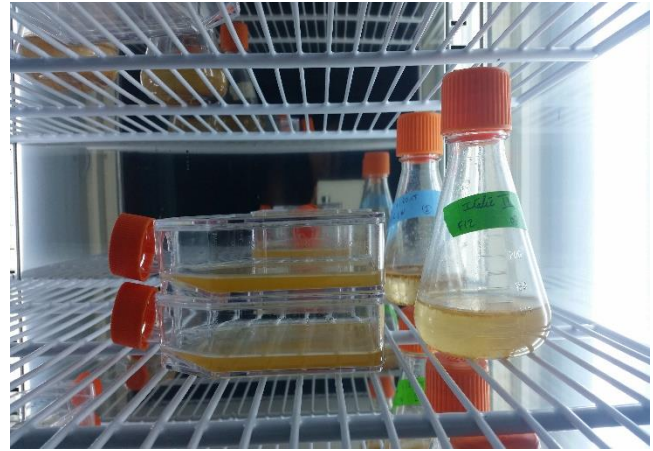


Figure 12 : En haut à gauche, spécimen d'*A. viridis* en aquarium. En haut à droite, *Symbiodinium* en culture. En bas, schéma de séparation des différents compartiments disponibles chez *A. viridis*

1.4.3. Objectifs du projet de doctorat

Durant la dernière décennie, une attention particulière a été demandée aux scientifiques sur le développement de marqueurs permettant la gestion des récifs coralliens. Néanmoins, malgré le développement des techniques de séquençage à haut débit qui ont connu un réel essor durant ces dernières années, ainsi que les nombreuses études portant sur la caractérisation des processus cellulaires impliqués dans la rupture de la symbiose, les acteurs moléculaires impliqués dans la mise en place et la rupture de la symbiose restent à définir dans leur grande majorité. Dans ce contexte, la compréhension des mécanismes permettant le maintien de la symbiose, ainsi que la caractérisation des nouveaux acteurs moléculaires au niveau de l'interface symbiotique entre les deux partenaires paraissent primordiaux.

Mon projet de recherche doctoral a donc pour principal objectif une meilleure compréhension des mécanismes cellulaires et moléculaires impliqués dans la relation symbiotique à l'état stable et lors de la rupture de l'association symbiotique, aussi bien au niveau de l'hôte animal que de son symbiote (Figure 13). Par diverses approches histologiques, biochimiques et moléculaires, ce projet avait pour but de définir de nouveaux outils afin d'identifier des cibles nouvelles dans les mécanismes de fonctionnement de la symbiose à l'état stable, mais également de mieux définir les processus mis en jeu dans la rupture de la symbiose. À terme, les résultats obtenus ont pour objectif de déterminer de nouvelles voies de signalisation impliquées dans les divers processus de la symbiose.

Une première partie de mon travail (Chapitre 2) nous a permis de mieux caractériser la structure cellulaire et subcellulaire de notre modèle d'étude (*A. viridis*), tant au niveau de l'organisme entier que celui de l'interface symbiotique. Lors de cette étude, nous avons déterminé les processus cellulaires et subcellulaires impliqués lors de la régulation de la symbiose en conditions stables et lors d'une limitation nutritionnelle de l'organisme. Nous avons également étudié les processus cellulaires intervenant lors d'un stress hyperthermal entraînant la rupture de la symbiose. Ce premier chapitre comporte aussi l'étude d'un nouveau procédé chimique de rupture de la symbiose, récemment mis au point chez les Scléractiniaires (Wang et al., 2012) et dont les mécanismes n'avaient jamais été décrits. Dans une seconde étude, nous avons utilisé un marqueur couramment utilisé dans la détection des plaques thécales chez les Dinoflagellés (Fritz and Triemer, 1985; Fujise et al., 2014a), le CalcoFluor White. Ce marqueur permet de révéler la présence de

cellulose. Il a également été utilisé dans une étude précédente pour corrélérer le cycle de synthèse de la cellulose (constituant majeur des plaques thécales) chez des Dinoflagellés avec les stades du cycle cellulaire (Kwok and Wong, 2003). En utilisant ce marqueur nous avons déterminé *in hospite* les modifications de la composition de la paroi des symbiotes en condition symbiotique et lors des différents stress étudiés dans la première étude.

Une deuxième partie de mon projet (Chapitre 3) a permis de définir le répertoire chez *A. viridis* des gènes *npc1* et *npc2*, gènes impliqués dans le trafic intracellulaire du cholestérol, puis de caractériser leur localisation et leur régulation au niveau transcriptomique et protéomique, à l'état stable et lors de la rupture de la symbiose. Les résultats obtenus nous permettent de proposer un nouveau marqueur de stress chez les Cnidaires symbiotiques (NPC2-d) mais également un marqueur de la membrane pérисymbiotique (NPC1) chez l'anémone de mer et deux espèces de coraux.

Par la suite, ce marqueur de la membrane pérисymbiotique nous a permis d'élaborer un protocole de préparation des symbiotes isolés, de façon à caractériser la membrane pérисymbiotique. Ce travail sera développé dans la dernière partie de mon manuscrit (Chapitre 4). Cette étude a l'ambition d'initier la caractérisation la plus complète à ce jour des protéines recrutées au niveau du symbiosome. Les résultats obtenus permettront également la caractérisation de nouvelles voies de signalisation dans l'étude de la symbiose Cnidaire-Dinoflagellés.

- 1 Caractérisation de la structure cellulaire et subcellulaire chez *A. viridis* (Chapitre 2)
- 2 Détermination du stade du cycle cellulaire du symbiote (Chapitre 2)
- 3 Localisation et régulation au niveau transcriptomique et protéomique des NPC (Chapitre 3)
- 4 Caractérisation du protéome du symbiosome (Chapitre 4)

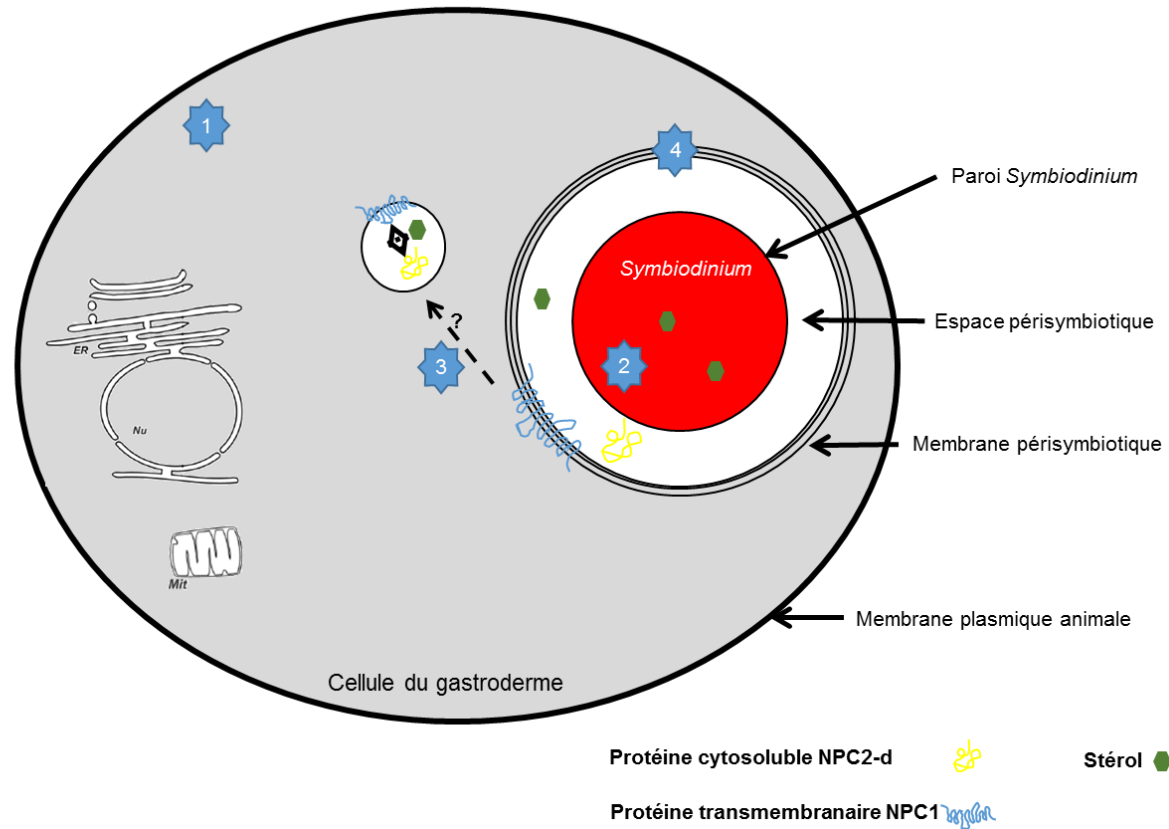


Figure 13: **Schéma bilan des différentes thématiques abordées durant mon projet doctoral.** Le schéma ci-dessus représente une cellule de gastroderme isolée possédant un symbiote (en rouge), entouré de la membrane périssymbiotique. La flèche en pointillés représente le transport hypothétique du stérol d'origine photosynthétique par les protéines NPC.

Chapitre 2 : Caractérisation du modèle : organisation cellulaire et subcellulaire, et régulation du cycle cellulaire des symbiotes

2.1. Contexte scientifique et problématique de l'étude

L'organisation des tissus chez les Anthozoaires a été largement décrite lors de précédentes études notamment par les travaux de Fautin, publiés en 1991 (Fautin and Mariscal, 1991). Cet ouvrage détaille l'organisation cellulaire et subcellulaire des Anthozoaires, par comparaison entre plusieurs espèces. Même si une organisation générale de ce groupe a pu être définie, la variété des formes sur le plan organisationnel et la grande plasticité démontrée par les organismes de ce groupe, nécessite une description plus approfondie de notre modèle d'étude. Il s'agira dans un premier temps de préciser l'organisation au niveau cellulaire et subcellulaire d'*A. viridis* à l'état symbiotique stable. L'objectif est de disposer d'une base de comparaison afin de comprendre les mécanismes d'interaction qui pourront être observés aussi bien dans des conditions normales de symbiose que lors de la rupture de cette dernière. Diverses réponses cellulaires ont été décrites chez les Cnidaires lors d'une rupture de symbiose, telles que la nécrose (Dunn et al., 2002), l'autophagie et l'apoptose (Dunn et al., 2007), l'exocytose (Steen and Muscatine, 1987) et le détachement de la cellule gastrodermique (Brown et al., 1995). Certains de ces mécanismes ont également été décrits lors de l'étude de la régulation de la population *in hospite* des symbiotes (Titlyanov et al., 1996) et en réponse à un stress nutritif (Fitt, 2000).

De façon complémentaire, de nombreuses études ont présenté une intime corrélation entre le cycle cellulaire de l'hôte et celui de son symbiote (Dimond et al., 2013; Wilkerson et al., 1988). Les travaux pionniers sur la relation symbiotique entre l'hydre et les chlorelles ont montré un contrôle de l'hôte sur le cycle cellulaire de ses symbiotes (Smith and Muscatine, 1999). Même si les mécanismes impliqués dans cette régulation sont encore à définir (en revue dans Davy et al., 2012a), la plupart des études ont pour le moment mis en évidence une limitation par l'hôte de la disponibilité de certains éléments indispensables à la photosynthèse des symbiotes (Fitt, 2000; McAuley, 1985; McAuley, 1987). Ainsi, l'hôte a la capacité de maintenir une partie de la population de ses symbiotes dans le stade G1/G0, correspondant à la phase de croissance et contrôle ainsi le passage des symbiotes dans la phase S de réplication. Ainsi, une partie minoritaire se trouve en phase S/G2/M, correspondant à la phase impliquée dans le cycle de division cellulaire. Néanmoins,

il n'existe pas d'informations sur le contrôle de l'hôte sur le cycle cellulaire des symbiotes lors d'épisodes de stress entraînant la rupture de la symbiose.

Dans ce contexte, les objectifs des études présentées dans ce chapitre consistent dans un premier temps à :

i) Préciser l'organisation cellulaire et subcellulaire de notre modèle d'étude *A. viridis*. Nous insisterons sur l'organisation cellulaire des deux compartiments (épiderme et gastroderme) ainsi que la mésoglée et l'interface symbiotique à l'état stable.

ii) Réaliser un suivi des modifications engendrées au niveau subcellulaire pour poser des hypothèses sur les mécanismes initiés lors de différents stress impliqués dans la rupture de la symbiose.

Il s'agira également dans un deuxième temps de :

iii) Mettre en place une approche méthodologique afin de définir *in hospite* le statut métabolique des symbiotes à l'état stable et le stade du cycle cellulaire des symbiotes

iv) Suivre le contrôle de l'hôte sur ses symbiotes en conditions de rupture de symbiose en utilisant comme proxy le stade du cycle cellulaire des symbiotes.

2.2. Approche expérimentale

Afin d'étudier les mécanismes cellulaires impliqués dans la rupture de la relation symbiotique chez *A. viridis*, nous avons choisi au préalable de caractériser l'état symbiotique stable. Ce travail préliminaire nous a paru déterminant afin de mieux appréhender les modifications observées lors de conditions qui perturbent l'état symbiotique. Nous avons également étudié les différents mécanismes observés lors d'un stress nutritionnel n'entraînant pas la rupture de la symbiose. En effet, si ce type de stress ne revêt pas d'utilité directe dans la compréhension des mécanismes qui entraînent le blanchissement massif des coraux, il pourrait nous en apprendre davantage sur les mécanismes de régulation tant au niveau de l'hôte que du symbiote, lors de l'association symbiotique. Nous avons ainsi étudié deux stress entraînant la rupture de la symbiose. Premièrement, nous avons suivi la réponse au niveau cellulaire lors d'une augmentation de la température de l'eau de 10°C. Ce type de stress est le plus communément étudié dans la problématique de blanchissement des coraux. Néanmoins, les mécanismes cellulaires et la régulation du cycle cellulaire des symbiotes lors d'un stress hyperthermal chez *A. viridis* n'ont pas été décrits à ce jour. Le deuxième stress étudié concerne la rupture de symbiose par incubation sérielle d'*Anemonia viridis* dans du menthol. Cette méthode de blanchissement, récemment mise au point par Wang et ses collaborateurs chez deux espèces de corail (Wang et al., 2012), a montré son utilité dans l'obtention d'individus aposymbiotiques. Le point remarquable est que ce protocole entraîne le blanchissement, sans à priori impacter les caractéristiques physiologiques des coraux (photosynthèse), qui restent comparables à celles d'organismes symbiotiques. Néanmoins, ce type de blanchissement n'a jamais été expérimenté chez un Actiniaire et aucune étude n'a permis de déterminer les mécanismes moléculaires et cellulaires impliqués.

Afin de mettre en œuvre ces différents suivis, plusieurs mises au point méthodologiques et expérimentales ont dû être effectuées. Premièrement, nous avons adapté le protocole de blanchissement au menthol sur les spécimens d'*A. viridis*. A terme, cette nouvelle technique permettra d'obtenir rapidement et avec un taux de succès de 100% des individus aposymbiotiques d'*A. viridis*. Nous avons prélevé différents types d'échantillons au cours d'une cinétique de 60 jours pour réaliser le suivi à la fois d'individus contrôles et d'individus traités avec les trois types de stress décrits ci-dessus. Deux points de prélèvement, quatre heures et cinq jours post-stress, correspondent respectivement à l'observation de l'initiation des stress appliqués et au

déclenchement d'une réponse transcriptomique importante dans le cas du stress hyperthermal chez *A. viridis* (Moya et al., 2012). Le point de prélèvement à 15 jours correspond à l'arrêt du stress menthol et le point à 22 jours correspond à l'arrêt du stress température. Le point de prélèvement à 59 jours correspond au point final, quatre semaines post-stress. Afin de suivre les mécanismes cellulaires impliqués dans les différentes conditions entraînant la rupture de symbiose, nous avons prélevé à chaque point de la cinétique, (i) un échantillon biologique permettant le suivi de la population de symbiotes, (ii) un échantillon que nous avons fixé et observé par microscopie électronique, (iii) un échantillon permettant l'analyse globale des métabolites sur coupes de tissus, et (iv), des échantillons permettant de suivre la réponse transcriptomique et protéomique globale des spécimens d'*A. viridis*. Afin de suivre l'évolution *in hospite* des modifications moléculaires dans les différentes conditions décrites ci-dessus, nous avons donc mis au point une technique d'inclusion à base de carboxyméthylcellulose (CMC). De façon globale, ce milieu d'enrobage permettra d'effectuer par la suite des expériences de localisation *in situ* (hybridation *in situ*, immunolocalisation), mais également des études en protéomique et métabolomique sur coupes de tissu, ces dernières étant exigeantes en termes de neutralité du milieu d'enrobage.

Nous avons également cherché à développer un nouvel outil afin de (v), déterminer le statut métabolique des symbiotes *in hospite* dans des conditions stables et lors de stress entraînant la rupture de la symbiose. Pour cela, nous nous sommes appuyés sur le couplage du cycle de synthèse de la cellulose avec les stades du cycle cellulaire chez les Dinoflagellés (Kwok and Wong, 2003; Kwok and Wong, 2010). Ces études ont montré que la synthèse de la cellulose, impliquée dans la mise en place des plaques thécales, avait lieu lors de la phase G1, période du cycle cellulaire associée à la phase mobile des Dinoflagellés et au métabolisme actif des lipides et des glucides. Un premier pic de cellulose est alors atteint à la phase S et marque le maximum de croissance de la cellule, et correspond au passage de celle-ci dans la phase de division. Un autre pic de cellulose est alors mis en évidence lors de l'initiation de la cytokinèse, nécessaire à la formation de la paroi cellulaire des cellules filles (Figure 14) (Kwok and Wong, 2003). Il faut également noter que la concentration de cellulose retrouve un niveau minimum uniquement après deux heures dans la phase G1. Dans cette dernière étude, les auteurs ont utilisé le CalcoFluor White (CFW), initialement proposé par Fritz et Triemer (Fritz and Triemer, 1985) pour marquer la présence des plaques thécales sur des organismes du groupe des Dinoflagellés (Figure 14A). En effet, le CFW interagit avec les béta-polysaccharides tels que la cellulose (Herth and Schnepf, 1980; Hughes and

McCully, 1975), constituant majoritaire des plaques thécales (Loeblich and Sherley, 1979). Ainsi, l'intensité de fluorescence du CFW a pu être partiellement corrélée avec les stades du cycle cellulaire des Dinoflagellés (Figure 14C). Cette étude montre que l'intensité maximum est observée au cours des phases S/G₂/M de division. Ce fluorochrome peut alors être utilisé pour marquer des Dinoflagellés ayant atteint le stade de croissance nécessaire pour passer en phase de réplication.

Ce type de marquage a également été utilisé sur des cultures de *Symbiodinium* dans le but de mettre en évidence l'alternance des phases mobile et coccoïde lors d'un cycle journalier (Fujise et al., 2014a). Les *Symbiodinium* en phase mobile présentent alors un marquage positif au CFW alors que les cellules en phase coccoïdes présentent une absence de marquage.

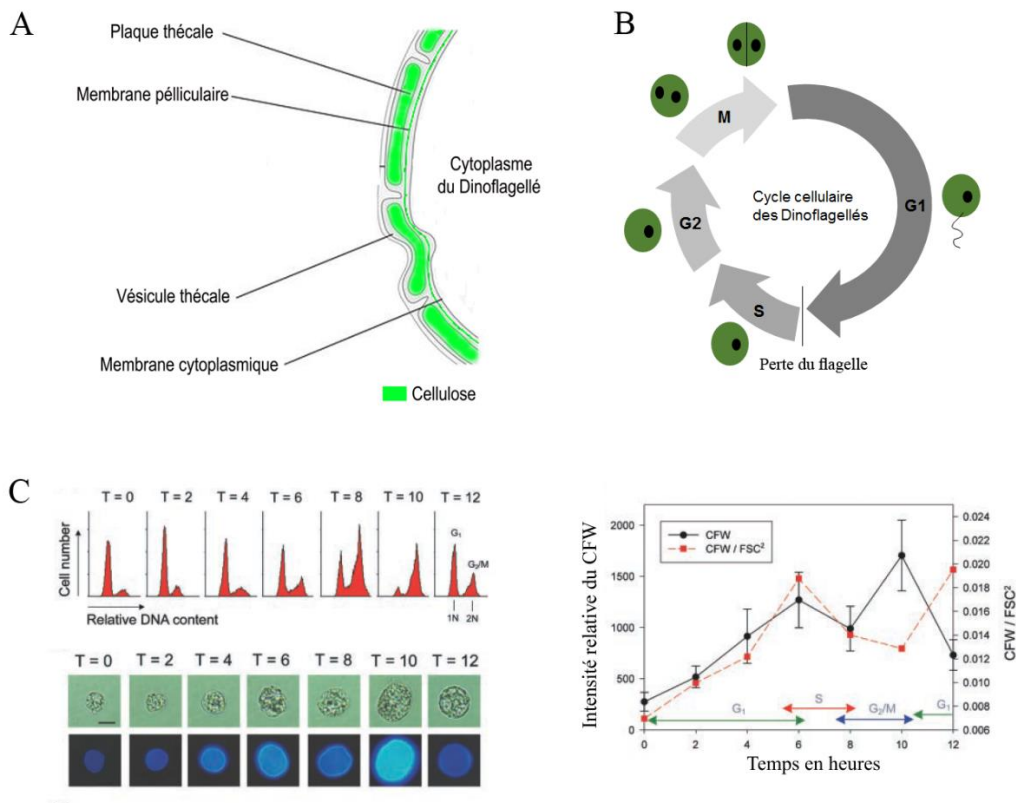


Figure 14: (A) Schéma de localisation des plaques thécales chez les Dinoflagellés (B) Schéma du cycle cellulaire des Dinoflagellés (C) Corrélation entre le stade du cycle cellulaire des Dinoflagellés et l'intensité de fluorescence du CFW.

Nous avons donc suivi la modification du cycle de la synthèse de cellulose en utilisant le marquage CFW comme indicateur de l'activité métabolique et du stade du cycle cellulaire des symbiotes *in hospite*, en condition stable de symbiose mais également lors des différents types de rupture de symbiose précédemment étudiés. En effet, la technique se basant sur la détermination de l'index mitotique par distinction des cellules de symbiote en doublet ou non (Costa et al., 2013; Wilkerson et al., 1988), ne permet pas de séparer les symbiotes en phase G2 des symbiotes en phase G1 mais seulement de distinguer entre des cellules en phase G1/G0/S/G2 et des symbiotes en phase M tardif, seul stade où la cellule est en cytotcinèse. Pourtant, il apparaît que le passage de la phase G1 à S est activement contrôlé par l'hôte (Falkowski et al., 1993; Wilkerson et al., 1988) via l'apport de nutriments (Blank and Muscatine, 1987; Neckelmann and Muscatine, 1983; Rees and Ellard, 1989) permettant à la cellule d'obtenir une taille seuil nécessaire au passage vers le cycle de division. Nous avons alors développé ce marquage aussi bien sur des cellules de symbiotes isolés que sur des coupes de tissus d'*A. viridis*, afin de comparer ces paramètres dans des conditions de symbiose stable avec ceux obtenus lors de la rupture provoquée de l'association. Comme de nombreuses études ont suggéré un contrôle de l'hôte sur le cycle cellulaire des symbiotes (en revue dans Davy et al., 2012), il sera pertinent de pouvoir suivre l'évolution du pourcentage de symbiotes dans les différents stades du cycle cellulaire au cours de différentes conditions expérimentales.

2.3. Résultats

2.3.1. Publication n°1 (soumise)

Dani, V., Priouzeau, F., Pagnotta, S., Carette, D., Laugier, J. P. and Sabourault, C. Thermal and menthol stress induce different cellular events during sea anemone bleaching. (Soumise) *Symbiosis*.

Problématique: Quels sont les mécanismes cellulaires impliqués dans les différents types de rupture de symbiose chez *A. viridis* ?

Outils méthodologiques : Observations par microscopie électronique de coupes de tentacules d'*A. viridis*

Principaux résultats : Selon le type de stress appliqué, le mécanisme principal impliqué dans la rupture de la symbiose au niveau cellulaire est différent. Lors de l'augmentation de la température, les mécanismes d'apoptose et de nécrose sont principalement activés. Lors du stress menthol, c'est la voie de l'autophagie qui est principalement observée.

Perspectives : Déterminer les acteurs moléculaires impliqués dans les différents types de rupture symbiotique (stress par augmentation de la température ou incubation dans le menthol) par analyses du transcriptome et du protéome.

Thermal and menthol stress induce different cellular events during sea anemone bleaching

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Additional key words: symbiotic interaction, cnidarian bleaching, autophagy, symbiophagy, apoptosis, necrosis

Running title: cellular mechanisms of symbiosis disruption

Abstract

Cnidarian-dinoflagellate symbiosis disruption and subsequent bleaching are major concerns, especially regarding their ecological consequences on coral reefs and temperate coralligenous communities. Cnidarian bleaching is caused by a variety of environmental stressors, such as elevated seawater temperature associated with global climate change, and by pollutants, such as herbicides and metals. Several cellular events have been described to explain symbiosis dysfunction and bleaching. Excess or damaged *Symbiodinium* symbionts are removed through a variety of mechanisms, including exocytosis, apoptosis, necrosis and autophagy. However, few studies have compared in the same species the relative involvement of these mechanisms, according to the stress inducing the bleaching. In this study, we used two different treatments — temperature and menthol— to induce bleaching in the sea anemone *Anemonia viridis*. By monitoring the ultrastructural tissue modifications, in control specimens we observed a basal rate of *in situ* symbiont digestion —or symbiophagy— induced by starvation. Symbiophagy was strongly induced in menthol-treated specimens and was the main cellular process of bleaching, whereas apoptosis and necrosis predominated in hyperthermal-induced bleaching. These results suggested a host effect through autophagy in menthol-treated specimens. These observations also suggested that symbiont removal may result from reengagement of the phagosomal maturation process in the host. These overall data demonstrate that several *Symbiodinium* cell removal mechanisms coexist and that stressors can activate one or more of these pathways, depending on the stress type, intensity or duration.

Introduction

Cnidarian–dinoflagellate endosymbioses have a key role in marine biodiversity as they form both the trophic and structural foundation of coral reef ecosystems, as well as diverse temperate habitats. The dinoflagellate symbionts, *Symbiodinium* spp., fix large quantities of inorganic carbon and translocate most of their photosynthates to support host metabolism, growth and reproduction. In return, the dinoflagellates have access to a stable and protected habitat, as well as essential nutrients, such as carbon, nitrogen and phosphorus, in part by recycling host waste products (Davy et al. 2012). However, the survival of the cnidarian-dinoflagellate association is threatened by global climate change and multiple stressors, including elevated temperature and ocean acidification, leading to symbiosis breakdown and cnidarian bleaching. Bleaching is a possibly-reversible mechanism that corresponds to a loss of color resulting from the loss of *Symbiodinium* cells and of their photosynthetic pigments (Weis 2008). Bleached individuals have a drastically reduced survival rate and fitness, as well as an increased susceptibility to diseases (Hoegh-Guldberg et al. 2007). Nevertheless, recolonization of the cnidarian host by the same or another *Symbiodinium* clade may happen and allow the host to recover.

In stable symbiotic condition, cnidarians host their unicellular dinoflagellate symbionts within the gastrodermal cell layer. The dinoflagellates may be maternally transmitted or acquired from the surrounding seawater, mainly by phagocytosis (Muscatine et al. 1975). They reside in an intracellular symbiotic compartment and the whole entity is defined as the symbiosome. The symbiosome membrane complex consists of a series of membranes of algal origin and an outer host-derived membrane (Wakefield and Kempf 2001). Conversion of the initial phagosomes into symbiosomes, mainly by the arrest of phagosomal maturation (Hill and Hill 2012), is required for symbiosis maintenance (Hohman et al. 1982; Davy et al. 2012). Indeed, Chen and coworkers demonstrated that *Symbiodinium* survive in symbiosomes by somehow manipulating endosomal trafficking (Chen et al. 2003; Chen et al. 2004), a mechanism that has also been described in many intracellular parasitic interactions, such as interactions between Mycobacteria or Apicomplexans and animal cells (Koul et al. 2004; Schwarz et al. 2008).

Many investigations have been performed to clarify the cnidarian bleaching process, which is complex and involves several different mechanisms, depending on the type, degree and duration of environmental stress. Several stressors have been studied, especially elevated seawater

temperature, which is the most significant factor leading to cnidarian bleaching (for review see Weis 2008 and Wooldridge 2013). Other stressors, such as ultraviolet radiation, cold stress and several chemical bleaching inducers, have also been tested. These studies have demonstrated that multiple cellular pathways are involved in the loss of symbionts, including *Symbiodinium* cell exocytosis (Steen and Muscatine 1987; Downs et al. 2013), gastrodermal cell detachment (Gates et al. 1992; Sawyer and Muscatine 2001), *in situ* dinoflagellate degradation (Brown et al. 1995), apoptosis (Dunn et al. 2002; Dunn et al. 2004; Richier et al. 2006; Dunn et al. 2007; Tchernov et al. 2011) and necrosis of both *Symbiodinium* (Strychar et al. 2004a; Strychar and Sammarco 2009) and host cells (Dunn et al. 2002; Dunn et al. 2004). More recently, Downs et al reported a process referred to as “symbiophagy”, which corresponds to autophagic activity within the host-derived symbiosome resulting in the digestion of the resident dinoflagellate (Downs et al. 2009). This process was described by homology to xenophagy, which corresponds to the conversion of the vesicle that envelopes intracellular bacteria from a niche for the pathogen to a phagolysosome that degrades the bacteria. This phenomenon is thought to be involved in various bleaching processes (Downs et al. 2009).

Autophagy is the generic name used for cellular processes that result in the degradation of cytosolic components inside lysosomes. Autophagy is a form of programmed cell death (PCD) and is linked with other membrane trafficking pathways and apoptosis (Cuervo 2004). This evolutionarily-conserved pathway is an important cellular process involved in the removal of degraded organelles (Chera et al. 2009), but also of microbial invaders (Koul et al. 2004; Wileman 2013). Indeed, when a non-self-target has been identified, the phagosomal structure fuses with lysosomes and is subjected to the action of hydrolytic enzymes. This field is the subject of intense research, and guidelines have been established to monitor autophagy in several experimental systems (Klionsky et al. 2012). Nevertheless, it is sometimes difficult to interpret results because no absolute criteria for determining autophagic status are applicable in every biological or experimental context. Regarding cnidarians, autophagic degradation within host cells was observed in symbiotic sea anemones submitted to thermal stress. This process is characterized by the presence of active autophagic structures (APSs), which are described as dense irregular structures containing partially digested cytoplasmic material (Hanes and Kempf 2013). Both whorls and APSs were also observed in gastrodermal cells of the coral *Pocillopora damicornis* exposed to high temperatures (Downs et al. 2009). In addition, these authors identified a new xenophagic-like

process that they termed symbiophagy. At an ultrastructural level, symbiophagy was described by the presence of specific structures, such as vacuolization around symbionts and increased vacuolar matrix volume (Downs et al. 2009). This process is thought to induce the conversion of symbiosomes into phagolysosomes, resulting in the degradation of dinoflagellate symbionts.

Apoptosis, another form of PCD, is a crucial process involved in the elimination of damaged or infected cells, and is highly conserved throughout evolution, from vertebrates (Lasi et al. 2010) to cnidarians (Dunn et al. 2006). It is initiated by a cascade of stages that are triggered by intrinsic or extrinsic signals (Rubinstein and Kimchi 2012). These stimuli activate cysteine proteases of the caspase family via different pathways. Upon activation, upstream caspases propagate death signals by activating downstream —or effector— caspases in a cascade-like manner. This cascade results in cell death via a series of host controlled cellular events, including blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, fragmentation of DNA and formation of apoptotic bodies that contain packaged cellular debris. Some of these morphological alterations have been previously described in sea anemones (Dunn et al. 2004; Richier et al. 2006) and corals (Lesser and Farrell 2004), as well as in their resident *Symbiodinium* ((Dunn et al. 2002; Strychar et al. 2004a; Dunn et al. 2004; Strychar et al. 2004b; Sammarco and Strychar 2013). They are characterized by condensation of the cytoplasm, nucleus and organelles, and the presence of multiple electron-dense apoptotic bodies (Dunn et al. 2002). In opposition to symbiophagy, no vacuolization surrounding the symbiont has been observed in ultrastructural studies (Dunn et al. 2004; Strychar et al. 2004b). While apoptosis has been described in both host and symbiont cells in cnidarians exposed to seawater temperature above their normal levels, hosts have higher temperature tolerances than their dinoflagellate symbionts (Strychar and Sammarco 2009). Indeed, the photosynthetic machinery is highly temperature-sensitive and its dysfunction results in energetic uncoupling of electron transport and reactive oxygen species (ROS) production (for a review, see Weis 2008). It is likely that impaired dinoflagellate symbionts enter a cell death process, such as apoptosis or necrosis, before being released from their host (Strychar et al. 2004b; Sammarco and Strychar 2013). However, recent results also suggest that the enzymatic regulation of cellular redox status depends of both the nature of the stressor and the species involved. For example, investigations related to antioxidant responses to heat and light stress in the coral *Stylophora pistillata* and its resident *Symbiodinium* demonstrate that photooxidative stress in these algae may not be a prerequisite for coral bleaching (Hawkins et al. 2015).

In contrast to autophagy and apoptosis, necrosis was initially described as an uncontrolled cell death mechanism that is triggered by external stimuli and causes cell lysis (Leist 2001). However, a necrosis-like PCD has also been depicted and usually involves caspase-independent signaling pathways (Proskuryakov et al. 2003). Necrosis involves cell swelling, cell membrane disruption, presence of lysed cellular material and absence of nuclear chromatin condensation. Necrosis can also occur as the final step of apoptosis. Host cell necrosis has been observed in symbiotic cnidarians subjected to hyperthermal stress, mainly within the symbiont-containing gastrodermal tissue (Dunn et al. 2002; Dunn et al. 2004). In these studies, necrosis was characterized at an ultrastructural level by large vacuolization of the cytoplasm, internal degradation of the cellular content, and the absence of condensed chromatin and apoptotic bodies in both the host cells (Dunn et al. 2002; Dunn et al. 2004) and their dinoflagellate symbionts (Dunn et al. 2002; Strychar et al. 2004a; Strychar et al. 2004b; Sammarco and Strychar 2013).

A gentle bleaching method involving serial menthol incubations of two scleractinian coral species was recently proposed in order to generate “healthy” aposymbiotic specimens for experimental purposes (Wang et al. 2012). Menthol was initially used to anesthetize cnidarians and was occasionally found to bleach symbiotic specimens. This technique seems promising because menthol-induced bleached specimens were shown to have comparable physiological performances as their symbiotic counterparts with or without nutrient complementation, depending on the coral species (Wang et al. 2012). As hyperthermal-induced bleaching usually decreases symbiotic cnidarian fitness and increases mortality, the menthol protocol is a good alternative to generate and study aposymbiotic specimens. However, while this protocol has been successfully tested on the sea anemone *Aiptasia* (Matthews et al. 2015), the cellular mechanisms that lead to bleaching in menthol-treated cnidarians have yet to be determined.

In this study, we attempted to fill this gap by studying the effect of menthol on the sea anemone *Anemonia viridis*. We characterized the cellular events involved in menthol-induced bleaching, compared to symbiotic sea anemones subjected to hyperthermal-induced bleaching. The possible occurrence of cell death mechanisms such as autophagy, apoptosis and necrosis was determined based on previously reported cellular ultrastructural characteristics. Our findings provide further insight into both host and symbiont responses regarding mechanisms of *Symbiodinium* cell removal that occur throughout the stress-induced bleaching process. We showed

that different bleaching mechanisms, including apoptosis, necrosis and autophagy, can be involved in the same species, i.e. the sea anemone *A. viridis*, in response to different stressors.

Methods

A. viridis culture conditions

Mediterranean *A. viridis* (Forskål, 1775) specimens were collected during the autumn season in 19°C shallow waters at Villefranche-sur-Mer (France). The only *Symbiodinium* clade hosted by *A. viridis* is the “temperate A” clade, which is mainly acquired by vertical transmission (Visram et al. 2006; Casado-Amezúa et al. 2014). Sea anemones were maintained in laboratory-controlled conditions where they were acclimated for one month before experimentation. Seawater was maintained at $19 \pm 0.5^\circ\text{C}$ in aquaria and renewed weekly. Sea anemones were fed *Artemia* once a week. Light was provided at a constant irradiance of $180 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ on a 12/12 h light/dark cycle with a metal halide lamp (HQI-TS 400 W, Philips). To avoid contamination of the protein and RNA samples used for another study, stressed specimens were starved throughout the stress experiment (days 0 to 22), except for control specimens that were maintained under standard laboratory conditions. At the end of the stress experiment (day 22), aposymbiotic specimens were maintained under control temperature, feeding and water renewal conditions.

A. viridis treatments

Symbiotic *A. viridis* specimens (n=3) were subjected to hyperthermal stress (+10°C) for 22 days (as described in Moya et al. 2012). The temperature was suddenly increased to 29°C and the specimens were kept under these conditions for 22 days, with continuous monitoring of the seawater temperature and daily seawater renewal.

For the menthol treatment, symbiotic *A. viridis* specimens (n=3) were incubated in 0.58 mM menthol for 22 days under a 8/16 h exposure/resting cycle and normal temperature, with daily seawater renewal. A 1000X menthol stock solution was prepared in absolute ethanol. After each menthol exposure, tanks were rinsed to flush out the menthol and sea anemones were kept in normal seawater for the resting period.

Symbiotic *A. viridis* specimens (n=3), referred to as starved controls, were subjected to starvation for 22 days under the control temperature and water renewal conditions. Two other

specimens, which were referred to as controls, were maintained in normal conditions and sampled like the stressed specimens.

For each specimen, tentacles were sampled and processed immediately at the following days: d0, d0+4h, d5, d10, d15, d22 and d59. At each sample time, two tentacles were used for *Symbiodinium* density quantification and two others were fixed for transmission electronic microscopy analysis (see below). All sampling was performed at 10:00 am to avoid circadian rhythm variations (Levy et al. 2011).

***Symbiodinium* density quantification**

For each time point, two tentacles per individual were sampled, dried and weighed using a precision balance (Precisia, Model 202A, Sweden). Samples were incubated for 90 min in 1M NaOH solution at 37°C in a drying chamber (Jouan, EB28, France) to allow animal tissue digestion and *Symbiodinium* isolation (Zamoum and Furla 2012). An aliquot of this homogenate (40 µl) was diluted and used to determine the *Symbiodinium* cell density using a Neubauer-Hauser hemocytometer (VWR, Fontenay-sous-Bois, France) under a light microscope (Leica, Wetzlar, Germany). Ten replicates were quantified for each sample and the results were weight normalized. The data were first checked for normality and heteroscedasticity of sample variance, then *Symbiodinium* quantifications were tested using repeated ANOVA measurements with the Tukey multiple comparison post hoc test. For these analyses, a p-value of <0.05 was considered statistically significant. All analyses described above were conducted using GraphPad InStat Version 3 software (GraphPad Software, San Diego, CA).

Scanning electron microscopy (SEM)

Tentacles for SEM analysis were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer/artificial seawater, then cryo-fixed in Leica EM HPM100. The samples were transferred in a cryo-transfer system PP3000T Quorum, cryo-fractured, freeze dried and coated with platinum. They were then observed on a FEI Quanta FEG 250 SEM at the CCMA facility (Nice, France).

Transmission electron microscopy (TEM)

A. viridis tentacles were fixed for 2 h at room temperature with 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4)/artificial seawater, then washed with 0.1 M cacodylate buffer (pH 7.4) and postfixed with 1% osmium tetroxide in cacodylate buffer containing 1% potassium ferrocyanide, according to Dani et al. (2014). The samples were embedded in Epon resin after dehydration using an acetone/water solution and then acetone. Ultrathin sections (70–80 nm) of mid-tentacle portions were cut using a diamond knife mounted on an ultramicrotome (Ultracut S, Leica) and placed on copper TEM grids coated with formvar film. To increase the contrast, the grids were treated with conventional uranyl acetate stain followed by lead citrate. Samples were observed under a JEOL JEM 1400 transmission electron microscope equipped with a CCD camera (Morada, Olympus SIS) at the Centre for Applied Microscopy (CCMA, University of Nice Sophia Antipolis, Nice, France). At least three independent sample observations were conducted for each condition.

TEM micrographs were used to monitor ultrastructural changes related to cell death mechanisms. First, tentacle tissue sections from healthy symbiotic specimens of *A. viridis* were observed to serve as a basis for interpretation of ultrastructural changes that were identified by comparison to tissues exposed to hyperthermal stress or menthol treatment for various time periods. The possible occurrence of cell death mechanisms was assessed based on cellular ultrastructural criteria that are presented in Figure 1. Such morphological characteristics were previously used to describe autophagy, apoptosis and necrosis in symbiotic cnidarians (Dunn et al. 2002; Strychar et al. 2004a; Dunn et al. 2004; Downs et al. 2009; Tchernov et al. 2011; Sammarco and Strychar 2013; Hanes and Kempf 2013). Both *A. viridis* cells and dinoflagellate symbionts displayed classic signs of autophagy, such as irregular electron-dense autophagic structures that resulted from the degradation of organelles and then retention of indigestible material, vacuolization around the symbionts, disorganization of thylakoids and degradation of *Symbiodinium* cell membranes (Fig. 1). Apoptotic symptoms in *Symbiodinium* cells within *A. viridis* tentacles included the development of electron-dense apoptotic bodies, condensation of organelles and/or nuclear chromatin, and cell shrinkage (Fig. 1). In contrast, cells were considered necrotic when they displayed intracellular debris or aggregate bodies, organelle rupture and cell swelling with or without rupturing (Fig.1).

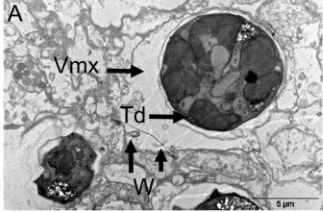
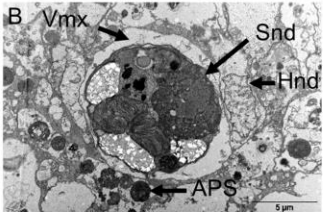
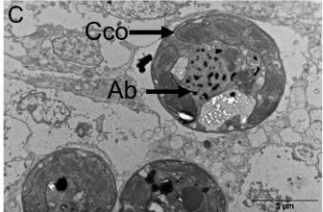
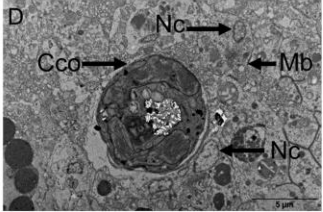
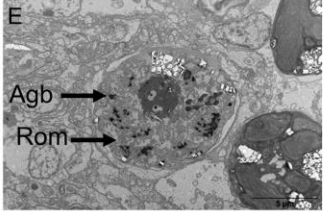
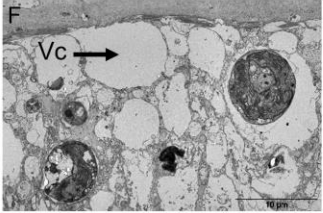
	Identification criteria	References
A U T O P H A G Y	<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;">  <p>Symbiophagy</p> <ul style="list-style-type: none"> • Vacuolar matrix (Vmx) • Thylakoid disorganization (Td) • Symbiont nucleus dissolution (Snd) </div> <div style="width: 45%;"> <ul style="list-style-type: none"> • Downs et al., 2009 </div> </div> <div style="display: flex; justify-content: space-between; margin-top: 10px;"> <div style="width: 45%;">  <p>Host</p> <ul style="list-style-type: none"> • Autophagic structures (APS) • Autophagic whorl (W) • Host nucleus dissolution (Hnd) </div> <div style="width: 45%;"> <ul style="list-style-type: none"> • Downs et al., 2009 • Hanes and Kempf., 2013 </div> </div>	
A P O P T O S I S	<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;">  <p>Symbiote</p> <ul style="list-style-type: none"> • Condensation of cytoplasm and organelles (Cco) • Apoptotic bodies (Ab) • Nuclear chromatin condensation (Nc) • Cell shrinkage </div> <div style="width: 45%;"> <ul style="list-style-type: none"> • Sammarco and Strychar., 2013 • Strychar et al., 2004 • Dunn et al., 2004 • Dunn et al., 2002 • Tchernov et al., 2011 </div> </div> <div style="display: flex; justify-content: space-between; margin-top: 10px;"> <div style="width: 45%;">  <p>Host</p> <ul style="list-style-type: none"> • Nuclear chromatin condensation (Nc) • Membrane blebbing (Mb) • Cell shrinkage • Condensation of cytoplasm and organelles (Cco) </div> <div style="width: 45%;"> <ul style="list-style-type: none"> • Dunn et al., 2004 • Dunn et al., 2002 </div> </div>	
N E C R O S I S	<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;">  <p>Symbiote</p> <ul style="list-style-type: none"> • Electron-dense aggregate bodies (Agb) • Ruptured organelle membrane (Rom) • Degraded organelles </div> <div style="width: 45%;"> <ul style="list-style-type: none"> • Sammarco and Strychar., 2013 • Strychar et al., 2004 • Dunn et al., 2004 • Dunn et al., 2002 </div> </div> <div style="display: flex; justify-content: space-between; margin-top: 10px;"> <div style="width: 45%;">  <p>Host</p> <ul style="list-style-type: none"> • Vacuolated cytoplasm (Vc) • Rupture of organelle membranes (Rom) • Cell swelling </div> <div style="width: 45%;"> <ul style="list-style-type: none"> • Dunn et al., 2004 • Dunn et al., 2002 </div> </div>	

Figure 1: **Identification criteria used to defined autophagic, apoptotic and necrotic processes observed by TEM in *A. viridis* host and *Symbiodinium* cells.** These morphological characteristics were previously used to describe host autophagy, symbiophagy, apoptosis and necrosis in symbiotic cnidarians (references provided) and are shown on *A. viridis* tentacle sections. a-e, scale bar = 5 μm; f, scale bar = 10 μm.

Screening of symbiophagic morphological signs

Symbiophagic morphological signs, as defined in Figure 1, were determined on TEM images from the same anatomical region of sea anemone tentacles. A xenophagic structure was described as a large vacuolated phagosome with a double-membrane enwrapping intracellular pathogens (Mizushima et al. 2010). In cnidarians, a symbiophagic structure was defined as a large vacuolated symbiosome containing a high volume of vacuolar matrix, which forms a large zone (0.5 to 2 μm) between the symbiosome or vacuolar membrane and the *Symbiodinium* cell wall (Downs et al. 2009). In our case, we compared these vacuolated symbiosomes to healthy symbionts (Fig. 2f), where symbiosome membrane and *Symbiodinium* cell wall were closely associated. Indeed, there was a tiny to undetectable volume of vacuolar matrix between the vacuolar membrane and the *Symbiodinium* cell wall. The relative number of *Symbiodinium* cells experiencing symbiophagy was determined from TEM images of sea anemones maintained under control conditions and starved-control conditions and hyperthermal and menthol-treated specimens, from day 0 to day 59. For heat-stressed and menthol-treated specimens no data were available after the bleaching threshold (day 10), as a consequence of the drastic decline of the symbiont population. The results were tested using the Kruskal–Wallis one-way analysis of variance by ranks with a Dunn multiple comparison post hoc test. For these analyses, a p-value of <0.05 was considered statistically significant. All the analyses described above were conducted using GraphPad InStat Version 3 software (GraphPad Software, San Diego, CA).

Results

A. *viridis* tentacle tissue description

A. viridis presented a classic diploblastic tissue organization, with an outer epidermis separated from the gastrodermis by a fibrous mesoglea layer (Fig. 2). TEM micrographs of tentacle highlighted the organizational features of the epidermis (Fig. 2a). Different cell types were found, including cnidocytes and mucus-secreting cells, as well as an extensive nerve plexus. Epidermal muscle cells containing longitudinal myonemes were functional antagonists to circular myonemes contiguous to the mesoglea (Fig. 2a). Interestingly, collagenous fibrils within mesoglea had the same organizational pattern as muscles, and were visible in both longitudinal and cross sections

(Fig. 2b). Most cells found in the mesoglea looked like granular amoebocytes (Fig. 2b-c), which are thought to be involved in mesogleal collagen production, phagocytosis, differentiation into other cell types, and in the cellular innate immune response (Larkman 1984; Fautin and Mariscal 1991). Different types of cellular junctions, including septate junctions (Wood 1959) were observed in the gastrodermal tissue layer (Fig. 2d). *Symbiodinium* cells were hosted within gastrodermis (Fig. 2a), in a phagosome-derived vacuole constituting the symbiosome. The symbiosome membrane complex was found to be a multi-lamellar structure consisting of one outer host-derived symbiosome membrane, plus a number of inner membranes of algal origin (Fig. 2e). Healthy symbionts (Fig. 2a and f, Fig. S1) were roughly spherical in shape and had multi-lobed well-developed chloroplasts with distinct lamellae (Fig. 2e). Depending on the micrograph, they usually displayed other distinct cellular components, such as a pyrenoid body surrounded by a white starch sheath and a nucleus with condensed chromosomes (Fig. 2f). Additional organelles were observed, such as dense accumulation bodies, lipid reserves and vacuoles that contained crystalline material.

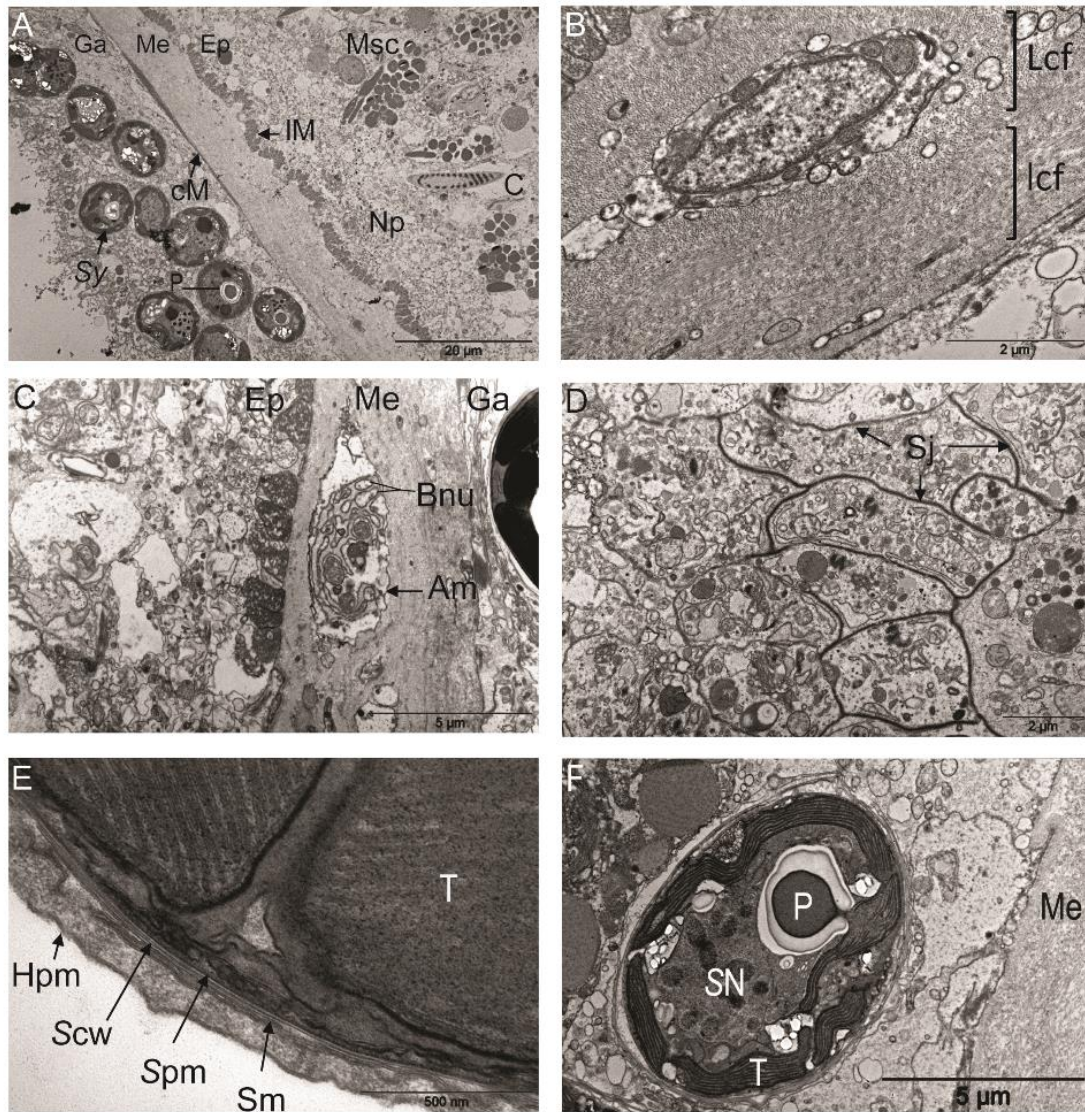


Figure 2: **Transmission electron micrographs of *A. viridis* tentacle cross sections.** (a) General view of a whole tentacle tissue section with the epidermis (Ep), mesoglea (Me) and gastrodermis (Ga) harboring unicellular dinoflagellate *Symbiodinium* symbionts (Sy). IM, longitudinal myonemes; cM, circular myonemes; Np, nerve plexus; C, cnidocyte; Msc, mucus-secreting cell; scale bar = 20 μm . (b) Focus on mesoglea showing lateral collagen fibers (lcf) and longitudinal collagen fibers (Lcf) adjacent to the gastrodermis and epidermis, respectively; scale bar = 2 μm . (c) A circulating ameobocyte-like cell (Am) within mesoglea, containing a bilobed nucleus (Bnu) and several types of granules; scale bar = 5 μm . (d) Focus on the apical end of the gastrodermis with cellular septate junctions (Sj); scale bar = 2 μm . (e) Higher magnification of an isolated symbiotic gastrodermal cell showing the different membranes: host plasma membrane (Hpm), symbiosome membrane (Sm), *Symbiodinium* cell wall (Scw), thylakoid (T) and *Symbiodinium* plasma membrane (Spm); scale bar = 500 nm. (f) Morphological structure of a symbiotic *Symbiodinium* cell, showing the nucleus (SN), multi-lobed thylakoids (T) and the pyrenoid body (P); scale bar = 5 μm .

Monitoring of *Symbiodinium* morphology and density during starvation

In normal symbiotic specimens, most symbiosomes displayed a host vacuolar membrane that was tightly attached to the algal membranes (Fig. S1). The volume of vacuolar matrix between the vacuolar membrane and the dinoflagellate cell wall was very limited. However, we also noticed the presence of a minor proportion of symbiophagic structures as defined in Figure 1, displaying vacuolization of symbiosomes and disorganized thylakoids (Fig. S1a and c).

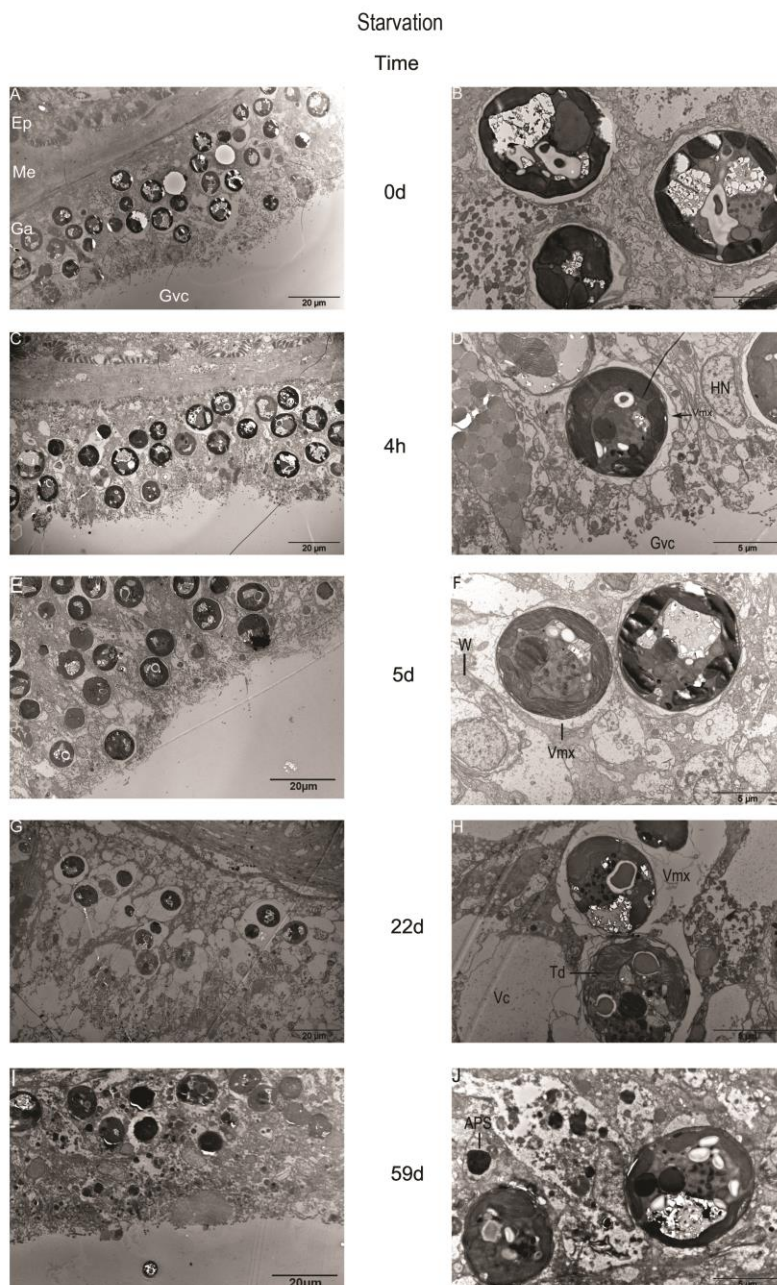


Figure 3: **Morphological structure of *A. viridis* specimens during starvation.**

Tentacle cross sections were performed and observed by TEM at the following sampling times: 0 (a,b), 4 h (c,d), 5 days (e,f), 22 days (g,h) and 59 days (i,j). Animals were starved from days 0 to 22. General views of the gastrodermal tissue layer (a, c, e, g, i) and focus on in hospite *Symbiodinium* cells (b, d, f, h, j). Ep, epidermis; Me, mesoglea; Ga, gastrodermis; Gvc, gastrovascular cavity; Vmx, vacuolar matrix; Td, thylakoid disorganization; HN, host nucleus; Vc, vacuolated cytoplasm; APS, autophagic structures; W, autophagic whorls; Am, amoebocyte. The observations revealed an activation of symbiophagy and host autophagy processes during starvation period.

Symbiophagy was also observed in the experimental control specimens that had been starved (referred to as “starved control”). These symbiophagic structures presented a large vacuolar space between the host vacuolar membrane and algal membranes (0.5 to 3 μm), and a high volume of vacuolar matrix (Fig. 3d). The proportion of symbiophagic structures remained stable at around 10 % throughout the experiment in the control specimens (Fig. 4), while it increased significantly in the starved-control specimens during starvation (Fig. 4). For example, degraded *Symbiodinium* cells were observed in tentacle cross sections of starved-control specimens, especially at day 22 (Fig. 3g-h). As soon as the sea anemones were fed again (after day 22), the proportion of symbiophagic structures decreased (day 59), until returning to the control level observed at d0 (Fig. 3i-j, Fig. 4). Nevertheless, the overall *Symbiodinium* cell population remained stable throughout the experiment (Fig. 5), probably because healthy *Symbiodinium* cell division *in hospite* (Fig. S2) may offset digestion or expulsion of non-functional resident symbionts. Other noteworthy structures included granular amoebocytes, which were also found to be closely associated with symbiosomes (Fig. 3j).

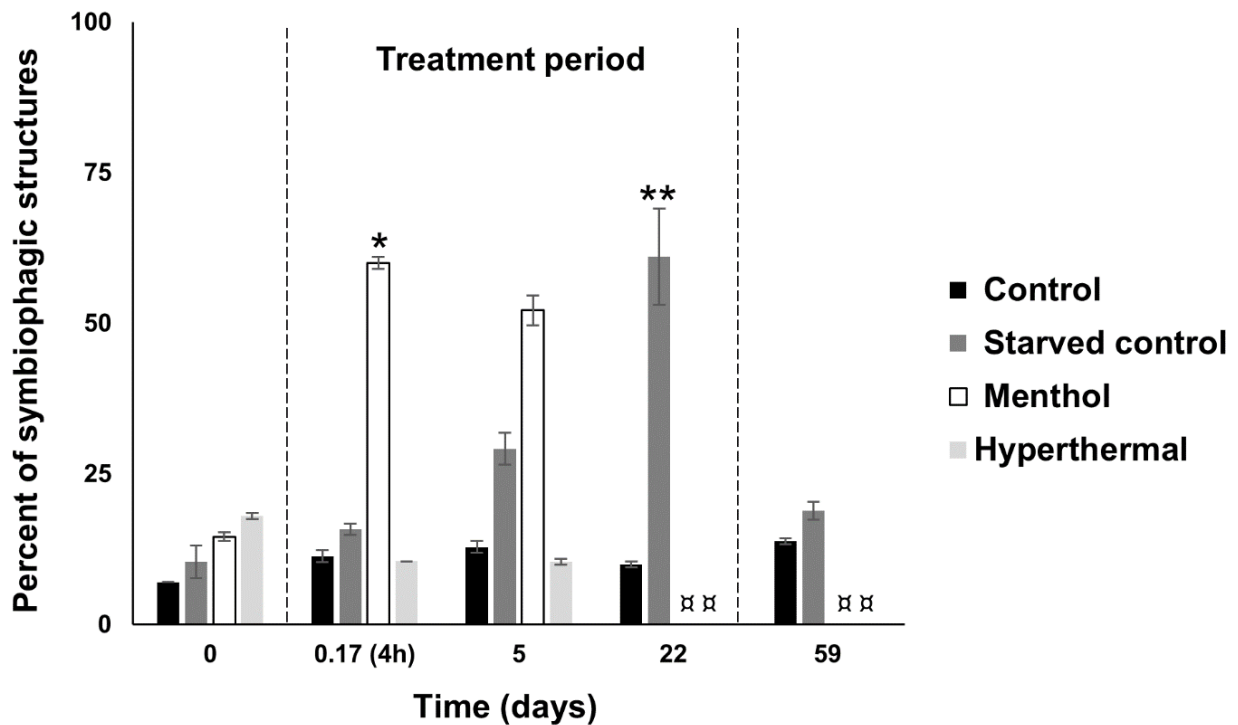


Figure 4: **Percent of symbiophagic structures in hospite in control, starved control, hyperthermal and menthol-stressed specimens.** Numbers of intact and symbiophagic structures were determined on TEM images for each sampling time. The percentage of symbiophagic structures remained stable in the control and hyperthermal-treated specimens and increased gradually during the starvation period in the starved control specimens and during menthol stress. Dotted lines indicate the treatment period. The results are given as means \pm SEM. Statistical analyses were performed using Kruskal–Wallis one-way analysis of variance by ranks with the Dunn multiple comparison post-hoc test; significant values are indicated for $p < 0.05$ (*) and $p < 0.01$ (**). \square indicate absence of data (bleached specimens).

SEM of tentacles from starved-control specimens showed that most symbionts were tightly embedded in the host gastrodermal tissue (Fig. 6a-c). While no visible thecal plates were observed at the surface of *Symbiodinium* cells, symbionts appeared to be covered by a granular-like film (Fig. 6b). We also found symbionts in larger vacuoles that were surrounded by actin-like filament networks (Fig. 6d). By analogy to the TEM results, we hypothesized that these vacuolated symbiosomes may correspond to potentially impaired resident symbionts. These SEM observations combined with the TEM findings suggested that the actin-like cytoskeleton network may

participate in symbiophagic structure formation, or that impaired symbionts were isolated in vacuoles and driven out of the host cell by cytoskeleton filaments (exocytosis).

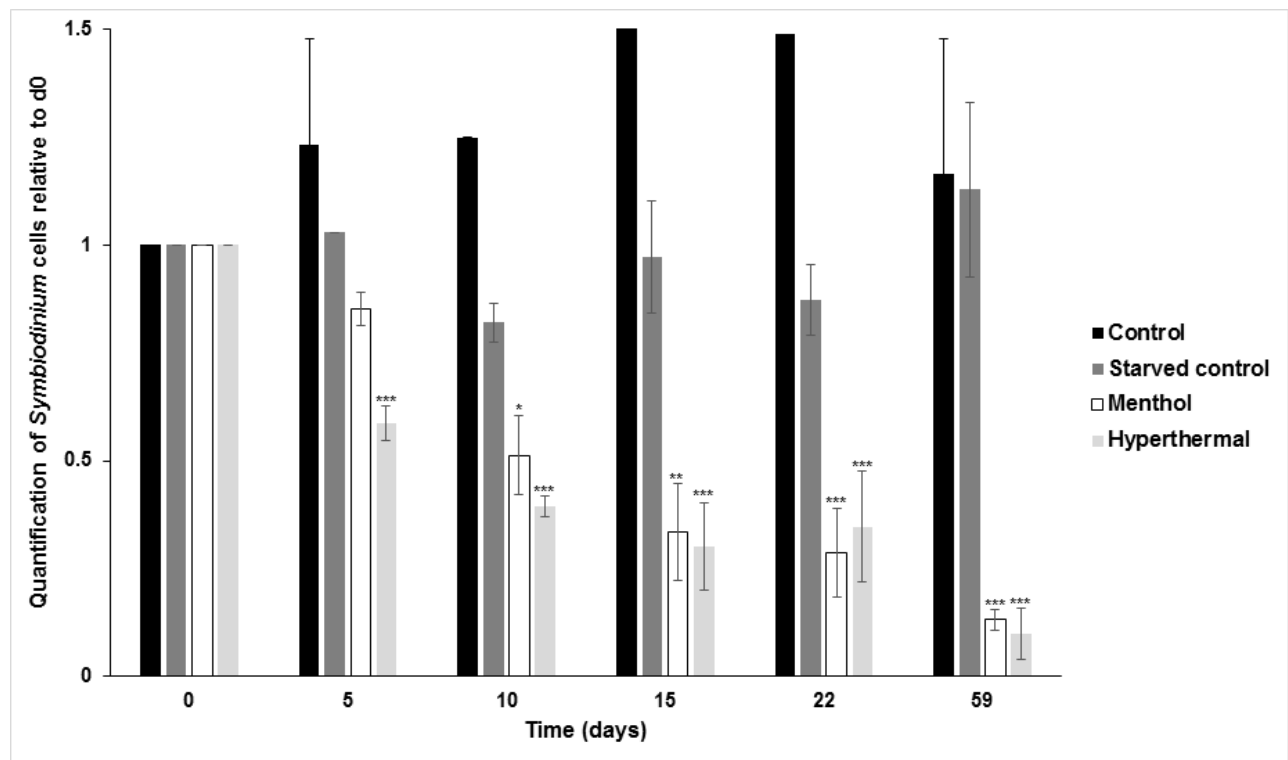


Figure 5: **Quantification of *Symbiodinium* cells relative to d0 within tentacles throughout the bleaching experiments.** The in hospite *Symbiodinium* population was determined by cell counting in control specimens (n=2), starved control specimens (n=3) and in specimens subjected to hyperthermal stress (n=3) or menthol stress (n=3). The results are given as means \pm SEM. Statistical analyses were performed using a repeated measures ANOVA with the Tukey multiple-comparison post-hoc test; significant values are indicated for $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

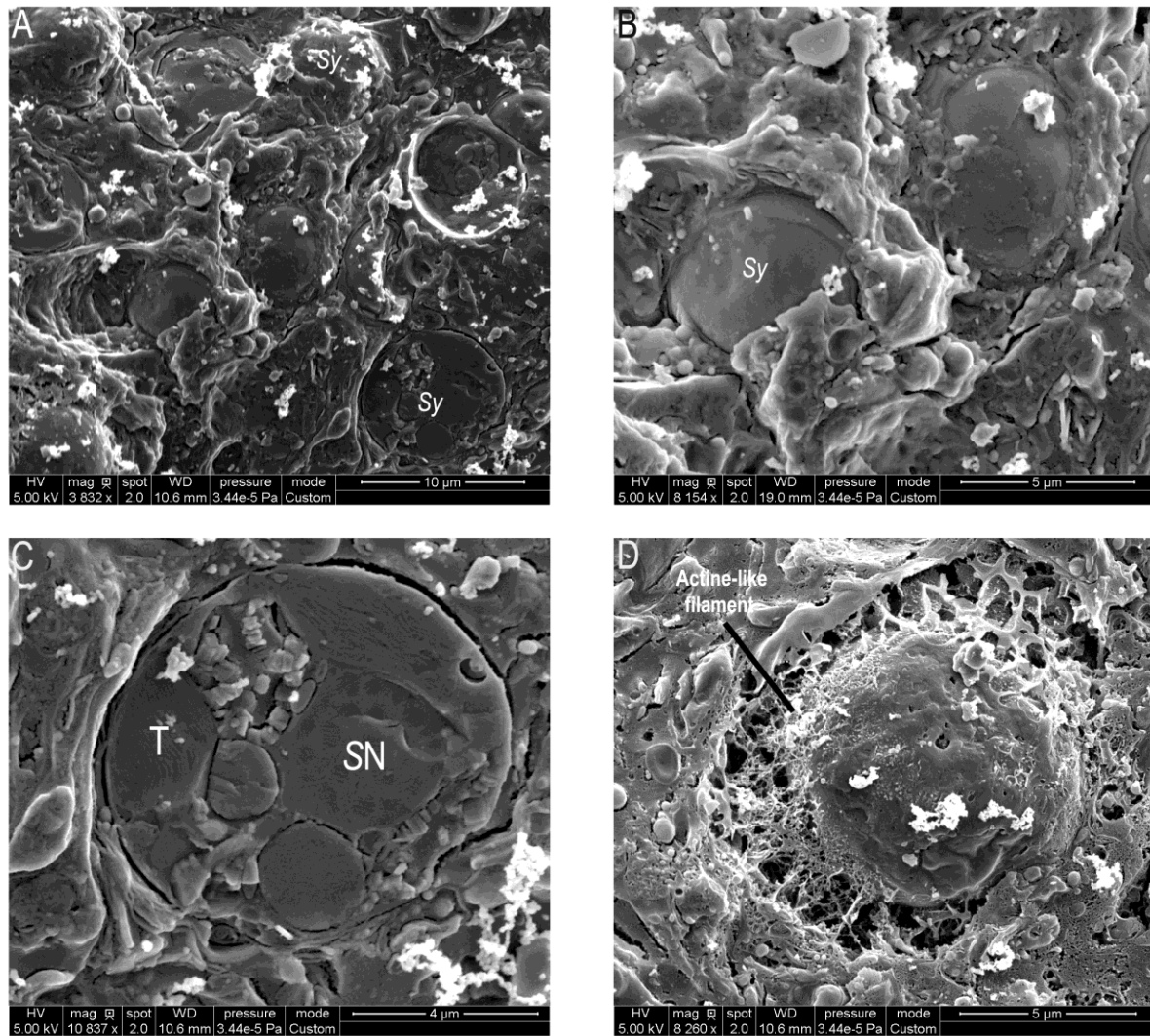


Figure 6: **Scanning electron microscopy of *A. viridis* tentacle cross sections.** A tentacle from a control specimen was cryo-fractured and observed by SEM, showing the gastrodermal tissue hosting numerous symbionts (a); scale bar = 10 μm . Higher magnifications showing surface of *in hospite* symbionts (b) and a cryo-fracture of a healthy *Symbiodinium* cell (c); scale bar = 5 μm for (b) and scale bar = 4 μm for (c). A symbiosome surrounded by an active actin-like network (d). *Symbiodinium* nucleus (SN), thylakoid (T); scale bar = 5 μm .

Thermal-induced bleaching mechanisms

A group of sea anemones were exposed to hyperthermal stress (+10°C) for 22 days to induce bleaching. *Symbiodinium* cell counts indicated that the loss of symbionts was time-dependent and became different from the initial population at day 5 ($p < 0.001$), with 50% loss (limit of the bleaching state) reached at day 10 (Fig. 5). The sea anemones were placed back in control conditions (normal seawater temperature and normal feeding) after 22 days, but symbiont loss continued to increase until 90% loss of the initial *Symbiodinium* population was reached after 59 days (bleached specimens).

The TEM observations (Fig. 7) showed condensation of organelles and formation of apoptotic bodies inside *Symbiodinium* cells, which corresponded to an active apoptotic process, even after a few hours (Fig. 7c-d). Furthermore, while the epidermal structure remained unchanged (data not shown), gastrodermal tissue was markedly affected and destructured at day 22 and thereafter (Fig. 7g-h). Indeed, the necrosis-like morphology of both host and *Symbiodinium* cells was more prevalent towards the end of the stress period (Fig. 7g-j). We also observed less gastrodermal lipid reserves towards the end of the stress period, i.e. at day 22 and later (Fig. 7g-i), than at the beginning of the experiment (Fig. 7a-f), or than in starved-control specimens (Fig. 3). This gastrodermal tissue remodeling was detected from the first days of stress, for example at day 5 (Fig. 7e-f). Nevertheless, most of the remaining *Symbiodinium* cells still present after 22 days of hyperthermal stress seemed quite healthy (Fig. 7g). They could be considered as “resistant” symbionts of the “temperate A” clade, while other *Symbiodinium* cells appeared to be completely degraded (Fig. 7h). Other *Symbiodinium* cell depletion processes were observed during the heat-induced bleaching experiment, including exocytosis at the apical side of gastrodermal cells (Fig. 8a) and *in situ* necrosis of *Symbiodinium* cells (Fig. 7h). We also observed vesicles containing a high density of granules adjacent to symbiosomes (Fig. 7i and j). Symbiophagy was observed at a rate that did not exceed those of the control specimens (Fig. 4). Based on these observations, we showed that heat stress induced several mechanisms of symbiont release, such as apoptosis, necrosis and exocytosis, in a time-dependent manner.

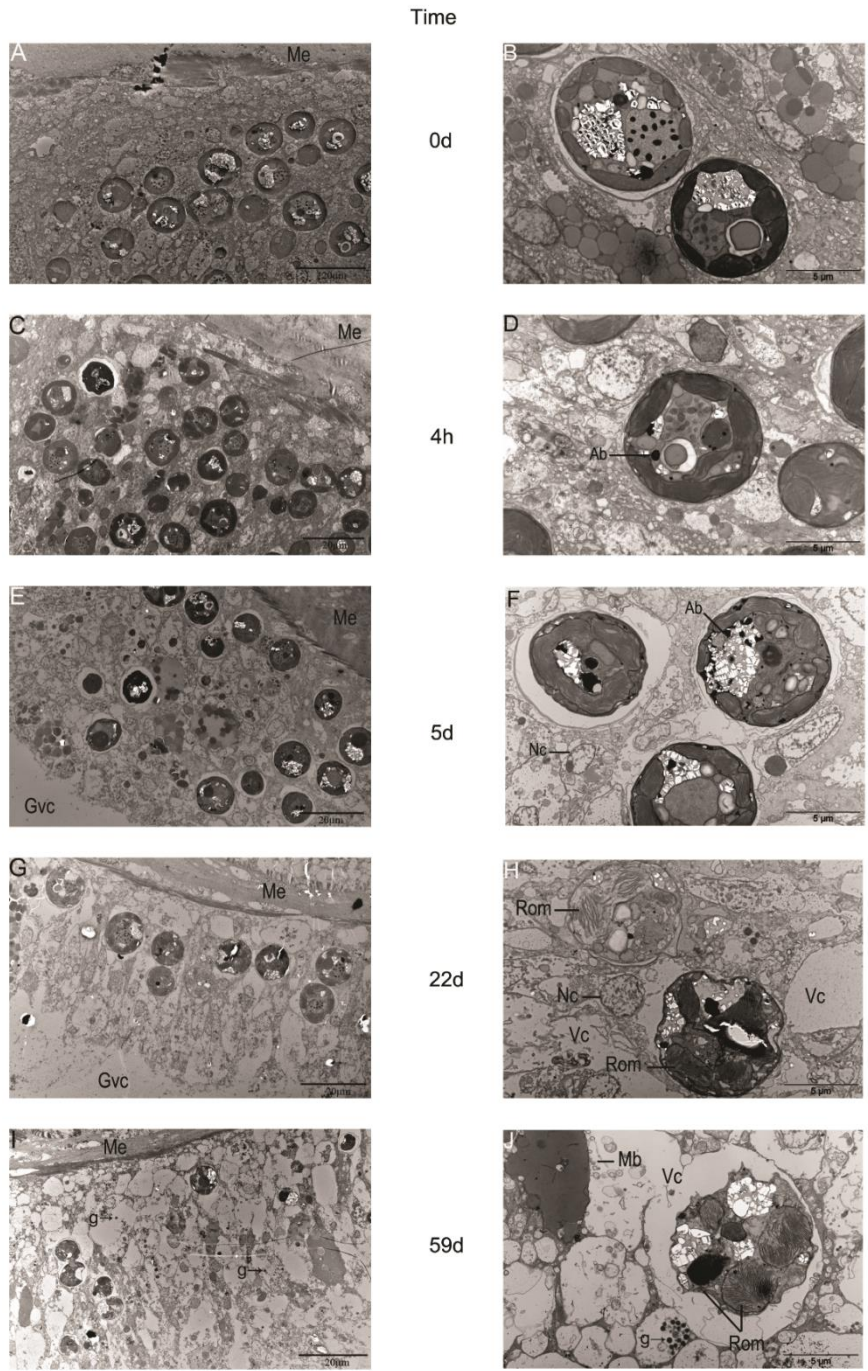


Figure 7: Transmission electron micrographs of *A. viridis* tentacle cross sections throughout hyperthermal-induced bleaching.

Tentacle cross sections were performed and observed by TEM at the following sampling times: 0 (a,b), 4 h (c,d), 5 days (e,f), 22 days (g,h) and 59 days (i,j). Animals were subjected to +10°C thermal stress until complete bleaching. General views of the gastrodermal tissue layer (a, c, e, g, i) and focus on in hospite *Symbiodinium* cells (b, d, f, h, j). Apoptotic bodies were rapidly observed at the beginning of the stress period (c,d). After 5 days of stress (e,f), the gastrodermal tissue integrity was impacted and we observed nuclear condensation and numerous apoptotic bodies. After 22 days of stress (g,h), we observed large vacuolization of host cells and necrotic-like symptoms. After 59 days of stress (i,j), the gastrodermal tissue was highly impacted. Mesoglea (Me), gastrovascular cavity (Gvc), ruptured organelle membrane (Rom), membrane blebbing (Mb), vacuolar matrix (Vmx), apoptotic bodies (Ab), hypothetical melanin granules (g), nuclear chromatin condensation (Nc) and vacuolated cytoplasm (Vc).

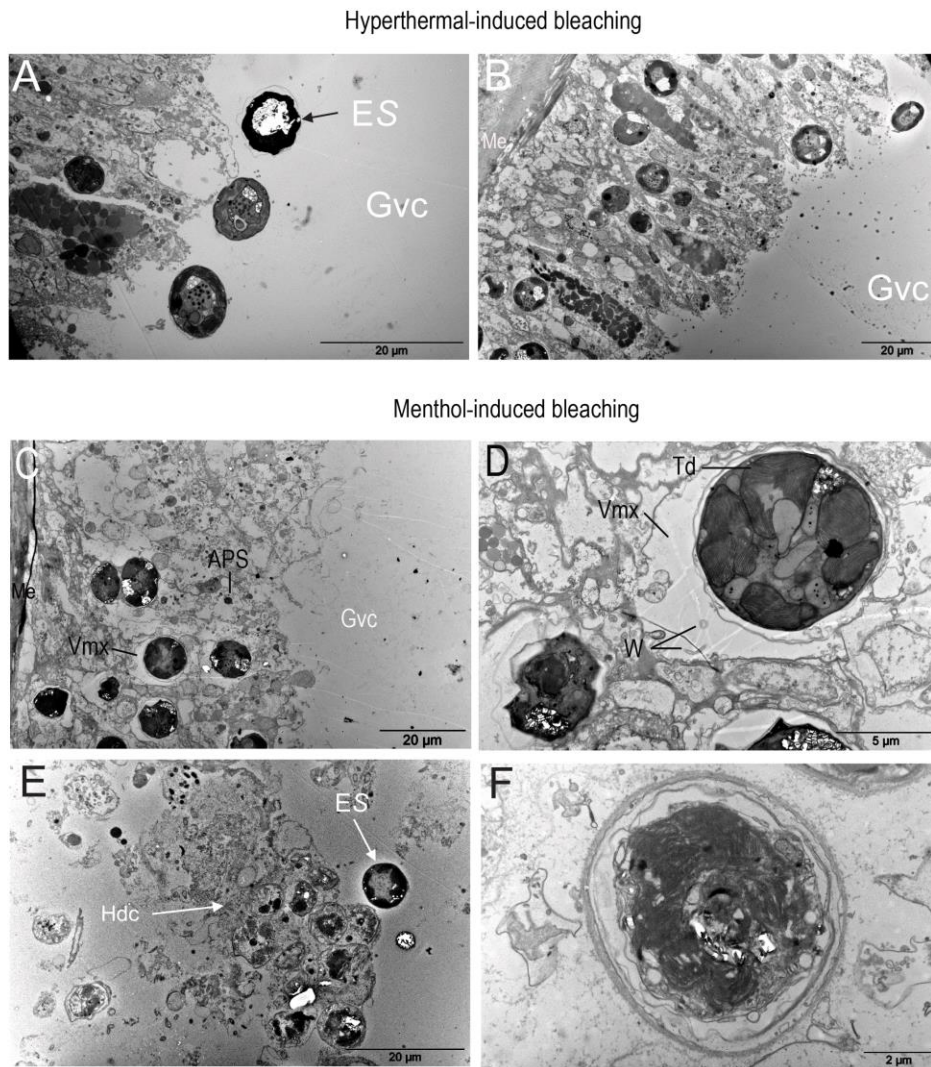


Figure 8: Morphological structures corresponding to the main cellular responses induced throughout thermal- or menthol-induced bleaching. TEM observations of tentacle cross sections (a-d) or expelled *Symbiodinium* cells (e,f). (a) Mechanism of expulsion of *Symbiodinium* cells by exocytosis at the apical end of the gastrodermal tissue layer in a hyperthermal-stressed specimen at day 15; scale bar = 20 μm . (b) General view of *Symbiodinium* expulsion and tissue degradation in a hyperthermal-stressed specimen at day 15; scale bar = 20 μm . (c) Advanced autophagosomal state of symbiosomes *in hospite* in a menthol-stressed specimen at day 10; scale bar = 20 μm . (d) Focus on the symbiosome membrane complex during the autophagosomal process in a menthol-stressed specimen at day 10; scale bar = 5 μm . (e) Expelled *Symbiodinium* (ES) and a host degraded cell (Hdc) after massive pellet expulsion in a menthol-induced bleaching experiment at day 15; scale bar = 20 μm . (f) Focus on a degraded symbiont expelled from a menthol-treated specimen at day 15; scale bar = 2 μm . Gvc, gastrovascular cavity; Vmx, vacuolar matrix; Td, thylakoid dissolution; W, autophagic whorl; APS, active autophagic structures.

Menthol-induced bleaching mechanisms

The second group of sea anemones was treated with menthol using a 8/16 h exposure/resting cycle. While a continuous incubation in 0.58 mM menthol caused high mortality (data not shown), a repeated 8/16 h menthol exposure/resting cycle successfully induced bleaching (Fig. S3). The decrease in the *Symbiodinium* cell density started later than for heat-stressed sea anemones (Fig. 5). Indeed, no significant decrease was quantified until day 10, where evidence of massive symbiont loss was observed ($p < 0.05$), mainly by the presence of digestive *Symbiodinium* pellets around the mouth. While menthol-induced bleaching was more sudden than temperature-induced bleaching (Fig. 5), menthol-treated animals seemed comfortable and coped with this stress without mortality, in contrast to the mortality which was sometimes observed during temperature-induced bleaching.

Nevertheless, the TEM observations indicated extensive vacuolization of symbiosomes at treatment onset (Fig. 4 and Fig. 9c), and the presence of numerous active autophagic structures (Fig. 9e). These structures suggested the induction of symbiophagy, especially at 4 h and day 5 (Fig. 4), where early or late phagolysosomes were observed (Fig. 9e-f). Indeed, closer examination of symbiosomes revealed phagolysosome-like morphological structures, such as a high volume of vacuolar matrix and the formation of autophagic structures and autophagic whorls (Fig. 8c-d). We also noticed active autophagic structures in gastrodermal cells from day 5 to the end of the experiments (Fig. 9e, g, and i), indicating that the autophagic processes also occurred in the host. While the gastrodermal tissue appeared to be less damaged (Fig. 9e, g and i) than in heat-stressed sea anemones, very few *Symbiodinium* cells were found at day 59 (Fig. 9i). Moreover, the few remaining symbionts still found in gastrodermal tissue were all degraded and we could not find "resistant" *Symbiodinium* cells as described in the thermal-induced response. We therefore postulated that menthol treatment mainly induces both host autophagy and symbiophagy *in hospite*.

Nevertheless, evidence of *Symbiodinium* cell depletion was noted at day 15 by the expulsion of mucus-containing symbiont pellets. TEM examination of these pellets showed numerous degraded dinoflagellates as well as degraded host cells (Fig. 8e-f). We also noticed the presence of a few intact expelled symbionts (Fig. 8e). Such mucus-containing pellets were not observed around the mouth of heat-stressed specimens, although exocytosis of symbionts was detected by TEM in these animals (Fig. 8a-b).

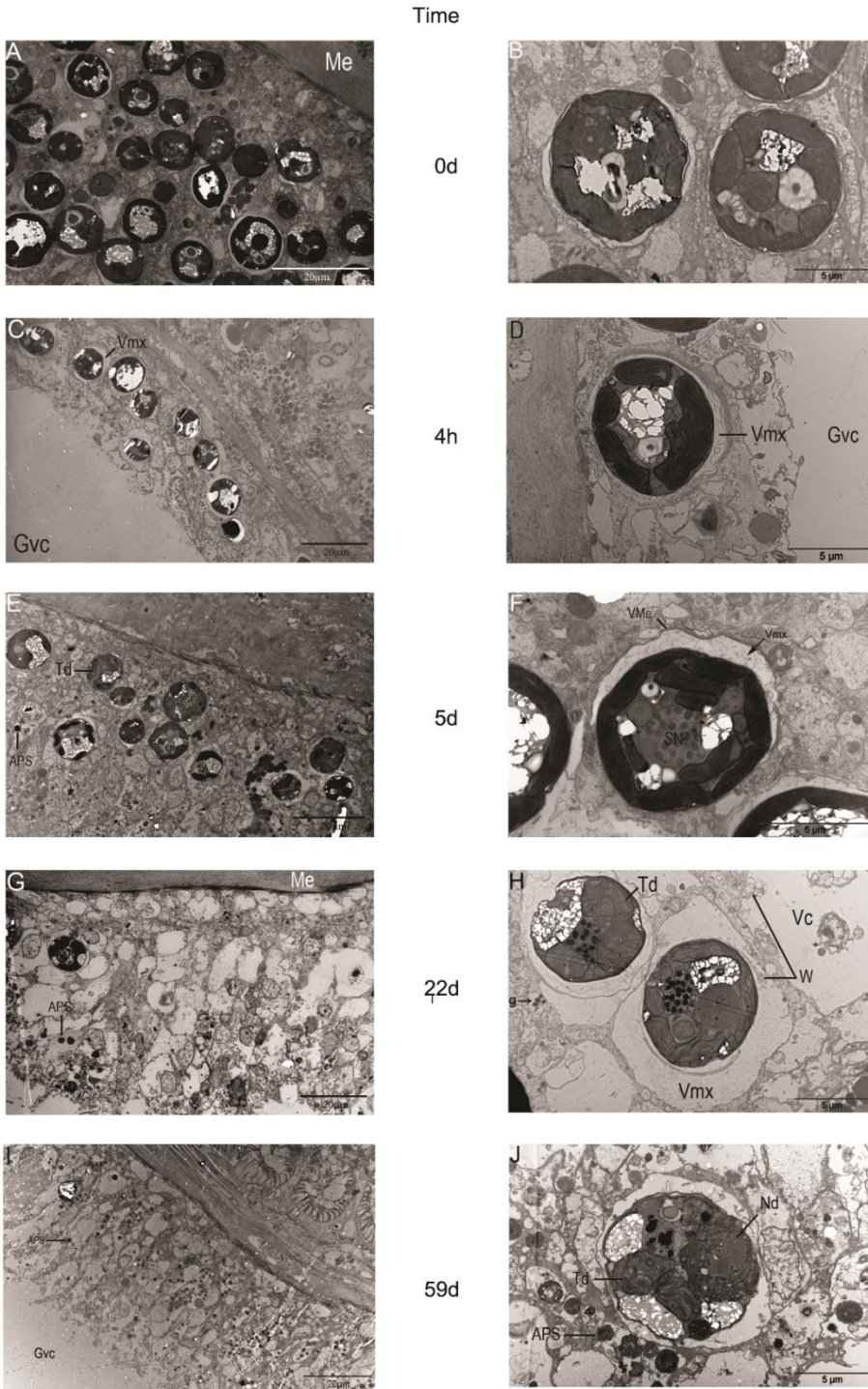


Figure 9: **Transmission electron micrographs of *A. viridis* tentacle cross sections throughout menthol induced bleaching.** Tentacle cross sections were performed and observed by TEM at the following sampling times: 0 (a,b), 4 h (c,d), 5 days (e,f), 22 days (g,h) and 59 days (i,j). Animals were subjected to menthol treatment. General views of the gastrodermal tissue layer (a,c, e, g, i) and focus on in hospite *Symbiodinium* cells (b, d, f, h, j). 4 h after the first menthol incubation (c,d), we observed a rapid increase in autophagic structures around *Symbiodinium* cells. After 5 days of stress (e,f), almost all *Symbiodinium* cells displayed early or late symbiophagy (presence of high volume of vacuolar matrix). After 22 days of stress (g,h), we observed a very low number of remaining *Symbiodinium* cells within the gastrodermis and large induction of autophagic processes in both host and symbiont. After 59 days (i,j), gastrodermal tissue was slightly impacted but almost no more *Symbiodinium* cells

remained. Mesoglea (M), gastrovascular cavity (Gvc), ruptured organelle membrane (Rom), membrane blebbing (Mb), vacuolar matrix (Vmx), vacuolated cytoplasm (Vc), disorganization (Td), nuclear dissolution (Nd), vacuolar matrix (Vmx), hypothetical melanin granules (g), autophagic whorl (W) and active autophagic structures (APS).

Discussion

Numerous studies have described *Symbiodinium* cell removal processes, including expulsion and active degradation of symbionts, mainly in the stress and bleaching context. However, there is now substantial evidence that symbiont expulsion or degradation commonly occurs in healthy specimens (Davy et al. 2012). In our experiments, we always noticed a small proportion of degraded *Symbiodinium* cells in *A. viridis* specimens maintained under normal seawater temperatures (Fig. 4 and Fig. S1).

Several hypotheses have been proposed to explain recurrent but reduced symbiont loss, including: i) sorting and elimination of inappropriate symbionts from host tissues, ii) control of *Symbiodinium* density and regulation of the host-symbiont biomass ratio, and iii) adaptation to different environmental conditions, such as seasonal changes (Cunning et al. 2014). The cnidarian innate immune system and membrane trafficking are both thought to be involved in these processes. For example, it was proposed that the cnidarian immune system plays a central role in the regulation of host-symbiont interactions and that immune systems have evolved to fulfil the need to control the resident beneficial microbes (Bosch 2013). There is no adaptive immune system in cnidarians, but their innate immune system is very efficient and not very different from that of other invertebrates. It consists of a large repertoire of pattern recognition receptors and effector components that trigger a humoral and/or cellular response, including inflammation, cytotoxic responses and melanization, antimicrobial peptide synthesis, and phagocytosis (for a review, see Toledo-Hernández et al. 2014). Some of these pathways have been described in the context of symbiotic interactions. In normal conditions, symbiont populations and host cells need to coordinate their growth to stabilize the symbiotic interaction. Cnidarian hosts have been shown to regulate the *Symbiodinium* cell cycle, while also inducing expulsion and/or degradation of excess

symbionts. For example, Baghdasarian and Muscatine concluded that the expulsion of dividing *Symbiodinium* cells is a primary process to regulate the symbiont density (Baghdasarian and Muscatine 2000). In cnidarians, the cellular response is triggered in part by amoebocytes, which are putative immune cells (Larkman 1984; Toledo-Hernández and Ruiz-Diaz 2014). Their role is not yet fully understood but they are thought to be involved in the response to infection and in wound healing (Mydlarz et al. 2008; Palmer et al. 2008; Palmer et al. 2011). Granular amoebocytes are motile cells found within the mesoglea. They are thought to have phagocytic capacities and may infiltrate stressed tissues (Palmer et al. 2008). They may also contain granules of phenoloxidase and reactive oxygen species (ROS) and release these cytotoxic compounds upon activation. Such circular amoebocytes were found within the mesoglea of *A. viridis* tentacles (Fig. 1b and c). Figure 3j also shows a structure resembling a granular amoebocyte that infiltrated the gastrodermal tissue. Moreover, while a few amoebocytes were observed in control specimens, both circulating amoebocytes and melanin granules were induced in stressed specimens (Fig. 7 and 9). Similar observations have been previously described in symbiotic cnidarians. For example, induction of a phenoloxidase-activating cascade and melanin synthesis, which is a key component of invertebrate immunity, have been demonstrated in corals in response to wound healing and stressors (Mydlarz et al. 2008; Palmer et al. 2008; Palmer et al. 2011). These authors also described a correlation between melanin detection and *Symbiodinium* cell depletion in pigmented areas of corals (Palmer et al. 2008).

Symbiophagy of symbiotic dinoflagellates was first described as a cellular mechanism for coral bleaching (Downs et al. 2009). As it is known that starvation induces autophagy (Klionsky et al. 2012; Chera et al. 2009), it is possible that a similar process may occur in symbiotic specimens to control the *Symbiodinium* density by inducing symbiophagy. Indeed, morphological criteria

suggested that most of the degrading symbionts in *A. viridis* control specimens were undergoing a symbiophagic process, which was enhanced by starvation (Fig. 3 and 4). SEM observations showed some symbiosomes wrapped in cytoskeleton structures (Fig. 6b). As cytoskeleton components are involved in microbial autophagy (Mostowy 2014), these observations suggested that these symbionts were probably subjected to vacuolization and symbiophagy. Moreover, this process was enhanced in control specimens during the starvation period, but there was a return to the basal level soon after the reintroduction of food (Fig. 3 and Fig. 4b). This mechanism might be considered as a host active digestion process to offset the nutritional deficiency. Indeed, digestion of resident *Symbiodinium* cells might contribute to supplying nutrients and help to cope with amino acid deprivation during starvation. These observations are somewhat consistent with the proposed role of the cnidarian host as an active “farmer” of its dinoflagellate photosynthetic symbionts (Wooldridge 2010). Similarly, in corals, Titlyanov and coworkers reported a diel periodicity of *Symbiodinium* degradation, which was induced during periods of host starvation (Titlyanov et al. 1996). This might also be considered as a normal process to balance the rate of symbionts undergoing division (Fig. S2), and therefore to maintain a constant *Symbiodinium* cell density, as seen in Fig. 5. Nevertheless, induction of autophagy was also described as a cellular adaptation to starvation and the main survival strategy in the non-symbiotic cnidarian *Hydra* (Chera et al. 2009), suggesting that autophagy also occurs within host cells independently of symbiont presence. Another possible explanation is that the apoptotic or symbiophagic structures of *Symbiodinium* cells seen on TEM micrographs were the result of a postphagocytic sorting process. Indeed, it has been proposed that cnidarian hosts mount an innate immune response against inappropriate *Symbiodinium* cells (Dunn and Weis 2009).

The cellular mechanisms underlying symbiont expulsion or degradation as a part of the bleaching process have been extensively studied (for reviews, see Weis 2008; Davy et al. 2012). Regarding symbiont expulsion, this includes symbiont exocytosis, host cell apoptosis, and host cell detachment. Processes that could result in degradation of symbionts include PCD of *Symbiodinium* cells, such as apoptosis or necrosis, as well as autophagic digestion of symbionts by the host cell (symbiophagy). As a first step in understanding the extent of each process, here we describe ultrastructural modifications in *A. viridis* tissues that occurred during heat-induced and menthol-induced bleaching.

In thermal-induced bleaching, major effects were detected within gastrodermal tissue, including general destructuration of the cell layer and damage in both host cells and symbionts. TEM observations suggested that there was no induction of symbiophagy during the hyperthermal treatment (Fig. 4). In contrast, the main processes that occurred in the chosen experimental conditions appeared to be apoptosis and necrosis, with the latter being considered as a late/final step of apoptosis. We also observed *Symbiodinium* exocytosis at the apical end of the tissue layer (Fig. 8a-b). These findings are consistent with previous studies of hyperthermal stress that described different cellular mechanisms of *Symbiodinium* removal, including exocytosis (Steen and Muscatine 1987; Gates et al. 1992; Brown et al. 1995; Downs et al. 2013; Fujise et al. 2014), *in situ* degradation (Brown et al. 1995), and host cell degradation and detachment (Dunn et al. 2002; Dunn et al. 2004; Richier et al. 2006; Dunn et al. 2007; Tchernov et al. 2011; Hanes and Kempf 2013). Previous investigations in *A. viridis* demonstrated that hyperthermal stress induced rapid oxidative stress and apoptotic events, including DNA fragmentation and caspase activities, mainly within the gastrodermal tissue (Richier et al. 2006). It is well documented that hyperthermal stress causes high ROS production, which probably modifies the immune response and/or cell

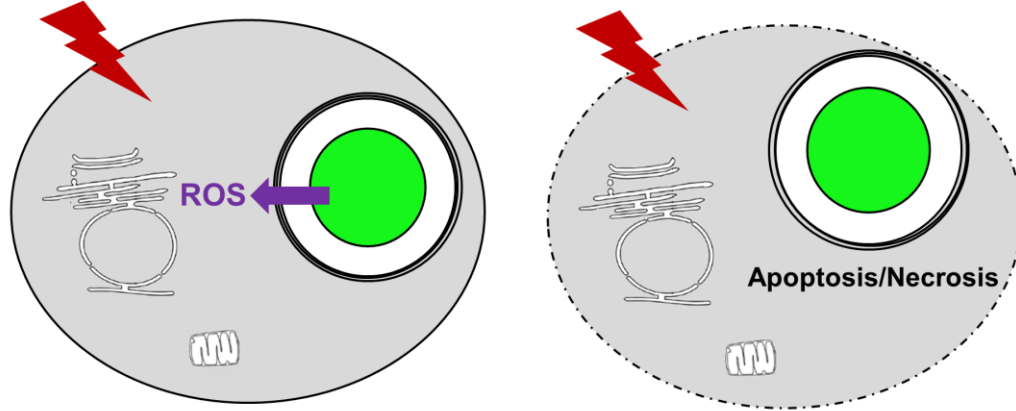
death/survival pathways (Weis 2008). Indeed, one of the first cellular events of the heat-induced bleaching cascade is photosystem dysfunction and energetic uncoupling of electron transport. In agreement with these previous studies (Strychar et al. 2004b; Ladrière et al. 2008, Richier 2006), we observed that *Symbiodinium* thylakoid membranes were damaged as early as 4 h after heat-stress exposure (Fig. 7d). We also noticed increased necrosis in both host and *Symbiodinium* cells throughout the experiment. We therefore concluded that heat-induced bleaching was the consequence of both *Symbiodinium* expulsion and cell death via apoptosis and necrosis (Fig. 10a). Nevertheless, we also found healthy symbionts hosted by adjacent cells, indicating the presence of “heat-resistant” *Symbiodinium* cells within the host tissue. After the stress period, the host might therefore regain symbionts by inducing the division of remaining heat-resistant *Symbiodinium* cells, as proposed by Wooldridge (Wooldridge 2010). Bleaching was also proposed to be an adaptive strategy of corals to cope with environmental disturbances, for example by shifting to a more adapted *Symbiodinium* clade. While *A. viridis* hosts only one symbiont clade, i.e. the ‘temperate A’ clade (Casado-Amezúa et al. 2014), intra-clade genetic diversity may play a role in holobiont plasticity.

Menthol exposure has been recently described as a gentle protocol to induce bleaching and to prepare aposymbiotic coral specimens (Wang et al. 2012). These aposymbiotic specimens displayed similar biochemical and physiological properties (e.g. respiration rates) as their symbiotic counterparts (Wang et al. 2012). Nevertheless, the cellular mechanisms that lead to symbiosis dysfunction in menthol-treated specimens are still unknown. In vertebrates, menthol can bind and activate transmembrane receptors of the transient receptor potential (TRP) family, especially TRPM8, which allows Na⁺ and Ca²⁺ ion entry into the cells (Bautista et al. 2007). There is no TRPM8 ortholog in cnidarian genomes (*Nematostella vectensis* and *Acropora digitifera*), but

sequences for other TRP receptors have been found in these genomes and in the *A. viridis* cDNA dataset (results not shown). Wang and coworkers described both exocytosis and digestion of *Symbiodinium* cells (Wang et al. 2012), two mechanisms that are potentially triggered by Ca^{2+} . When exposed to menthol, *A. viridis* specimens rapidly displayed symbiosomes with autophagic features resulting from the activation of symbiophagy as part of the host innate immunity. This hypothesis was confirmed by the observed *in situ* digestion of *Symbiodinium* at day 10, where most of the *Symbiodinium* cells were in an early autophagosomal state or digested (Fig. 4 and Fig. 9). Our findings suggest that menthol might promote phagosome maturation into phagolysosomes (Fig. 8c-d). As menthol-treated specimens easily recovered from the stress, we might hypothesize that symbiophagy is another strategy to maintain host metabolism and to cope with a bleaching episode. Nevertheless, numerous expelled pellets of both degraded host cells and dinoflagellates were observed at day 10 (Fig. 8e-f), which was correlated with the massive decline in the *Symbiodinium* population (Fig. 5). This findings suggested that other processes might occur and contribute to symbiont depletion.

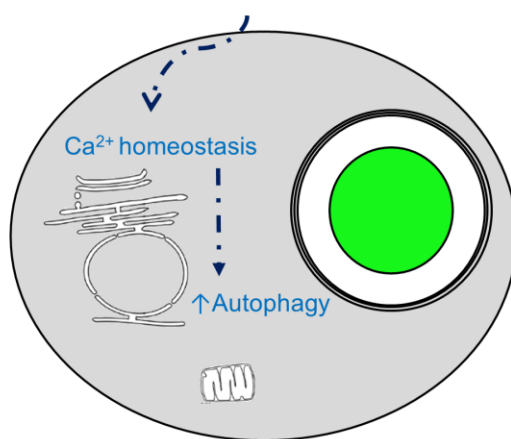
A

Heat stress



B

Menthol



Menthol

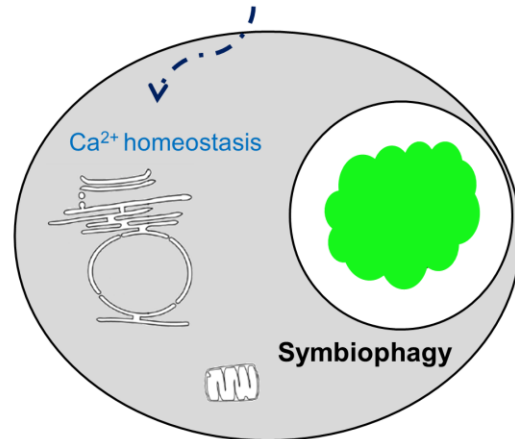


Figure 10: **Hypothetical scheme for mechanisms of symbiosis disruption.** (a) Hyperthermal stress first induced the release of ROS from the symbiont, leading to *Symbiodinium* expulsion, apoptosis or necrosis of the symbiotic cell. (b) Menthol treatment induced an increase in the intracellular Ca²⁺ concentration that triggered the symbiophagy.

We still are far from having a clear understanding of the cellular events that lead to symbiophagy induction. By analogy with vertebrates, we hypothesize that menthol-induced activation of a host TRP receptor might increase the intracellular calcium concentration. The modulation of calcium homeostasis, which is known to upregulate the autophagic pathway (Smaili et al. 2013), could therefore trigger *Symbiodinium* depletion through a phagolysosomal process. This hypothesis is also consistent with previous results from transcriptomic studies that suggested the disruption of calcium homeostasis in both corals and sea anemones during stress-induced bleaching (Desalvo et al. 2008; Moya et al. 2012). Several intracellular pathogens are able to arrest phagosome maturation to ensure their survival and a similar mechanism is proposed to explain how *Symbiodinium* may succeed in entering and surviving within gastrodermal host cells (Davy et al. 2012). For example, Chen and coworkers monitored the location of several Rab GTPases and demonstrated that *Symbiodinium* cells probably arrest phagosomal maturation in the early phagosome stage. Moreover, symbiophagy induction in symbiotic cnidarians was shown to be mediated by Rab7 activation, which was triggered by several cellular signals, including temperature stress and darkness (Downs et al 2009). We therefore propose that menthol might re-engage this phagosomal maturation process through Ca^{2+} -triggered mechanisms and therefore induce symbiophagy (Fig. 10b). Like other symbiotic associations, cnidarian-dinoflagellate symbioses can be regarded as dynamic interactions that shift along a continuum between mutualism and parasitism, depending on the environmental conditions (Davy et al. 2012; Lesser et al. 2013). Under stressful environmental conditions, for example, beneficial symbionts may shift to a more parasitic relationship. Changing their *Symbiodinium* partner or other microbial partners may therefore also allow cnidarians to better adapt to changing environmental conditions. In such a scheme, *Symbiodinium* cell sorting or depletion processes, such as those involving the innate immune system, may play a key role in symbiosis stability. Similar processes are very well described in parasitic infections of animal cells and comparative approaches would be a very powerful way to gain insight into some of the common pathways that pathogens and symbionts have developed in their interaction with their host (Schwarz et al. 2008).

The results of this study indicated that several mechanisms of symbiont depletion co-exist in both normal and stressful conditions. Exocytosis of healthy or degraded *Symbiodinium* cells, as

well as symbiophagy, were observed in healthy *A. viridis* specimens. Hyperthermal stress not only induced expulsion of damaged symbiont, but also cell death of both *Symbiodinium* and host cells, through apoptotic then necrotic processes. Conversely, menthol-induced bleaching seemed mainly to result from the induction of symbiophagy, which could be explained by the reengagement of the phagosomal maturation process in the host. Nevertheless, we cannot exclude that these responses are species-specific and that different overriding cell death processes may be activated in other symbiotic cnidarians in response to various experimental treatments. Host physiology and gastrodermal cell integrity may clearly be impacted by some of these mechanisms, especially apoptosis and necrosis. It is therefore important to gain further insight into the molecular pathways underlying these mechanisms and their interaction, if any, through time or degrees of stress. Indeed, although the term “bleached” defines one visual phenotype resulting from various stress responses, it can result from diverse variations of gene expression profiles, as already mentioned in a previous transcriptomic study of *A. viridis* (Ganot et al. 2011).

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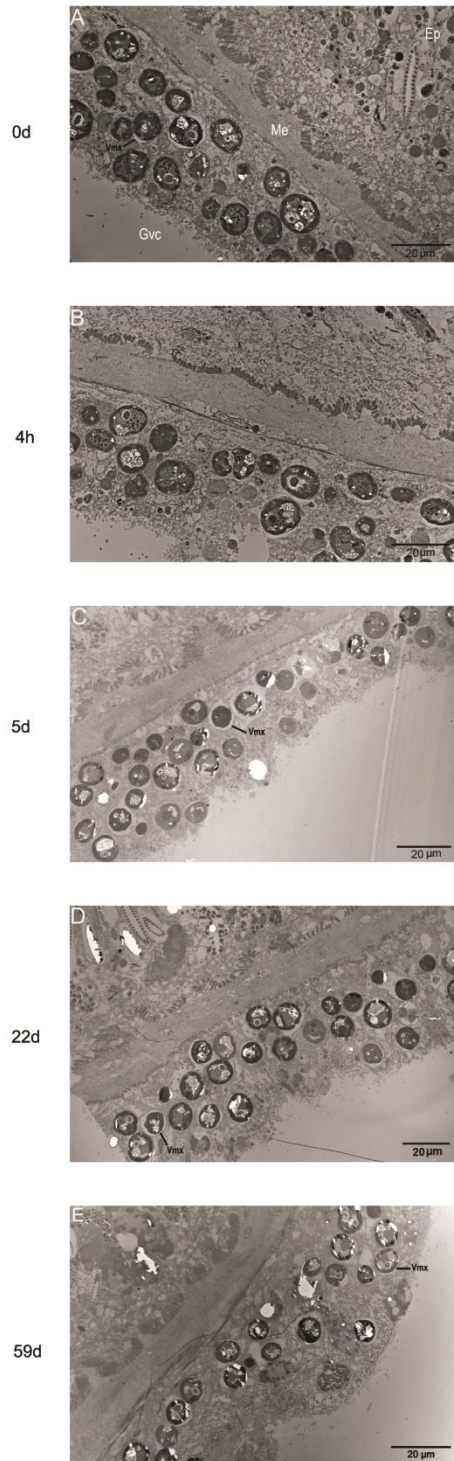


Figure S1: **Transmission electron micrographs of *A. viridis* tentacle cross sections in control specimens.** Tentacle cross sections were performed and observed by TEM at the following sampling times: 0 (a), 4 h (b), 5 days (c), 22 days (d) and 59 days (e). These observations showed a basal rate of symbiophagy. Epidermis (Ep), mesoglea (Me), gastrodermis (Ga), vacuolar matrix (Vmx) and gastrovascular cavity (Gvc).

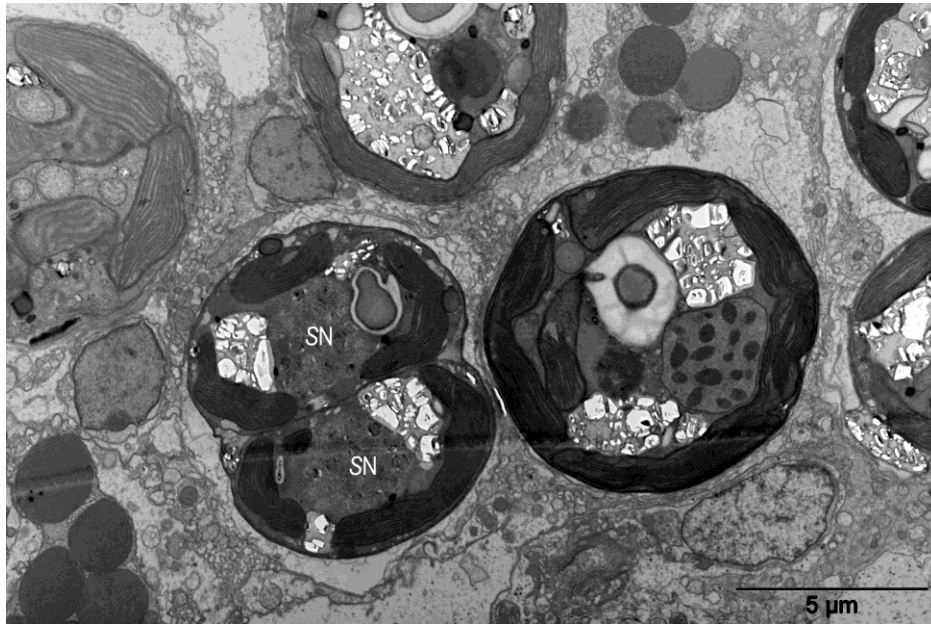


Fig. S2 Transmission electron micrographs of a *A. viridis* tentacle cross section in a starved-control specimen showing a *Symbiodinium* cell in active division at day 5. SN, *Symbiodinium* nucleus

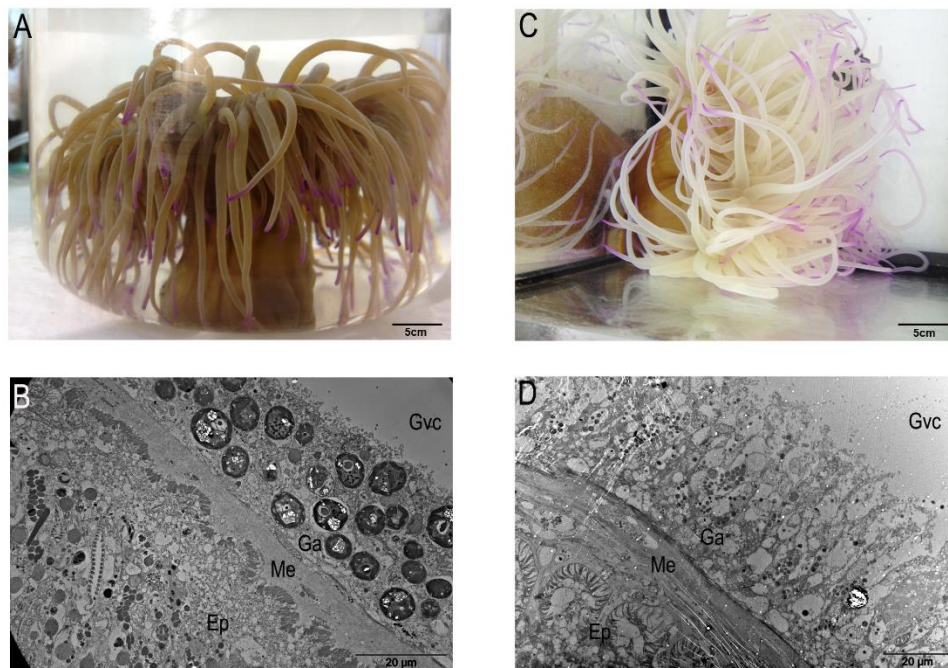


Fig. S3 Effects of menthol treatment on an *A. viridis* specimen. Visual observation of a symbiotic specimen (a) showing its brown color due to the presence of symbionts. TEM observation of a tentacle cross section showed the presence of numerous *Symbiodinium in hospite* (b). The same specimen after menthol-induced bleaching (c). TEM observation demonstrated the loss of symbionts from the gastrodermal tissue (d). Epidermis (Ep), mesoglea (Me), gastrodermis (Ga), vacuolar matrix (Vmx) and gastrovascular cavity (Gvc).

2.3.2. **Publication n°2** (en préparation)

Dani, V., Priouzeau, F., Pagnotta, S., Agnès Loubat., and Sabourault, C.

Cell-cycle state of *in hospite Symbiodinium* is dependent of symbiotic conditions in the cnidarian-dinoflagellate association (en préparation)

Problématique: Quel est l'impact de différents stress entraînant la rupture de symbiose chez *A. viridis*, au niveau de la régulation du cycle cellulaire des symbiotes *in hospite*?

Outils méthodologiques : Suivi du cycle cellulaire via l'utilisation du CalcoFluor White (CFW) comme proxy du cycle cellulaire des symbiotes.

Principaux résultats : Les différents stress utilisés induisent une réduction du nombre de symbiotes capables de passer de la phase G1/G0 de croissance vers les stades S/G2/M de division cellulaire.

Perspectives : Définir la régulation du cycle cellulaire des symbiotes *in hospite* par cytométrie de flux ; sur un cycle de 24h ; afin de définir les périodes de transition de phase en symbiose. Cela permettra de déterminer les points de prélèvements pour une étude visant à établir par cytométrie de flux, la régulation du cycle cellulaire des symbiotes lors d'épisode de stress.

Cell-cycle state of *in hospite Symbiodinium* is dependent of symbiotic conditions in the cnidarian-dinoflagellate association

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Running title: IN HOSPITE SYMBIODINIUM CELL-CYCLE REGULATION

Abstract

Despite the ecological importance of coral reef, the symbiotic interaction between cnidarians, such as corals and sea anemones, and the unicellular algae *Symbiodinium* is regulated by yet poorly understood cellular mechanisms. In this association, free living of motile stage *Symbiodinium* are acquired by phagocytosis and then surrounded by symbiosome membranes, originally derived from host plasma membranes during onset of the symbiosis. In this study, we used Calcofluor White staining to follow metabolic status and cell-cycle phase of *in hospite* *Symbiodinium* within tentacles of *Anemonia viridis*, *Acropora digitifera* and *Stylophora pistillata* adults. We showed that in *A. viridis*, during a stress leading to symbiosis disruption or during starvation, most of the *in hospite* symbiont population is maintained in the G1/G0 cell-cycle phase. We also observed a third population composed of *in situ* digested symbionts, overrepresented following a menthol stress, mainly in fecal pellets expelled in the late phase of the stress. This study thus provide a simple protocol to follow cell-cycle state of symbionts in the cnidarian-dinoflagellate association and intimate regulations that may underlie this partnership. This study also pinpoint the fact that the *Symbiodinium* cell-cycle is actively regulated by the symbiotic state of the cnidarian host.

Introduction

Despite the ecological importance of coral reefs, the mechanism that modulate the intimate association between the host cnidarian, such as coral and sea anemones, and the unicellular algae *Symbiodinium*, are regulated by yet poorly understood mechanisms of recognition and molecular communication (in review Davy et al. 2012). The endosymbiotic population of *Symbiodinium* can be acquired by vertical acquisition, transmitted by parents to the offspring or by horizontal transfer (Trench, 1987). The offspring must then acquire symbiont from the environment by internalization by a phagocytosis process (Hohman et al., 1982) of motile *Symbiodinium* in gastrodermal tissue layer of the host. In this mechanism, the motile stage which has a swimming capacity due to mobile thecal plates, then plays a key role for the establishment of coral-*Symbiodinium* symbiosis (Pasternak et al., 2004). In stable condition, the maturation of phagosome to lysosome are inhibited and phagosome membranes containing *Symbiodinium* mature in symbiosome membranes (Wakefield and Kempf, 2001) which are at the center of trophic exchanges and molecular communication between the two mutualistic partners. The outer membrane layer, originate by the host, is surrounding multiples inner membranes layers originate by the symbiont at the end of each cell cycle (Wakefield and Kempf, 2001; Wakefield et al., 2000). This mechanism lead to a mutualistic association between the two partner in stable condition but can turn into a parasitic state under variable environmental conditions leading to a breakdown of symbiosis, characterised by a drastic diminution of the symbiont population and so called bleaching (Hoegh-Guldberg et al., 2007). Even if prolonged bleaching cause a severe diminution of host fitness and can lead to the death of the organism, it is also an adaptive mechanism in order to change symbiont population to a more adapted *Symbiodinium* to the new environmental condition (Baker et al., 2004; Buddemeier and Fautin, 1993). Cellular mechanisms that drive mutualistic partnership has been a large field of investigation in the past years in the cnidarian-dinoflagellate association (see review Weis, 2008). However, the main mechanisms that regulate host-symbiont biomass have been largely studied in the *Hydra* symbiosis (McAuley, 1982b; McAuley, 1985; McAuley, 1986; McAuley and Muscatine, 1986), but rarely by direct assessing of symbiont cell-cycle *in hospite* (Dimond et al., 2013; Smith and Muscatine, 1999). Indeed, a majority of studies were conducted on *Symbiodinium* extracted from a symbiotic host and kept in culture several days before experimentation, leading to a bias by desynchronization of symbiotic cell-cycle phase (Fitt and Trench, 1983b; Fitt et al., 1981; Iglesias-Prieto et al., 1992; Kodama and Fujishima, 2012; Kwok and Wong, 2003; Smith

and Muscatine, 1999; Trench and Blank, 1987; Wang et al., 2008) and selection of *Symbiodinium* “adapted” to culture conditions. Nevertheless, using mitotic index, these studies clearly defined that the doubling time of *Symbiodinium* cells in culture is 5 to 10 times faster than *in hospite* symbiont (Davy et al., 1996; Hoegh-Guldberg et al., 1987; Jones and Yellowlees, 1997; Wilkerson et al., 1983; Wilkerson et al., 1988), indicating a control of the symbiont cell-cycle phase by the host. While the cellular mechanisms that underlie this regulation are largely unknown, it is clearly accepted that the host may regulate the symbiont density by actively inhibiting symbiont cell growth and division, blocking the symbiont population at the growth dependent checkpoint from the G1/S to the G2/M cell-cycle phase (Falkowski et al., 1993; Wilkerson et al., 1988). In dinoflagellate, the division phase occur between 06:00 and 10:00 whereas the G1/G0 phase is the major state during the resting diel periodicity (Fitt, 2000; Kwok and Wong, 2003). Four proposed regulation mechanisms resulting from study in the Hydra-Chlorella symbiosis are actually discussed (pH-stimulated release of maltose (McAuley, 1986), host regulation supply of “division factor” (McAuley and Muscatine, 1986), host-regulated supply of inorganic nutrients to the symbiont (Blank and Muscatine, 1987; Neckelmann and Muscatine, 1983; Rees and Ellard, 1989) and production of a density-dependant inhibitor by the symbiont himself (McAuley, 1982b; Muscatine and Pool, 1979; Rodriguez-Lanetty et al., 2006), but the host-regulation by nitrogen delivery to the symbiont emerge as the main important regulatory mechanism (Davy et al., 2012). All these studies used mitotic index to determine the cell-cycle (Baghdasarian and Muscatine, 2000; Horwitz et al., 2015; Smith and Muscatine, 1999; Wilkerson et al., 1988; Wooldridge, 2012), but this marker only allowed the distinction between the G1/S/G2 state characterized by one nucleus and dividing state specific to the mitosis characterized by two nuclei. Only one study, using direct assessment of both host and symbiont cell-cycle by flow cytometry, showed a partial evidence for a positive relationship in cell-cycle synchronisation (Dimond et al., 2013)

However, it has been shown that the cellulose synthesis is coupled to the cell-cycle progression in dinoflagellate (Kwok and Wong, 2003; Kwok and Wong, 2010). In the G1 cell-cycle phase, occurring during light-period, the dinoflagellates harbour cellulosic-based thecal plates. This phase corresponds to the growing phase with active carbohydrate and lipid metabolism, two major processes involved in the molecular communication during symbiosis (Gordon and Leggat, 2010). During the night phase, if the cellular growth size reach the checkpoint baseline, the dinoflagellate enter in the dividing cell cycle state characterized by deflagellation at the

initiation of the S/G2 cycle-phase. During the mitosis state, the cellulose content is maximum (Kwok and Wong, 2010) and allow the active dividing phase (Kwok and Wong, 2003). Recently, Fujise and co-workers (Fujise et al., 2014a) used the CalcoFluor White staining CFW to differentiate between motile and non-motile *Symbiodinium* in culture. Indeed, the CFW can bind to components in the underlying cell wall as cellulose, chitin, carboxylated polysaccharides, b-linked polymers (Hughes and McCully, 1975). Based on the presence of thecal plates; internal to the plasma membrane (Chen et al., 2012), present in the motile or G1/S cell-cycle phase of cultured *Symbiodinium* (Kwok and Wong, 2003), it is possible to differentiate population between coccoid and motile stage .As well, based on the coupling of the cellulose synthesis and cell cycle phase of dinoflagellate, it is possible to use the CFW as a proxy of metabolic state and cell cycle state of *Symbidinium*. Indeed, Kwok and coworkers showed that cellulose synthesis was cycled with cell-cycle in dinoflagellate, with a maximum cellulose concentration/CFW fluorescence occurring at the initiation of the replication phase to the end of the mitosis phase (Kwok and Wong, 2003; Kwok and Wong, 2010). The CFW staining could then be used to label dinoflagellates that reach the minimum size cell allowing the transition to the dividing cell-cycle phase.

In this study, we used the properties of the CFW to stain cellulose contents in dinoflagellate thecal plates (Fritz and Triemer, 1985; Fujise et al., 2014a) to determine cellulosic contents in both cultured *Symbiodinium* and *in hospite Symbiodinium* in symbiotic specimens of both the sea anemone *Anemonia viridis* and corals. This marker allowed us to determine the state and progression of symbiont between active symbiotic state related to the G1/G0 phase and the dividing state corresponding to S/G2/M cell-cycle phase. We also follow the regulation of cell-cycle of symbionts in specimens of *Anemonia viridis* submitted to a starvation experiment and both hyperthermal and menthol stress leading to bleaching. We also characterise the cell-cycle state of *Symbiodinium* population in expelled pellet in the menthol bleaching experiment. This study will then assess by fast process the control of the host on the *in hospite* symbiont population during several stress leading to disturbance of the symbiotic relationship.

Materials and methods

Anemonia viridis, *Acropora digitifera* and *Stylophora pistillata* culture conditions

Mediterranean *A. viridis* specimens were collected in shallow waters at Villefranche-sur-Mer (France). They were maintained in laboratory-controlled conditions where they were acclimated for one month before experimentation. Seawater was maintained at $19 \pm 0.5^\circ\text{C}$ in aquaria and renewed weekly. Sea anemones were fed with *Artemia* once a week. Light was provided at a constant irradiance of $180 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ on a 12/12 h light/dark cycle with a metal halide lamp (HQI-TS 400 W, Philips). To avoid contamination of the protein and RNA samples used for other purpose, all specimens were starved throughout the stress experiment (days 0 to 22), excepted for normal specimens that were maintained under standard laboratory conditions. Specimens of *A. digitifera* (FR14-068-02767-I) and *S. pistillata* (FR15-068-03752-I) were imported from the United-States and maintained in optimal conditions two weeks in Aquarium recifal, Antibes, FRANCE before experimentation.

Maintenance of cultured *Symbiodinium*

Three single-cell derived cell lines of *Symbiodinium* sp. (clade A') were originally isolated from a single specimen of *A. viridis*, and established as clonal *in vitro* cultures at the University of Nice (Marine Symbiosis lab, UMR 7138). The *Symbiodinium* cells were cultured in f/2 medium (Guillard and Ryther, 1962), supplemented with 1% antibiotic-antimycotic solution cocktail (Sigma). Cultures were routinely maintained at 24°C and 90% humidity, under a standard 12h/12h light/dark cycle using an irradiance of $200 \mu\text{mol quanta m}^{-2} \text{sec}^{-1}$ (JLB ; Solar ultra marin day) in a controlled cabinet (Witeg Labortechnik GmbH ; WiseCube model WGC-450).

Hyperthermal and menthol stress treatments

A part of the symbiotic *A. viridis* specimens (n=3) were subjected to hyperthermal stress ($+10^\circ\text{C}$). The temperature was suddenly increased to 29°C and the specimens were kept under these conditions for 22 days, with continuous monitoring of seawater temperature. Following bleaching, aposymbiotic specimens were maintained in the dark under the same temperature, feeding and water renewal conditions.

For the menthol treatment, symbiotic *A. viridis* specimens (n=3) were incubated in 0.58 mM menthol for 15 days under a 8/16 h exposure/resting cycle. Following bleaching, aposymbiotic specimens were maintained in the dark under the same temperature, feeding and water renewal conditions. Pellets of expelled *Symbiodinium* were collected at day 2, 3, 5, 9, 12, 15, 17 and 22 during the menthol experiment.

Some symbiotic *A. viridis* specimens (n=3) were subjected to starvation only (22 days) under control temperature and water renewal conditions, as an experimental control. Three other specimens were maintained in normal conditions and sampled like the stressed specimens.

For each specimen, tentacles were sampled at the following days: d0, d5, d15, d22 and d59. All samplings were performed at 10:00 am to avoid circadian rhythm variations (Levy et al. 2011) and to fit with the sorting division phase (Fitt, 2000; Kwok and Wong, 2003).

Determination and quantification of cellulosic contents in *Symbiodinium* cell wall.

For cultured *Symbiodinium*, 3ml of both pellet and supernatant were separately centrifuge at 350 x g for 3 minutes and then resuspended in 500 µl of filtered seawater 0,2µm (SWF). For *in hospite Symbiodinium*, a tentacle were cut at each time of the kinetic and epidermis tissue layer were separated from the gastrodermal tissue layer by micro-dissection then centrifuge at 500 x g for 3 minute to pellet *Symbiodinium*. For corals, *Symbiodinium* cell are separated from the tissue and skeleton using gentle brushing applied on the specimens. The sample is then centrifuge first at 100 x g for 30 seconds to pellet skeleton waste and the supernatant containing both tissue and *Symbiodinium* cells were centrifuged at 500 x g for 3 minutes three times to pellet *Symbiodinium* in SWF. Both *Symbiodinium* sample form *A. viridis* and corals were then passed back and forth three times in a 23G syringe to discard gastrodermal cell from *Symbiodinium* and resuspended in SWF. The freshly isolated *Symbiodinium* (FIS) were wash three times in SWF, centrifuged at 500 x g for 3 min to pellet *Symbiodinium* cells and suspended in 500µl of SWF.

For pellets expelled during menthol stress experiment, we centrifuged the pellets at 500 x g for 3 min three times to wash and separate expelled *Symbiodinium* from waste. *Symbiodinium* were then passed three times back and forth through a 23G syringe to discard gastrodermal cell from *Symbiodinium* and then wash three times in SWF and then pellet at 500 x g.

Both cultured, freshly isolated *Symbiodinium* and *Symbiodinium* extracted from pellets were immediately incubated in freshly prepared Calcofluor White M2R (F3543 Sigma) at a final concentration of 20µg/l for 30 minutes at room temperature in the dark. Samples were washed 3 times in SWF to eliminate excess of unspecific CFW staining and were trapped onto the polycarbonate filter (Isopore, 0.4 µm HTBP, HTBP01300, Millipore, Billerica, MA, USA) by gentle vacuuming, and rinsed with 10 ml of filtered sea water (0.2 µm filtered) to remove the extra and unbound stain in the sample. The filter was put onto a slide glass, seal with Fluoromount™ Aqueous Mounting Medium (F4680 Sigma) to prevent dehydration and oxidation, covered with a cover slip, and kept in the dark. Each condition was repeated on three independent samples. On each sample, total number of stained and unstained *Symbiodinium* cells were counted. To ensure the randomness of the cells counted, the area was selected only based on the autofluorescence of the chlorophyll contained in the *Symbiodinium* and then, absence or presence of Calcofluor White staining surrounding the *Symbiodinium* cell were assessed under specific filters. Fluorescence images were acquired using a Zeiss LSM 710 confocal laser-scanning microscope.

Stained *Symbiodinium* versus unstained were statistically analysed using one-way analysis of variance (ANOVA) and Tukey-Kramer post hoc test for the comparison between cultured *Symbiodinium* and in hospite *Symbiodinium* and for stress kinetics. For all these analyses, a p-value <0.05 was considered statistically significant. All the analyses described above were conducted using the software GraphPad InStat Version 3 (GraphPad Software, San Diego, CA).

Flow cytometric analysis on both cultured and *in hospite* *Symbiodinium*

Sample of both cultured and *in hospite* *Symbiodinium* were extracted from culture medium or fresh tissue and fixed in 70% EtOH overnight before propidium iodide (PI-A, laser excitation 561nm, BP filter 610/20 nm) incubation to predict cell-cycle phase of the biological sample. For CFW profile determination, sample was incubated 30 minutes before fixation. Cells were first sorted according to FSC related to the relative cell size of the sample. Cells were then sorted out according to chlorophyll autofluorescence (PET-Texas Red-A, laser excitation 561nm, BP filter 610/20 nm) and/or CFW staining (DAPI-A, laser excitation 405 nm, BP filter 450/40). Flow cytometry analysis and sorts were performed on a BD ARIA sorter and analysed using the BD FACS Diva software.

Fluorescent microscopy of CalcoFluor White on *in hospite Symbiodinium* during stress experiment

At each times of the kinetic, a tentacle of symbiotic *A. viridis* specimen was fixed overnight in 4% paraformaldehyde and embed in Carboylxmethlycellulose and cryofixed in isopentane at -120°C in liquid azote. Cryosections (12 µm) of tentacles were rinse in SWF and incubated 5 minutes in Calcofluor White at a concentration of 10 µg/ml at room temperature in the dark. Slides were rinsed 3 times in SWF and seal with Fluoromount™ Aqueous Mounting Medium (F4680 Sigma). Fluorescence images were acquired using a Zeiss LSM 710 confocal laser-scanning microscope.

***Symbiodinium* density quantification**

Samples were incubated for 90 min in 1M NaOH solution at 37°C in a drying chamber (Jouan, EB28, France) to allow animal tissue digestion and *Symbiodinium* isolation (Zamoum and Furla, 2012). An aliquot of this homogenate (40 µl) was diluted and used to determine the *Symbiodinium* cell density using a Neubauer-Hauser hemocytometer (VWR, Fontenay-sous-Bois, France) under a light microscope (Leica, Wetzlar, Germany). Ten replicates were quantified and results were weight normalized. Data were first checked for normality and heteroscedasticity of sample variance, then *Symbiodinium* quantifications were tested using a one-way analysis of variance by ranks Kruskal and Wallis with the Dunn multiples comparison post-hoc test. For these analyses, a p-value of <0.05 was considered statistically significant. All analyses described above were conducted using GraphPad InStat Version 3 software (GraphPad Software, San Diego, CA).

Transmission electron microscopy

The different *Symbiodinium* sample were fixed for 2 h at room temperature with 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4)/artificial sea water, then washed with 0.1 M cacodylate buffer (pH 7.4) and postfixed with 1% osmium tetroxide in cacodylate buffer containing 1% potassium ferrocyanide. The samples were embedded in Epon resin after dehydration using an acetone/water solution and then acetone. Ultrathin sections (70–80 nm) were made using a diamond knife mounted on an ultramicrotome (Ultracut S, Leica) and placed on copper TEM grids coated with formvar film. To increase the contrast, the grids were treated with conventional uranyl acetate stain followed by lead citrate. Samples were observed under a JEOL JEM 1400 transmission

electron microscope equipped with a CCD camera (Morada, Olympus SIS) at the Centre for Applied Microscopy (CCMA, University of Nice Sophia Antipolis, Nice, France). At least three independent observations were conducted for each condition

Results

Identification of motile and non-motile stage in cultured *Symbiodinium*

Both motile swimming cells and settled cultured *Symbiodinium* were extracted at 10:00 and stained independently with CalcoFluor White (Fig 1). As CFW stain thecal plates structures present at the motile stage of *Symbiodinium* in culture; we determine the life stage of the dinoflagellate cell population in both condition.

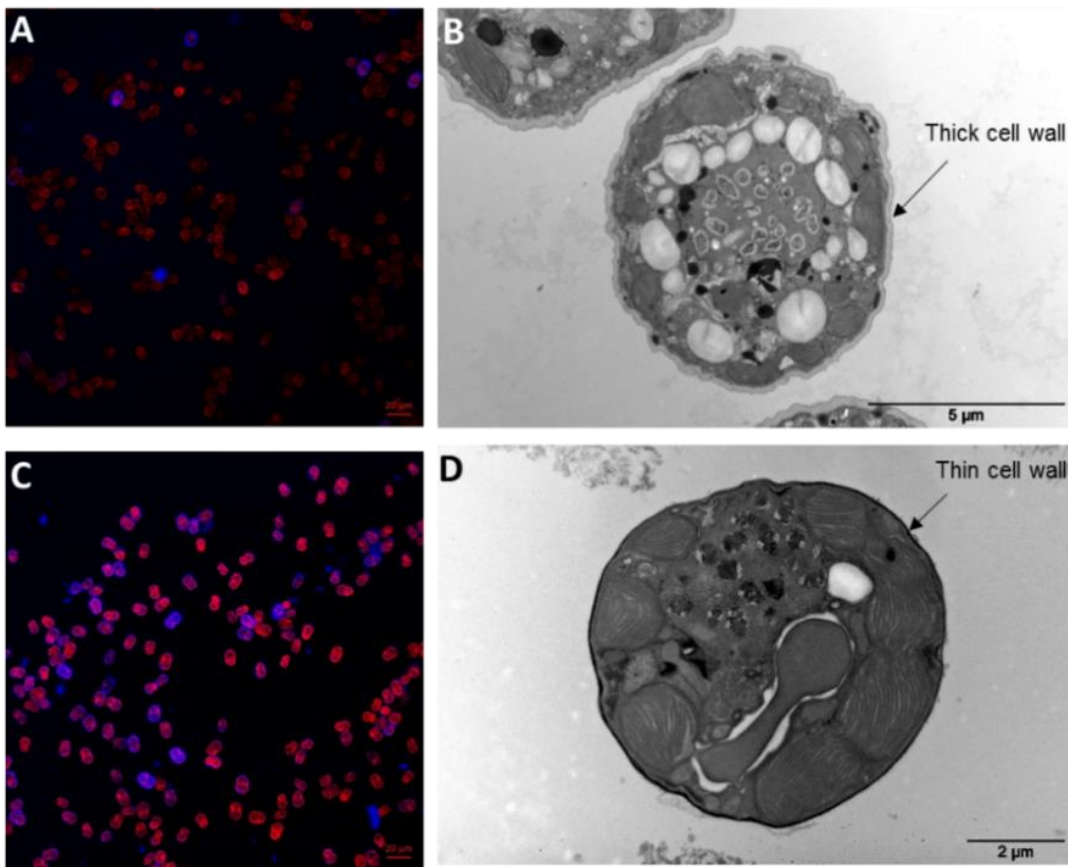


Figure 1: **Identification of motile and non-motile stage of *Symbiodinium* in culture condition**

(A) Pellet of cultured *Symbiodinium* with a coccoid like shape observed by confocal microscopy with the red autofluorescence of chlorophyll (B) TEM of a *Symbiodinium* cell in the pellet of cultured *Symbiodinium* with thick cell wall (C) Supernatant of cultured *Symbiodinium* with bilobed shape characteristic of motile stage observed by confocal microscopy with blue staining specific of CFW (D) TEM of a *Symbiodinium* cell in the supernatant of cultured *Symbiodinium* with thin cell wall.

In pellet, only 10% of the *Symbiodinium* cells were in motile stage and 90% of cells were in coccoid stage (Fig 1A, Fig 2). This result was confirmed by TEM showing thick cell wall surrounding the *Symbiodinium* cell (Fig 1B), characteristic of the coccoid stage. In contrast, 68% of the *Symbiodinium* cells were motile in the supernatant (Fig 1C, Fig 2), with a characteristic gymnodioid structure occurring when the *Symbiodinium* is flagellated. We confirm this result by thin cell wall surrounding the *Symbiodinium* in TEM observation (Fig 1D). This result also indicate that these two populations were statistically differentially represented in culture condition ($p < 0.001$). The cell-cycle state of supernatant *Symbiodinium* also indicate that the majority of cells were in G1/G0 cell-cycle (Fig. S1).

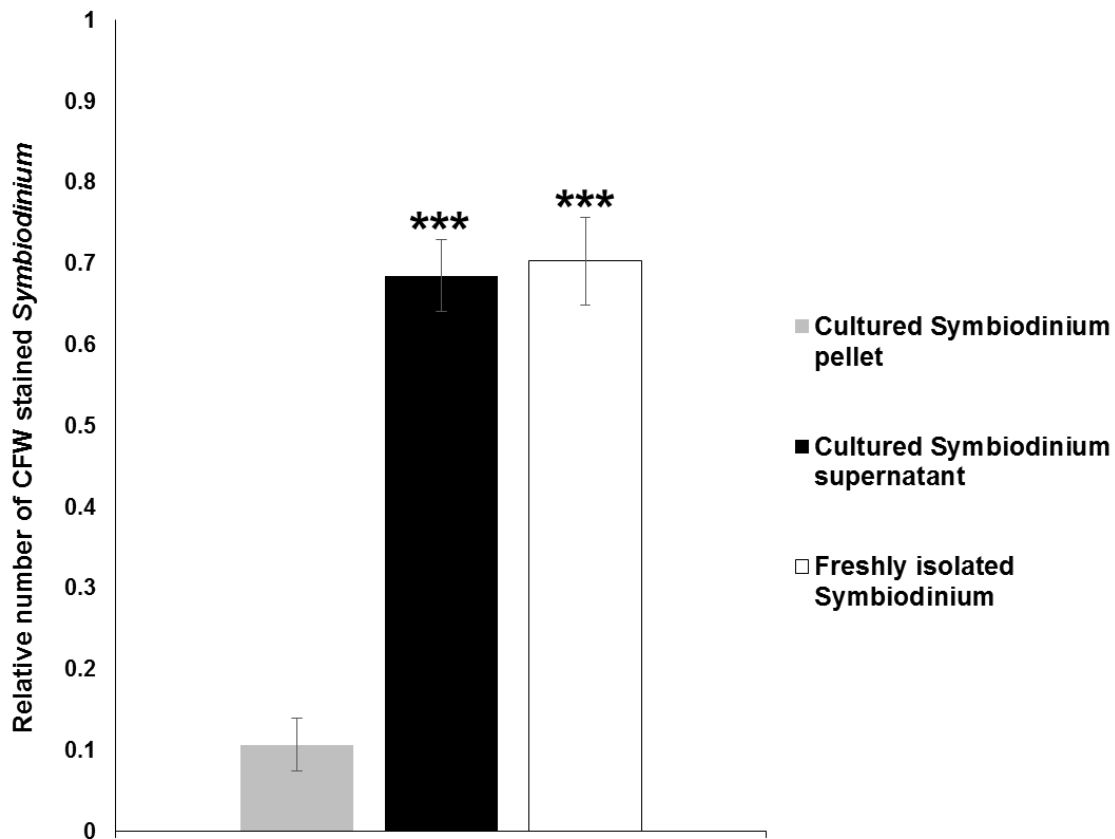


Figure 2: **Relative number of CFW stained *Symbiodinium* in both pellet and supernatant of cultured *Symbiodinium* and in *in hospite* *Symbiodinium* freshly extracted from a symbiotic control specimen.** Statistical analyses using One-way ANOVA and post-hoc multiple comparison analysis of variance Tukey-Kramer test revealed significant difference between pellet of cultured *Symbiodinium* and both supernatant cultured *Symbiodinium* and in hospite *Symbiodinium* at $p < 0.001$ ***. Errors bars represent standard errors of the means (\pm SEM).

Correlation between CFW fluorescence and cell-cycle phase of *in hospite* symbionts

To correlate cell-cycle state of *in hospite* *Symbiodinium* with CFW fluorescence, we combined both flow cytometric analyses and visualization by confocal microscopy of the different populations observed. The flow cytometric analyses were able to distinguish and sort out five different population based on both autofluorescence of the *Symbiodinium* and CFW fluorescence (Fig. 3A). We further determine the major cell-cycle phase of the different pattern by Propidium iodure (Fig. S2). The result reveals that P5 and P6 populations were mainly composed of *Symbiodinium* in the G1/G0 cell-cycle phase, whereas P7 and P8 populations were mainly composed by of symbionts in the S/G2/M cell-cycle phase. The P4 population were composed on CFW positive element without chlorophyll autofluorescence. We then observed by confocal microscopy the different sorted out population. Based on the CFW profile (Fig. 3B), we were able to distinguish between positive stained *Symbiodinium* cells in the S/G2/M cell-cycle phase, negative stained *Symbiodinium* cells in the G1/G0 cell-cycle phase and a third population composed of positive CFW stained elements, consisting of symbiophagic *Symbiodinium* cells. The CFW staining criteria will be used in this study to assess the cell-cycle of *in hospite* symbionts.

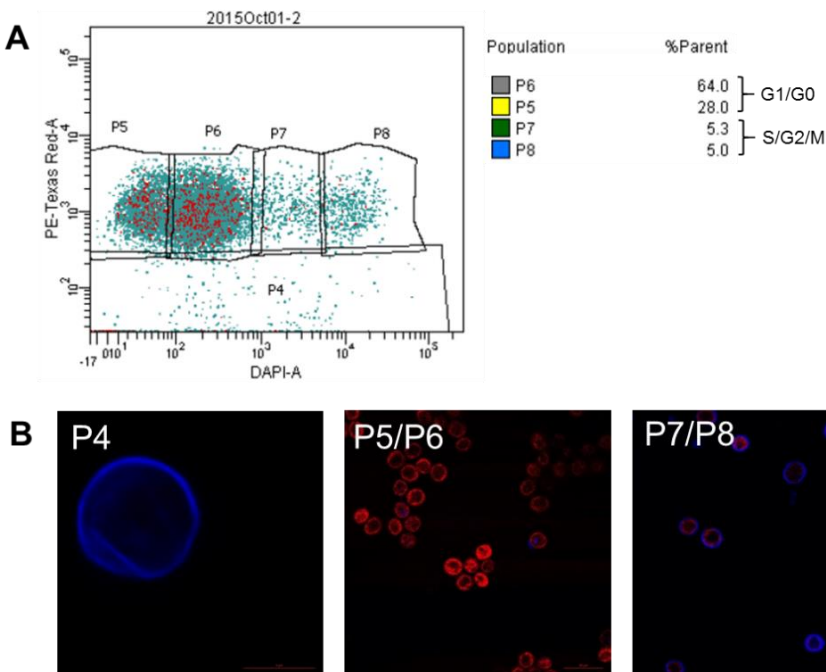


Figure 3: Identification of CFW profile in *in hospite* *Symbiodinium* cells. (A) Flow cytometric analysis of freshly extracted *Symbiodinium* based on chlorophyll autofluorescence and CFW staining showing five distinct populations. Observation by confocal microscopy (B) of P4 population showing digested *Symbiodinium* cells, *Symbiodinium* cells in G1/G0 cell-cycle phase corresponding to fraction P5 and P6 cell cycle state and *Symbiodinium* cells in S/G2/M cell-cycle state corresponding to

fraction P7 and P8. Cell-cycle state was determined by propidium iodure fluorescence (in annex)

Regulation of symbiont population in optimal symbiotic conditions

Concerning *in hospite Symbiodinium* population, we found three different patterns (Fig. 3B) corresponding to S/G2/M cell-cycle state *Symbiodinium* cells for blue stained cells, symbionts in the G1/G0 cell cycle phase for unstained cells (Fig. 3D) and autophagic symbionts characterized by absence of chlorophyll autofluorescence but bright CFW staining. As shown in Fig. 2, 70% of the *Symbiodinium* cells in symbiotic conditions were positively stained in *A. viridis* (Fig. 2) and 45% in both *A. digitifera* and *S. pistillata* (Fig. S3). These results indicate that a major part of the symbiont population was in the dividing state at 10:00, in symbiotic specimens. The stained population doesn't correspond to newly acquired symbionts because any of observed *in hospite Symbiodinium* harboured gymnodinioid structure characteristic of motile stage observed in cultured *Symbiodinium* (Fig. 1D). As the host maintained a stable symbiont population in control condition by active division of *Symbiodinium* cell (Fig. S4), the stained symbionts cells correspond to symbiont entering or sorting of division phase in the G2/M cell-cycle state. This result indicate that symbiont are present in a heterologous population in different cell-cycle phase, within the tissue of the host. Then, a part of the symbiont population is under active metabolic photosynthesis and the other part is under division to maintain a constant symbiont population. As expected, all symbiotic *Symbiodinium* cell harbour the same morphological shape with perisymbiotic membrane surrounding a thin cell wall.

Renewal of *Symbiodinium* population during starvation

Control specimens of *A. viridis* were subjected to 22 day of starvation under normal light and temperature conditions. CFW staining of *in hospite Symbiodinium* revealed a shift of symbiont cell-cycle state population during starvation period (day 0 to day 22) (Fig. 4). First of all, we show that the relative proportion of symbionts respectively in G1/G0 and S/G2/M cell-cycle phase remained almost stable and equally distributed from d0 to d5 (Fig. 4 and Fig. S5A-B). However, a third population composed of digested symbiont was significantly represented at day 5 (12%) compared to day 0 (2%) (Fig. 4) and were characteristic by the partially or totally absence of chlorophyll pigmentation, unregularly shape of the cell and presence of thick cellulosic staining surrounding the cell (Fig. S5).

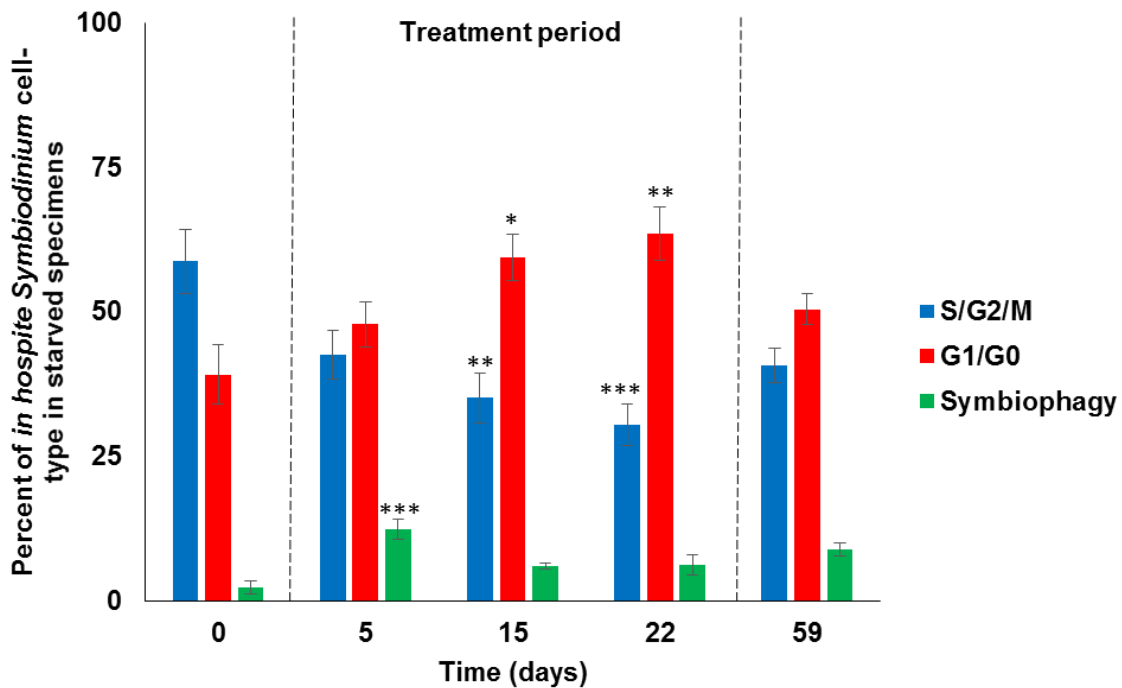


Figure 4: **Relative proportion of different cell-cycle phase of *in hospite Symbiodinium* population during the starvation experiment from day 0 to 22 (starved) and day 23 to 59 (fedded).** Statistical analyses using one-way ANOVA analysis of variance and Tuke-Kramer multiple comparisons post hoc test revealed significant difference between repartition of *Symbiodinium* cell-cycle phase during the kinetic. Statistical difference compared to day 0 were mentioned as $p < 0.05$ *, $p < 0.01$ ** and $p < 0.001$ ***. Errors bars represent standard errors of the means (\pm SEM).

According to nutrient deprivation, the number of *in hospite Symbiodinium* in S/G2/M dividing state decrease at d15 (35%) and d22 (30%) (Fig. 4 and Fig. S5C-D) as a consequence of limitation of metabolic exchange from the host to the symbiont, lowering the number of symbiont able to reach the minimal cell size to cross the S checkpoint. Consequently, the population of symbiont in G1/G0 cell-cycle phase increased significantly at day 15 (59%) and day 22 (63%) indicating active metabolic activity to fulfil nutrient depletion acquired by heterotrophic feeding. The remaining division of a subset of the algal population could explain why the total symbiont population remain stable during starvation stress (Fig. 5). Consequently to feeding reintroduction at day 23, we observed a return to basal level and distribution of both symbiont in G1/G0 and S/G2/M cell-cycle phase at d59 (Fig. 4 and Fig. S5E), with still some remaining digested symbionts (9%).

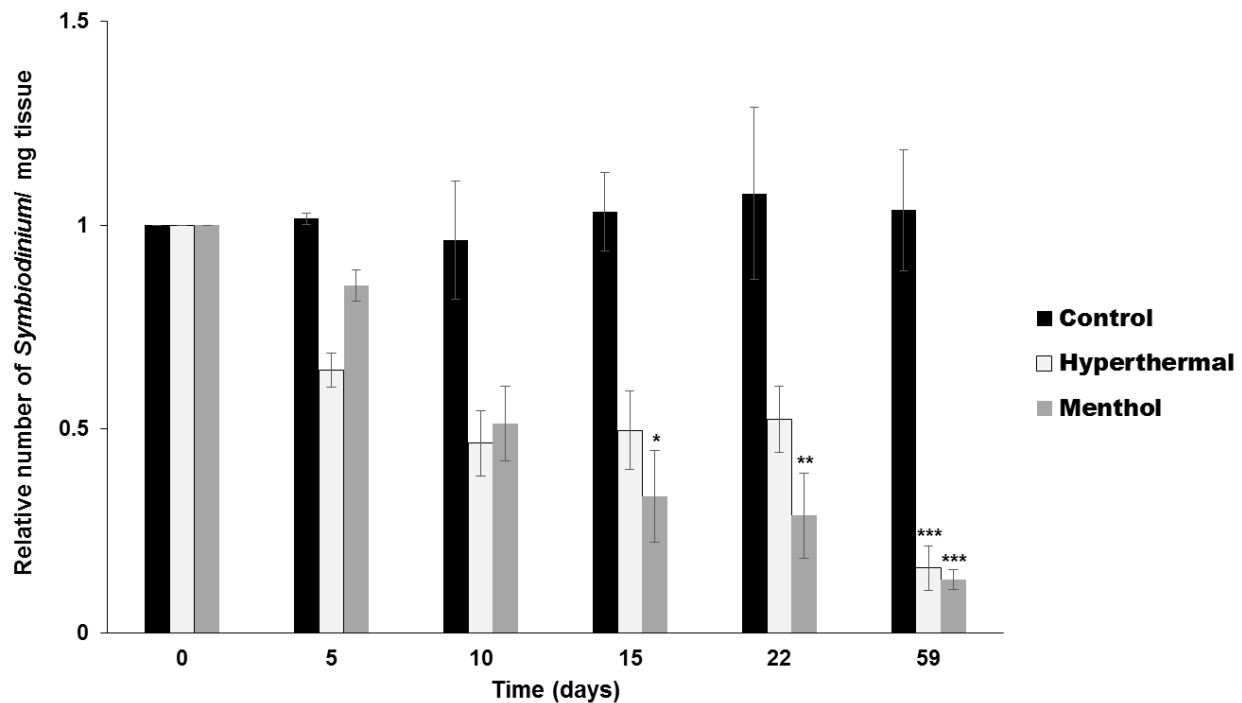


Figure 5: **Quantification of *Symbiodinium* cells relative to d0 within tentacles throughout the bleaching experiments and in the control specimens.** The *in hospite Symbiodinium* population was determined by cell counting in control specimens (n=3) and in specimens subjected to hyperthermal stress (n=3) or menthol stress (n=3). The results shows that bleaching threshold was reached at day 59 for hyperthermal stress and at day 15 for menthol treatment. The results are given as means \pm SEM. Statistical analyses were performed using a one-way analysis of variance by ranks Kruskal and Wallis with the Dunn multiples comparison post-hoc test; significant values are indicated for $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). Errors bars represent standard errors of the means (\pm SEM).

Cell cycle state of *in hospite* *Symbiodinium* population during a stress inducing bleaching

During the hyperthermal stress experiment (Fig. 6A and Fig. S7), we observed a rapid diminution of symbiont in the S/G2/M cell-cycle phase compared to the control experiment. At day 5, 67% of the symbionts observed were in G1/G0 phase. Indeed, the bleaching signal could rapidly block the communication and nutrient exchange between the two partners inducing an arrest of *Symbiodinium* growth necessary to reach the division state.

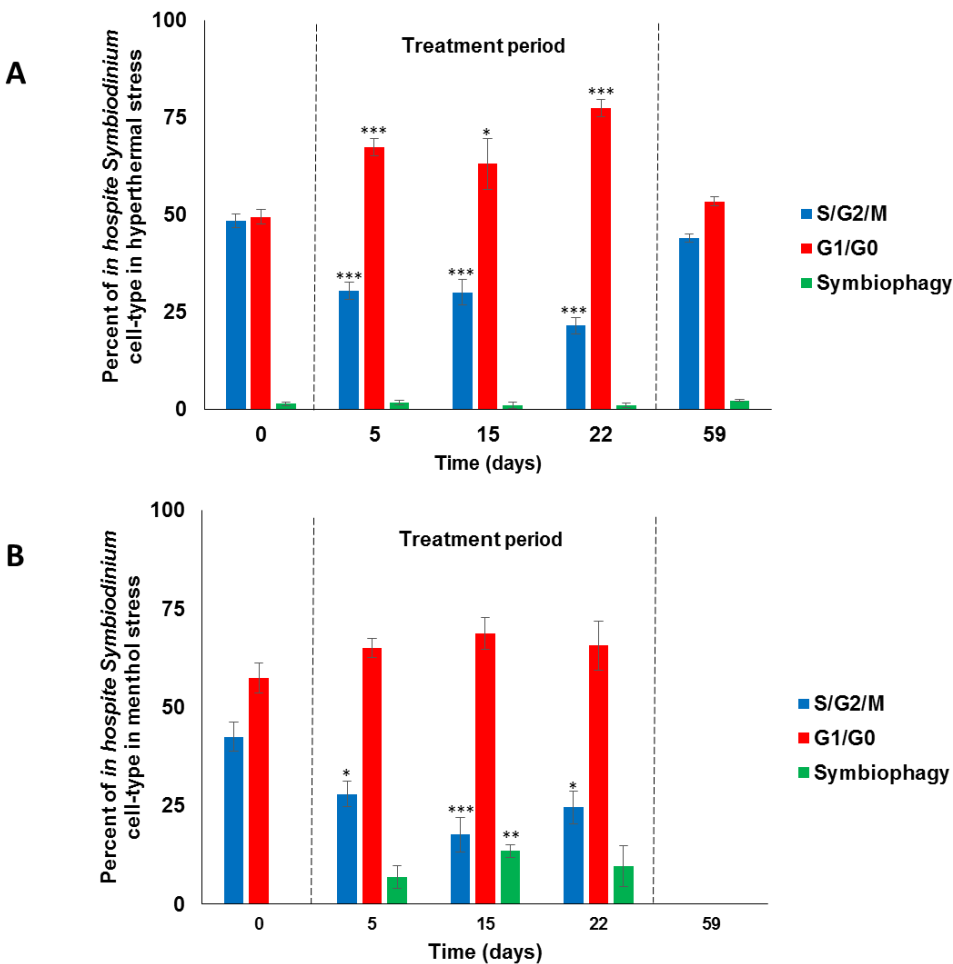


Figure 6: **Relative proportion of different cell-cycle phase of *in hospite* *Symbiodinium* population during the hyperthermal (A) and the menthol (B) bleaching stresses from day 0 to day 59.** Statistical analyses using one-way ANOVA analysis of variance and Tuke-Kramer multiple comparisons post hoc test revealed significant difference between repartition of *Symbiodinium* cell-cycle phase during the kinetic. Statistical difference compared to day 0 were mentioned as $p < 0.05$ *, $p < 0.01$ ** and $p < 0.001$ ***. Errors bars represent standard errors of the means (\pm SEM).

This observation were confirm by late time of stress, d15 and d22, showing a majority of *in hospite* symbionts respectively at 63% and 78% in G1/G0 cell-cycle phase (Fig. 6A and Fig. S7C, D). At d59, the repartition of the remaining symbiont in G1/G0 and S/G2/M returns nearly to basal level with a 1.2 ratio, that could be the result of partial recovery post bleaching episode or presence of “adapted” *Symbiodinium* under active symbiotic exchanges. We also observed a population of symbiont totally digest at all time of the experiment representing a percentage around 1 % of the total *in hospite* population.

Regarding the menthol bleaching experiment, we observed a more abrupt shift with only 28% of *Symbiodinium* in S/G2/M cell-cycle phase at day 5 (Fig. 6B). The efficiency of this fast bleaching method was confirmed regarding the few symbiont remaining *in hospite* at day 15 and day 22 (Fig. 5 and Fig. S8C-D), with respectively 18% and 25% (Fig. 6B). Because menthol bleaching reactivate phagosomal pathway, we were able to identify a third population of *in hospite* representing digested symbionts at day 5, day 15 and day 22 representing respectively 7%, 14% and 10% of the total population. At day 59, no more symbiont were observed in the tissue (Fig. 5 and Fig. S8E). As menthol-stressed specimen’s regularly expelled pellets of *Symbiodinium* around the mouth region during the stress episode, we characterize the cell-cycle state and integrity of the symbionts expelled when fecal pellets were observed (Fig. 7). Similarly as the heterogeneity of *in hospite* symbionts observed in symbiotic condition (Fig. 3 and Fig. S5), we observed a heterogeneity of expelled *Symbiodinium* with G1/G0 phase, S/G2/M phase and digested symbionts at each time of the kinetic (Fig. 8). TEM of *Symbiodinium* in a pellet expelled at day 5 confirm these three different state were observed by CFW staining (Fig. 8B, C, D). Noteworthy, so called digested symbiont could also result of autophagic symbionts as shown by severe *in situ* degradation of symbiont integrity (Fig. 8D). In the early time of stress (d2 and d3), the host release 20% of symbionts in S/G2/M phase, 40% of symbionts in G1/G0 phase and 40% of symbiophagic symbionts. This result indicate that 60% of the expelled symbiont in the early menthol bleaching are viable, indicating an active process of expulsion by the host. At day 5, the dominant population expelled was represented by the symbiont population in the S/G2/M phase, and in contrary the dominant population expelled was the symbionts in

G1/G0 cell-cycle phase at day 9. Starting at day 12 but highly significant at day 15, 17 and 22, we show that the large majority of the symbiont expelled were constitute of digested *Symbiodinium* with respectively 48%, 93%, 81% and 76%.

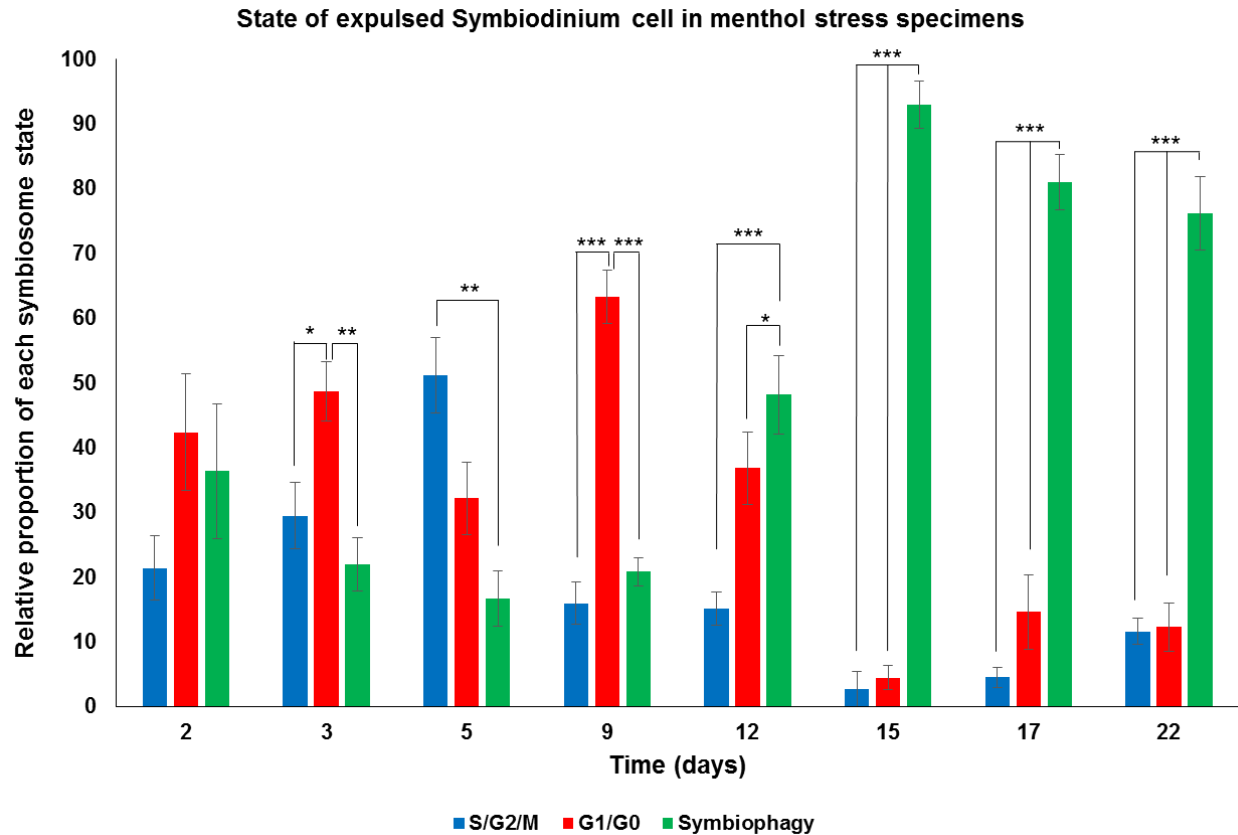


Figure 7: **Relative expression of the different cell-cycle phase a life stage of *Symbiodinium* observed in expelled pellets during menthol bleaching experiment.** Statistical analyses at each time independantly using one-way analysis of variance (ANOVA) and Tukey-Kramer post hoc test revealed significant difference between the different cell-cycle states of expelled *Symbiodinium* cells. A statistical difference of $p < 0.05$ were represented by *, $p < 0.01$ by ** and $p < 0.001$ by ***. Errors bars represent standard errors of the means (\pm SEM).

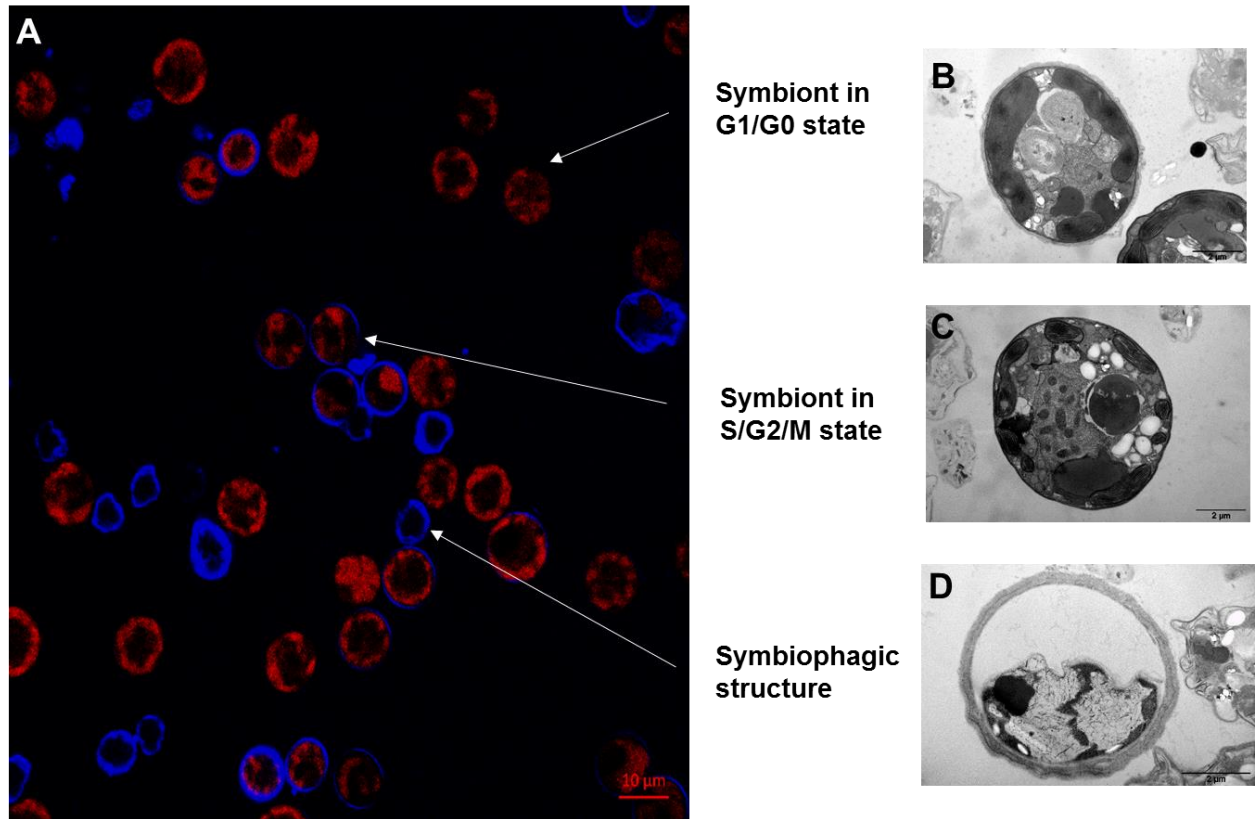


Figure 8: **Identification of different state of *Symbiodinium* expelled from *A. viridis* reveal by CFW** (A) Immunostaining of *Symbiodinium* found in pellets expelled during the menthol bleaching experiment showing the three different population observed by confocal microscopy. Transmission electron micrographs of (B) *Symbiodinium* in G1/G0 cell-cycle phase, (C) *Symbiodinium* in S/G2/M cell-cycle phase and (D) symbiophagic *Symbiodinium* still surrounded by thecal plates, observed in pellets expelled during the menthol bleaching experiment.

Discussion

Heterogeneity of *in hospite* symbiont population in stable symbiotic conditions

In free living *Symbiodinium*, the motile stage occurs during the G1/G0 cell-cycle phase and is characterised by the presence of motile thecal plate while coccoid stage is characteristic of both G2 and M phase (Kwok and Wong, 2003; Steele, 1975). Recently, Kwok and co-workers linked the cellulose synthesis with the cell-cycle state of dinoflagellate and were able to label the cellulosic contents by using the fluorescent marker CFW (Kwok and Wong, 2003; Kwok and Wong, 2010). In cultured *Symbiodinium*, we show that the population can be divided in two distinct population,

based on the capacity of the CFW to label or not the thecal plate of *Symbiodinium* cells (Fig. 1). In the pellet, a majority of the *Symbiodinium* cells harboured a typical coccoid cell-shape with a thick cell wall (Fig. 1a, b). On contrary, the *Symbiodinium* cells in the supernatant were in majority with a gymnodinioid structure, specific of the motile stage (Fig. 1c, d). By applying CFW staining, we were able to determine the percentage of cells with cellulosic compound representative of the motile stage (Fritz and Triemer, 1985; Fujise et al., 2014a). With 10% of cells positively labelled in the pellet conditions and 70% in the supernatant conditions (Fig. 2), we showed that the cultured *Symbiodinium* population was composed of a heterogenic population of cells in these two distinct cell-cycle state. Consideration about the motile state of *Symbiodinium* under investigation should be specified, especially in further studies dealing with proteomics and metabolomics in cultured *Symbiodinium*.

While newly acquired *Symbiodinium* in the coral-dinoflagellate are motile in free living state, the flagella is rapidly loose and resident symbiont *in hospite* are found only in coccoid stage (Steele, 1975b; Trench and Blank, 1987). In this study, we used CFW *in hospite* to assess cellulose synthesis as a proxy of both metabolic activity and cell-cycle of symbionts (Fig. 3). We show that *in hospite* symbionts were distributed in three distinct population (Fig. 2 and Fig. 3a), even if all *Symbiodinium* cells were in coccoid like cell-shape, with a perisymbiotic membrane surrounding the cell. Then, *in hospite* population of symbionts were composed by 70% of symbiont in dividing or newly divided state in *A. viridis* (Fig. 2) and nearly equally distributed in *A. digitifera* and *S. pistillata* (Fig. S2). This result is not consistent with previous studies indicating that a majority of the *in hospite* symbiont are controlled in G1/G0 cell-cycle state to control symbiont population (McAuley 1982; Wilkerson et al. 1988; Falkowski et al. 1993; Smith and Muscatine 1999). However, this result could be explain by the observation in a previous study that the major point of symbiont division occurs between 06:00 and 10:00 (Fitt, 2000). Further experimentation, including determination of the distribution of the cell-cycle state of the *in hospite* symbiont at different time over a day are actually under investigation. Nevertheless, the high proportion of S/G2/M symbionts observed in healthy specimens is an indicator of the positive relationship between the two partners, allowing a part of the symbiont to complete their life cycle. Indeed, G1/G0 cell-cycle phase of symbiont correspond to the active phase of carbohydrate and lipid metabolism, allowing metabolic exchanges in this mutualistic association (reviewed in Davy et al. 2012b). By controlling the G1/S major checkpoint in the cell-cycle of symbionts by nutriment

delivery (Blank and Muscatine, 1987; Neckelmann and Muscatine, 1983; Rees and Ellard, 1989), the symbiont doesn't reach the critical increase of size involved in cell division and enter in the G0 resting phase where they stay in active metabolic exchanges within the host. Nevertheless, observation of dividing symbiont (Fig. S3) and the major part of *in hospite* symbiont in the S/G2/M cell-cycle phase (Fig. 2), indicate that the host allow a part of the symbiont population to divide in order to maintain a constant symbiont population in a stable symbiotic state (Douglas and Smith, 1984; Falkowski et al., 1993; Pardy, 1981). Few symbiont in apoptosis or autophagy in stable condition (Fig. S3) also indicate that the host also use *in hospite* symbiont digestion to regulate the algal population (Dani et al., soumise à publication; Dunn et al., 2007).

Host maintain symbiont in G1/G0 cell-cycle phase to fulfil heterotrophic feeding during starvation

Starvation studies conducted on Hydra, showed that cell size of the symbiont increase to the size of typical division cell-cycle phase during nutrient deprivation (Chera et al. 2009; reviewed in Davy et al. 2012b). In the same manner, nutritional status of the host has been correlated with the length of the G1/G0 phase duration of symbiont in *Aiptasia pulchella* (Smith and Muscatine, 1999), indicating that growth and algal biomass increase *in hospite* during this phase. This study also indicate that the host-cell environment coordinate the relative rates of the heterotrophic and autotrophic requirement. In our model, we show that after 5 day of starvation, the cell-cycle ratio of symbiont in G1/G0 or S/G2/M doesn't significantly changed compare to day 0 (Fig. 4). Yet, we showed that a third population representing digested symbionts was statistically represented. This led us to hypothesis that the *A. viridis-Symbiodinium* symbiosis have a resilience delay capacity to face to starvation episode by digesting *in hospite* symbiont as described previously by Wooldridge in the farming theory (Wooldridge, 2010). Likewise, this mechanism could allow the host to maintain stable physiologic capacities. Then, the presence of a symbiont population in S/G2/M cell-cycle phase could allow a maintenance of stable *in hospite* symbiont population (Fig. 5). In a second phase occurring after 5 day of starvation, we show a shift of the symbiont population, majority composed of G1/G0 symbiont at day 15 and day 22 (Fig. 4). Thus, the nutriment limitation induce by an arrest of heterotrophic feeding could induce a diminution in metabolites transferred to the symbiont and block the growth of the symbiont needed to reach the replication phase. On the other side, the host could maintain a higher proportion of symbiont in the active metabolic

production phase in order to increase autotrophic transfer to compensate loss of heterotrophic feeding. Nevertheless, the presence of remaining symbiont in the dividing phase during all the experiment could be allowed by the host to maintain the stable algal biomass observed during the starvation experiment (Fig. 5). We also show that after nutriment reintroduction at t23, the proportion of symbiont in S/G2/M phase return at day 59 to standard levels, indicating that the nutriment state of the host is actively correlated with symbiont cell-cycle state. This statement was further confirm by TEM showing actively dividing symbiont during all starvation experiment (Dani et al., soumise à publication).

Symbiont transition from G1 to S/G2/M cell-cycle phase is arrest during stress leading to bleaching

During the hyperthermal and menthol stress experiment, symbiont cell-cycle population modifications were enhanced compared to the starvation experiment (Fig. 6). We show that at a different time scale, with a faster response in menthol than in the hyperthermal stress, the symbiont population rapidly shift to a dominant G1/G0 population from day 5 to day 22 (Fig. 6a). This time scale correspond to the increase of the temperature and menthol exposure. Concerning the hyperthermal stress, only few digested symbionts were observed during the stress period, which is consistent with the dominant mechanism involved in symbionts depletion (exocytosis and cell death), previously observed in *A. viridis* (Dani et al., soumise à publication). Besides, this period correspond in the diminution of the symbiont population to 52% of the initial population (Fig. 5). Indeed, the bleaching signal could rapidly block the communication and the nutrient transfer from the host to the symbiont inducing an arrest of the life cycle of the symbionts. The remaining *in hospite* symbiont could be “resistant” symbionts under both active division and active photosynthesis state as proposed by Cunning and co-workers (Cunning et al., 2015). Furthermore, even if a proportion of symbionts remain in the dividing phase, this was not sufficient to compensate the induction of *Symbiodinium* cell depletion induce by the hyperthermal bleaching stress. However, at day 59 (Fig. 6a), the proportion of symbiont in each cell-cycle state return to basal levels. The arrest of the bleaching stress and the reintroduction of feeding could allow the transition of the G1 to S phase thus allowing transition of the symbiont to the G2/M cell-cycle phase. This mechanism as previously been shown to be a way for the host to regain symbionts by inducing the division of the remaining adapted symbiont (Wooldridge, 2010). It would also be

interesting to genetically identify the resistant symbiont population to identify genetics adaptation to different environmental conditions.

Concerning the menthol bleaching experiment, we show an immediate response with a drastic shift of the population from S/G2/M to G1/G0 from day 5 to the end of the stress (Fig. 6b). Moreover, we reveal the presence of a third significant population represented by the digested/autophagic symbionts (Fig. 8) at day 15, corresponding to the bleaching threshold (Fig. 5). Even if symbiophagy has been previously described as a bleaching mechanism (Downs et al., 2009; Dunn et al., 2007; Hanes and Kempf, 2013), it is the dominant mechanism of bleaching in *A. viridis* specimens subjected to menthol (Dani et al., soumise à publication). This result could explain why menthol bleaching specimens preserve standard physiological performance (Wang et al., 2012). Moreover, as in hyperthermal stress, even if a proportion of symbionts remain in the S/G2/M cell-cycle state, the bleaching stress was stronger than the compensation capacity by division of the symbionts (Fig. 5). As in hyperthermal stress, the bleaching signal induced by the menthol incubation could rapidly block the molecular communication between the two partners. The result could be an arrest of the life cycle of the *in hospite* symbiont, then constrain in the G1/G0 cell-cycle state. Furthermore, we characterize the population of *Symbiodinium* expelled in pellets actively rejected by the host during the menthol-stressed specimens of *A. viridis* (Fig. 7). We show that this population was not composed majority by degraded or digested symbiont but by three distinct population easily distinguished by CFW-staining pattern (Fig. 8). In the early time of stress (d2 and d3), the host release 20% of symbiont in S/G2/M phase, 40% of symbiont in G1/G0 phase and 40% of autophagic/degraded symbionts. This result indicate that 60% of the expelled symbiont in the early menthol bleaching are viable, indicating an active process of expulsion. Thus, during the first day of stress, the host expelled dividing *Symbiodinium* cells as previously shown in *Aiptasia pulchella* (Baghdasarian and Muscatine, 2000). A previous study also reported that motile *Symbiodinium* cell were found in fecal pellets from sea anemone (Steele, 1975; Steele, 1977). At day 5, the dominant expelled population was the symbiont in the S/G2/M phase and further at day 9, the dominant population was the symbiont in G1/G0 phase (Fig. 7). Indeed, even if expulsion of dividing symbiont has been shown to be a dominant strategy to regulate algal population density (Baghdasarian and Muscatine, 2000) in some cnidarian species, it could also be a result of the inability of the host to accommodate with ingrowing new symbionts. Otherwise, the presence of a

proportion of viable symbiont in pellets underline the hypothesis of the release of viable symbiont with the capacity to establish a new partnership in another host (Steele, 1975). It should also be taken in account the high level of symbiont actively digested that remain at a constant level around 20% and those undetectable by CFW that are completely degraded or digested. However, under the bleaching line (<50% of initial algal population) that was cross by menthol specimens at day 15 (Fig. 5), the dominant population expelled was almost exclusively digested symbiont (Fig. 7). This result further confirm previous result obtained in (Dani et al., soumise à publication) showing that the menthol stress reactivate phagosomal maturation, leading to massive in situ digestion of symbionts.

Conclusions

In this study, we follow the cell-cycle of *in hospite* symbionts by adaptation of CFW staining as a proxy. We showed that both nutriment deprivation and bleaching stress blocked the cell-cycle transition of *in hospite Symbiodinium* to the dividing phase. We hypothesized that the immediate shutdown of metabolic transfer between the two partners is followed by a shift from a mutualism state; represented by symbiont population allowed to enter in division cell-cycle phase; to a major control of the life state of the symbiont by maintaining the symbiont in the G1/G0 cell-cycle phase. The repartition of the symbiont in the different cell-cycle state are then correlate to the host cell environment as demonstrate previously by (Smith and Muscatine, 1999). Metabolic exchanges are highly regulated and mediated by active transport at the perisymbiotic membrane (Dani et al., 2014; Davy et al., 2012; Ganot et al., 2011; Peng et al., 2010; Revel et al., soumise à publication), as well as processes involved in cell-cell recognition (Schwarz and Weis, 2003) and vesicle trafficking (Chen et al., 2003; Chen et al., 2004; Peng et al., 2010a; Revel et al., soumise à publication). Based on the preliminary result obtain in this study, further investigation will be conducted in order to characterize the proteome modification depending on the cell-cycle state of *in hospite* symbionts. As an exemple, the sterol transporter NPC1, expressed at the perisymbiotic membrane (Baumgarten et al., 2015; Dani et al., en préparation; Dani et al., 2014a; Lehnert et al., 2014) has been shown to be differentially expressed depending on the diel cycle in *in hospite* symbiont with a significant decrease of his expression during the night cycle in the coral *Acropora millepora* (Bertucci et al., 2015a).

These results altogether indicating that the perisymbiotic membrane proteome is probably remodelled depending on the cell-cycle state of the *in hospite* symbionts. The molecular characterisation of the symbiosome proteome could allow the identification of the molecular processes involved in the host control of *in hospite* *Symbiodinium*.

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Supplementary figures

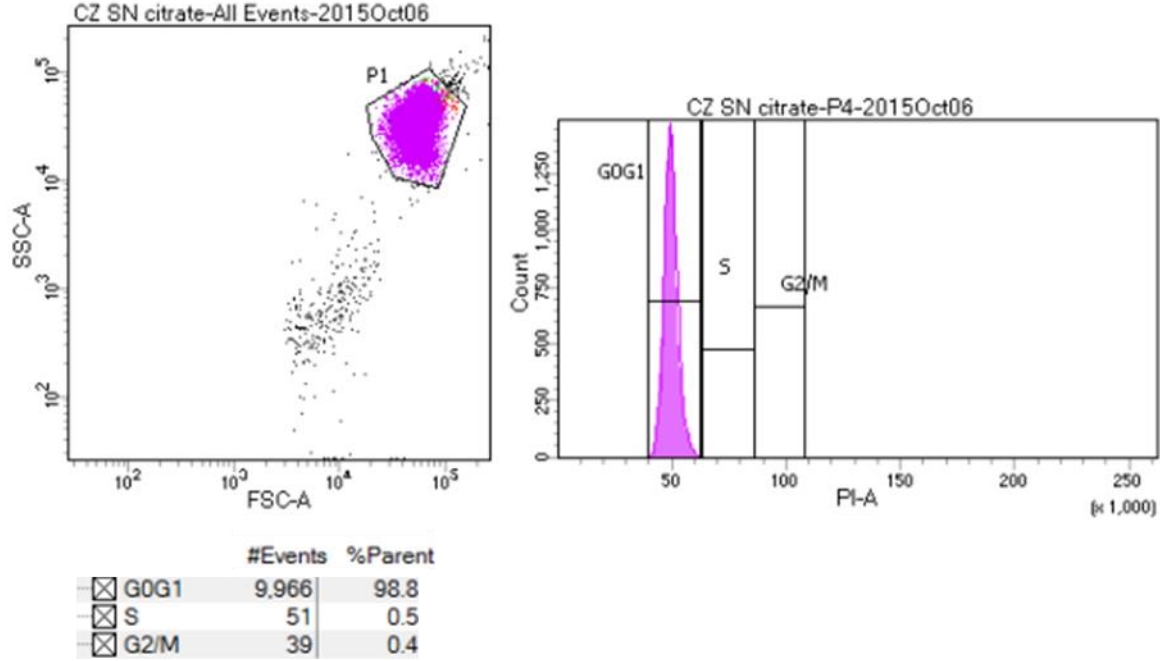


Figure S1: Identification of cell-cycle state of the supernatant fraction of cultured *Symbiodinium* by Propidium iodide.

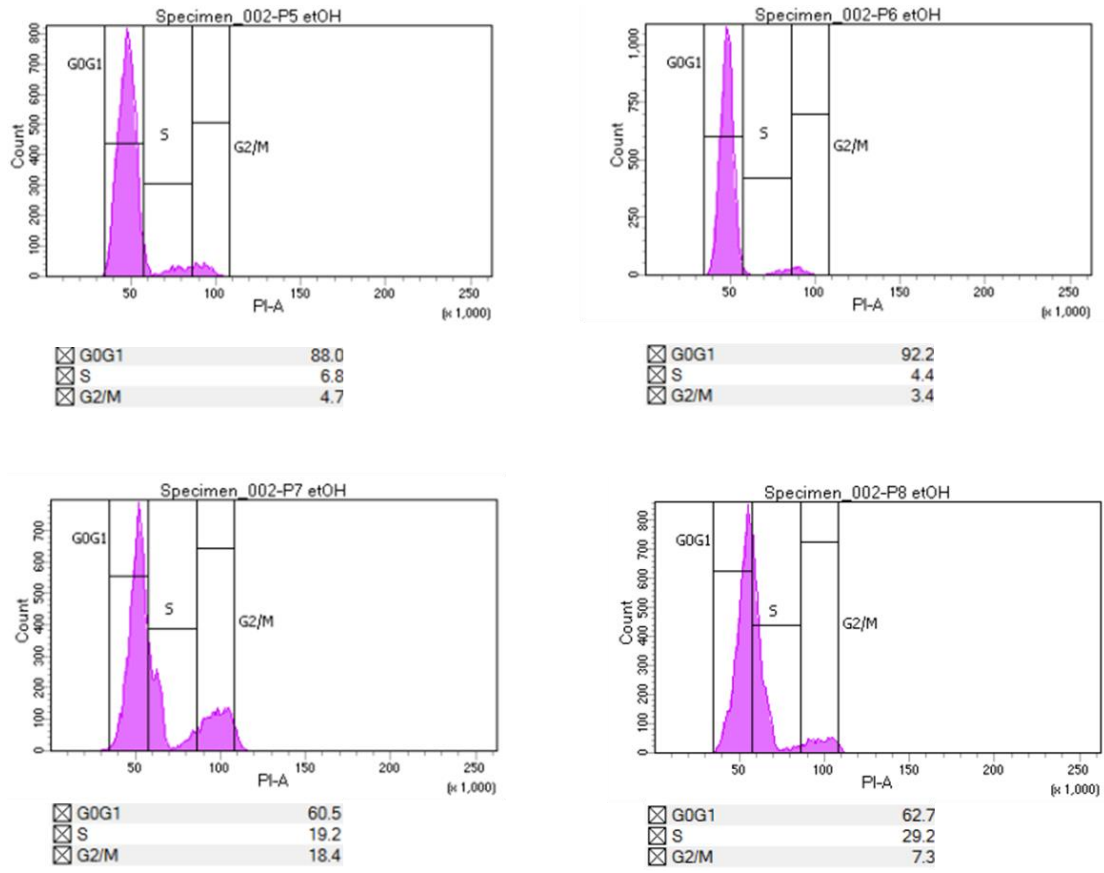


Figure S2: Identification of cell-cycle state of *in hospite Symbiodinium* by Propidium iodide. The different population correspond to the population sort out by the initial flow cytometric analysis in Figure 3.

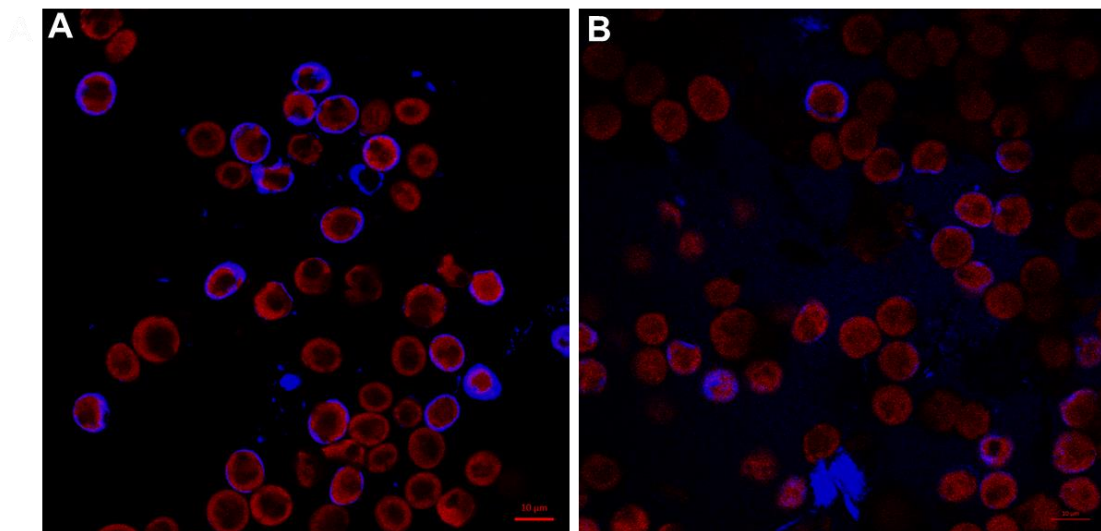


Figure S3: Identification of cell-cycle state of *in hospite Symbiodinium* reveal by CFW. We observed two different population: symbionts in the G1/G0 cell-cycle phase showing red autofluorescence of chlorophyll and symbionts in the S/G2/M cell-cycle state showing blue CFW staining surrounding *Symbiodinium* cells in *Acropora digitifera* (A) and *Stylophora pistillata* (B).

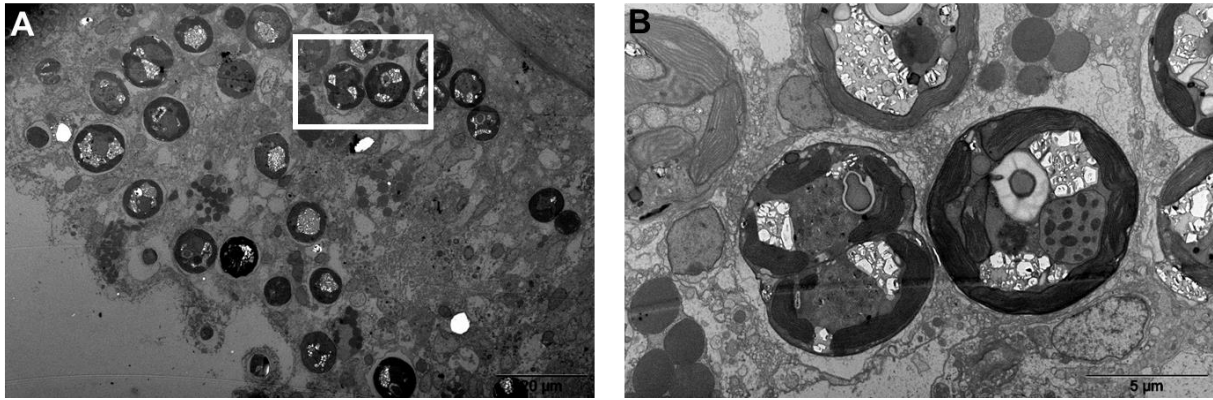


Figure S4: Constant renewal of *in hospite* *Symbiodinium* population by active symbiont division observed by transmission electron micrograph of *Anemonia viridis* tissue section of a symbiotic specimen. (A) Focus on gastrodermal tissue layer and higher magnification in the white box (B).

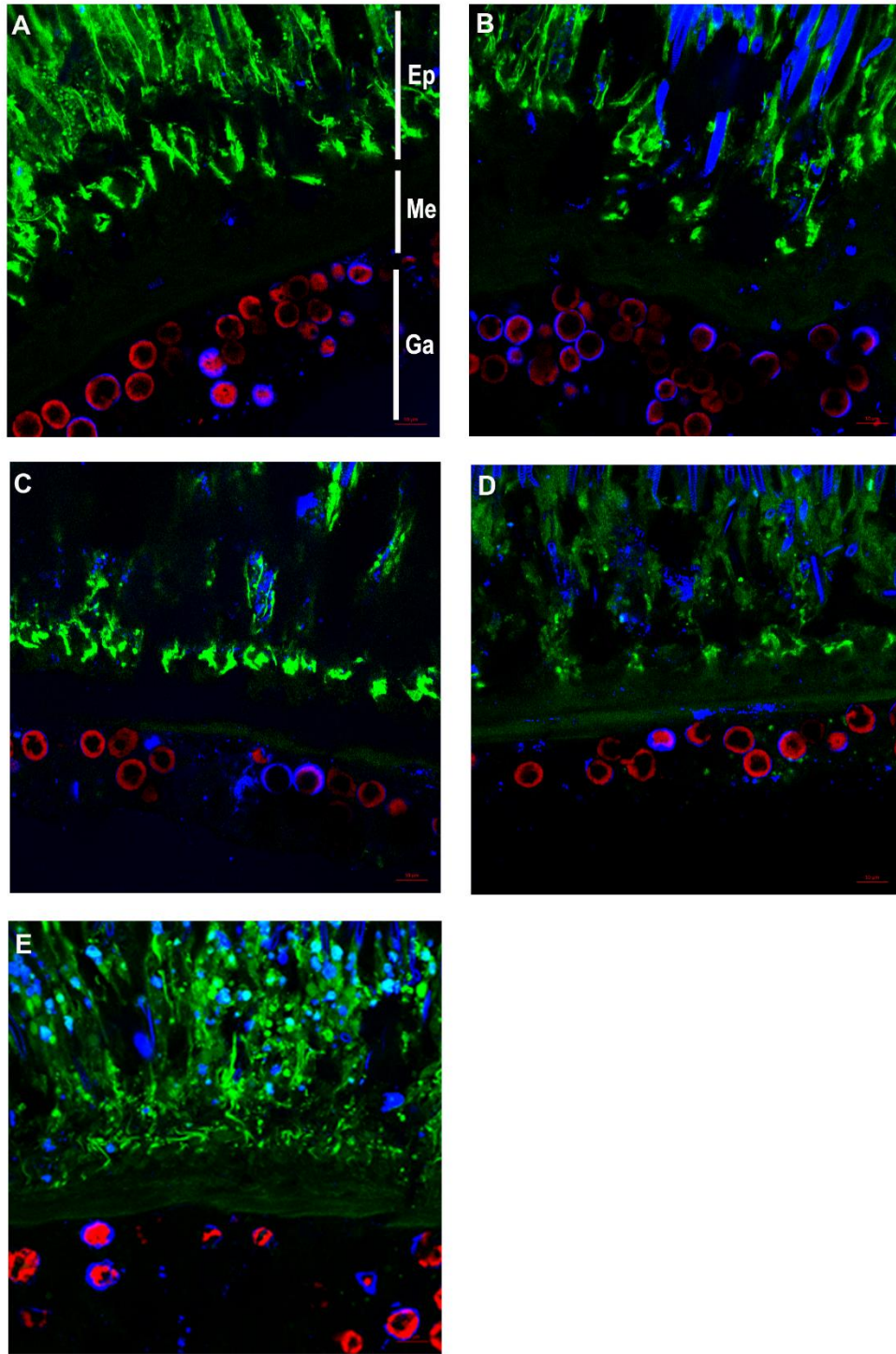


Figure S5: **Immunostaining of a starved specimen with CFW on tentacle sections of *A. viridis*.** We observed a shift of the symbiont population from the S/G2/M cell-cycle phase at early time of starvation (A-B) respectively d0 and d5 to a majority of symbiont in the G1/G0 cell-cycle phase at late time of starvation (C-D), respectively d15 and d22. Green (GFP autofluorescence); Red (chlorophyll autofluorescence) and Blue (CFW staining). Ep, epidermis layer; Me, mesoglea; G, gastrodermis layer.

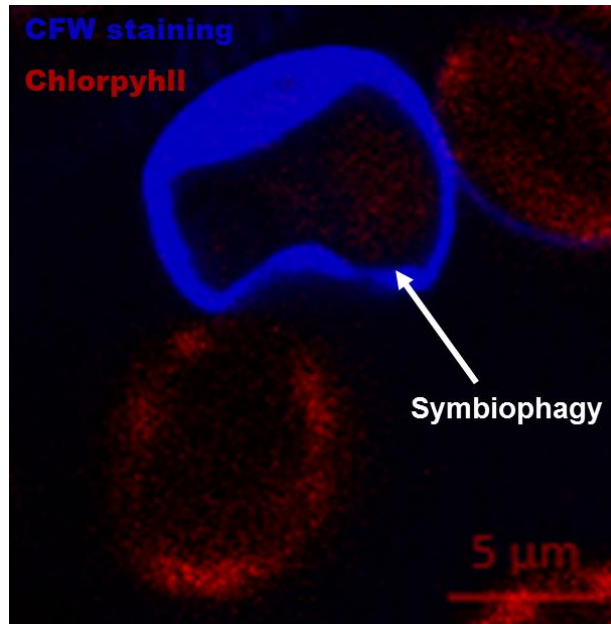


Figure S6: **Identification of symbiophagic *in hospite* *Symbiodinium* cell in tentacle section of *A. viridis* observed by confocal microscopy.** In red the autofluorescence of chlorophyll and CFW staining in blue.

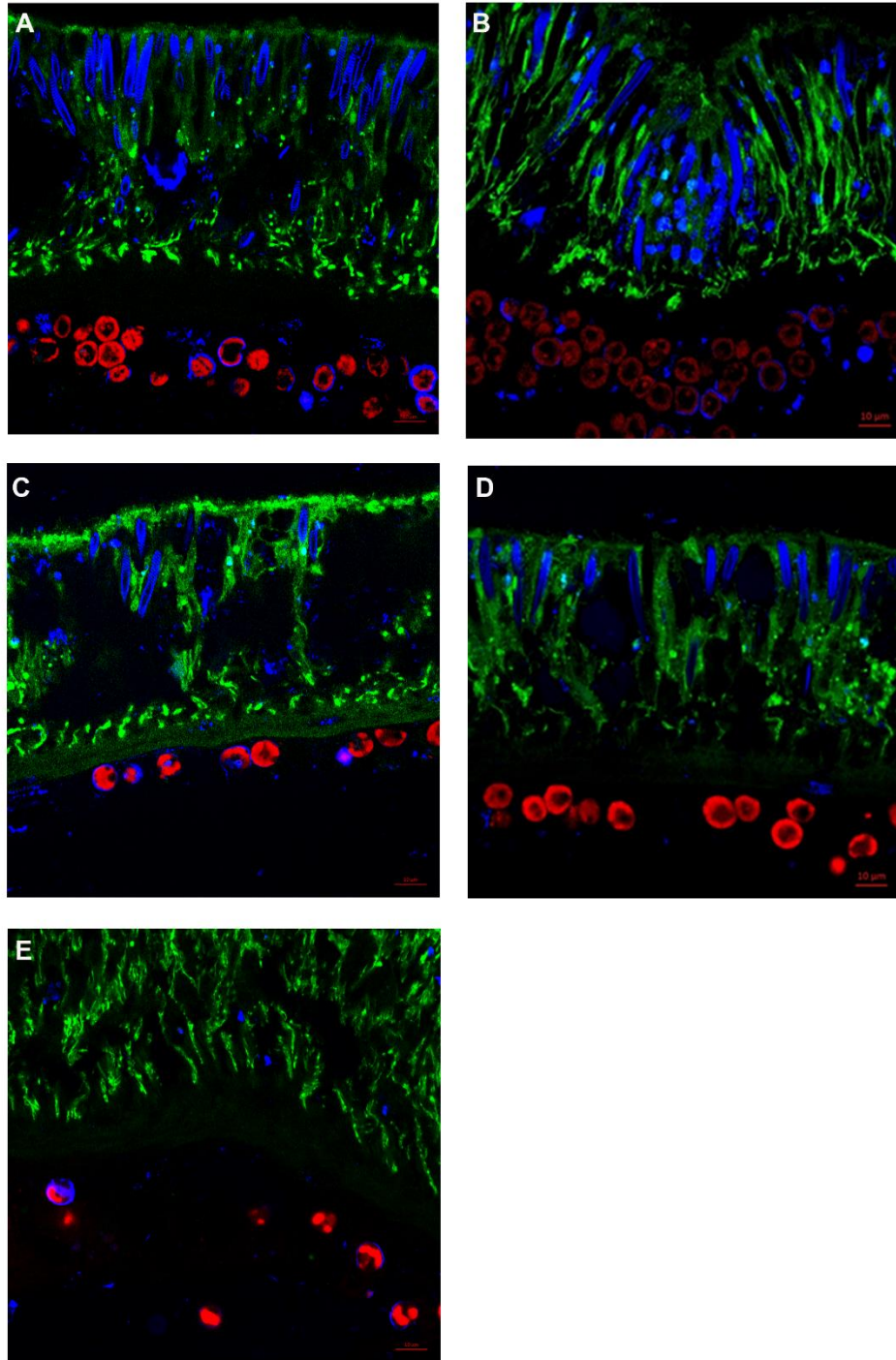


Figure S7: **Immunostaining of *A. viridis* tissue cryosection with CFW in specimens subjected to hyperthermal stress.** A majority of symbiont was blocked in G1 phase a day 0 (A) and we observed a shift of the symbiont from S/G2/M to G1/G0 phase at day 5 (B). In late time of stress (C-D-E) corresponding to day 15, day 22 and day 59, the majority of the symbiont was in the G1/G0 phase. Green (GFP autofluorescence); Red (chlorophyll autofluorescence) and Blue (CFW staining).

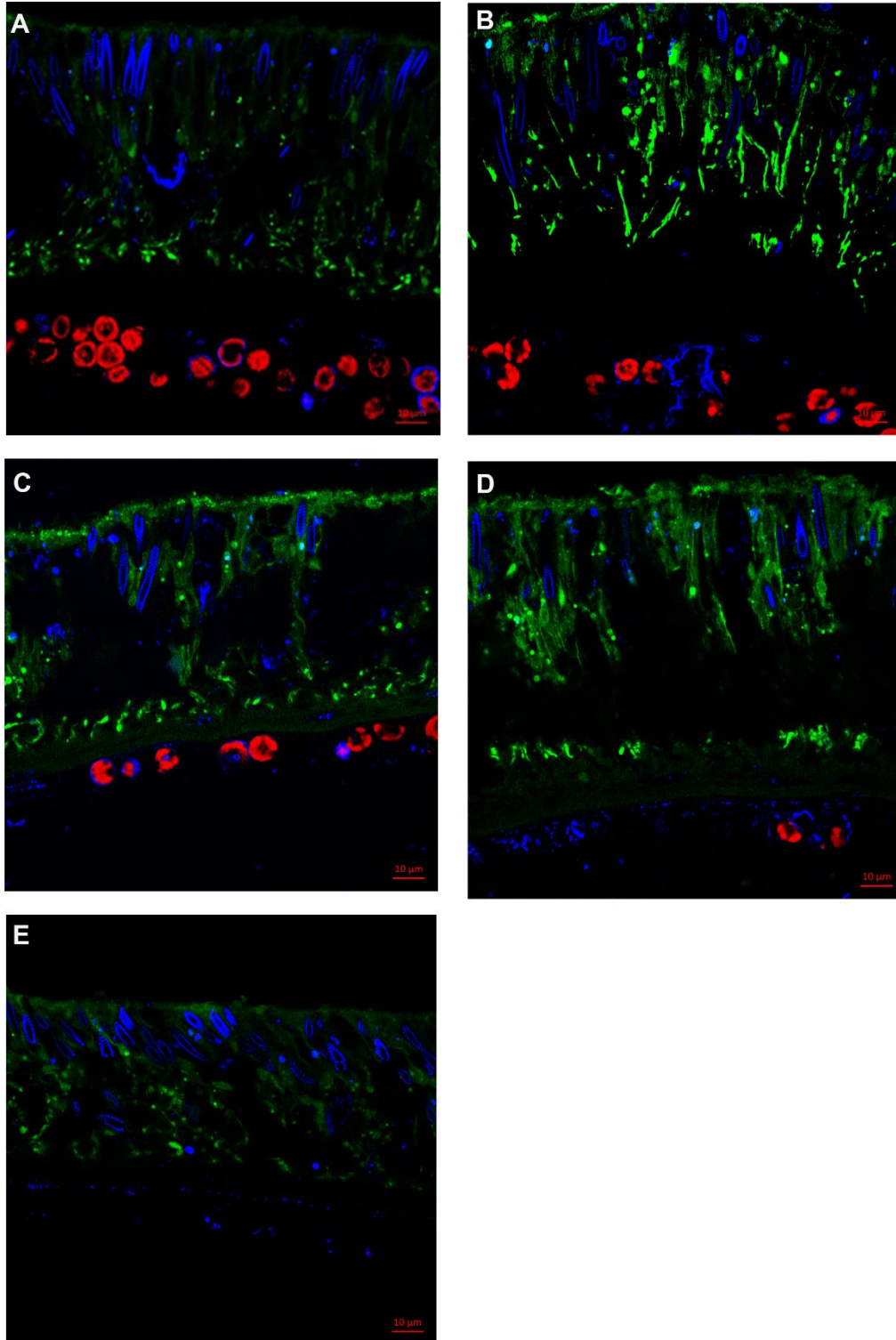


Figure S8: **Immunostaining of *A. viridis* tissue cryosection with CFW in a specimens subjected to menthol stress.** A majority of symbiont were blocked in G1 phase a day 0 (A) and we observed a shift of the symbiont from S/G2/M to G1/G0 phase that occurred before day 5 (B). In late time of stress (C-D-E) corresponding to day 15, day 22 and day 59, all remaining symbiont were in G1/G0 phase. Green (GFP autofluorescence); Red (chlorophyll autofluorescence) and Blue (CFW staining).

2.4. Discussion et Perspectives

Premièrement, la caractérisation de notre modèle biologique au niveau cellulaire et subcellulaire, lors de ces différents processus impliqués dans la rupture de symbiose, nous ont permis de mettre en évidence la présence de types cellulaires tels que les amœbocytes, des jonctions cellulaires et l'organisation de la mésoglye, encore très peu décrite chez ces organismes. Nous avons également, en collaboration avec le Centre Commun de Microscopie Appliquée de l'Université de Nice-Sophia Antipolis (CCMA), pu mettre en évidence la présence d'une modification du cytosquelette lors de l'induction de l'autophagie, par une technique de pointe en microscopie électronique de surface.

Ces résultats nous ont permis de confirmer nos modèles expérimentaux et les techniques employées. Le protocole de blanchissement au menthol sur *A. viridis* a été validé et permet de disposer d'un traitement contrôlé, rapide et efficace, conduisant à l'obtention d'individus aposymbiotiques avec un taux de réussite de 100%. Nous n'avons pas effectué de mesures des capacités physiologiques après le blanchissement, cependant nos observations concernant l'état général des animaux traités sont cohérentes avec les conclusions de l'article de Wang, qui montre que les individus aposymbiotiques possèdent des performances physiologiques comparables avec les individus symbiotiques (Wang et al., 2012). En effet, même si le stress par augmentation de la température est écologiquement pertinent dans le but d'étudier le réchauffement climatique actuel, celui-ci entraîne une mortalité post-blanchissement et même au cours de blanchissement dans les conditions de laboratoire. Ces observations indiquent également que tous les animaux ne répondent pas de la même façon, en fonction de leur histoire de vie. De plus, le stress température induit un blanchissement en deux phases. Une première diminution de la population des symbiotes intervient lors de l'induction du stress, suivie d'une deuxième lors de l'arrêt du stress et de retour à la condition de température contrôlée, provoquant un phénomène de stress supplémentaire. Au contraire, le traitement au menthol induit un blanchissement uniforme, présentant une perte graduelle ainsi qu'une disparition presque complète de la population *in situ* de symbiotes. Cependant, nous avons remarqué en fin de stress température la présence de symbiotes «résistants». Ainsi, après un épisode de blanchissement, l'hôte pourrait induire la division des symbiotes survivants et adaptés aux nouvelles conditions comme le propose Wooldridge dans une étude récente (Wooldridge, 2010). Nous avons caractérisé les mécanismes cellulaires et

subcellulaires impliqués dans le blanchissement au menthol. Nous avons ainsi montré au niveau histologique que ce stress induit une forte réponse de dégradation *in situ* des symbiotes, par un processus d'autophagie (Figure 15B). Il apparaît que la réponse autophagique de l'hôte soit induite et conduise à la fusion des lysosomes avec les phagosomes, résultant d'une levée de l'inhibition de la maturation des phagosomes (Hohman et al., 1982). Afin de connaître de façon plus précise les régulations impliquées dans ce mécanisme de blanchissement, nous avons également engagé un suivi au niveau transcriptomique et protéomique en collaboration d'une part avec le laboratoire Protéomique, Réponse Inflammatoire et Spectrométrie de Masse (PRISM) de l'Université de Lille 1 dirigé par le Professeur Michel Salzet, et d'autre part l'équipe Physiologie génomique des eucaryotes du Dr Pascal Barbry à l'Institut de Pharmacologie Moléculaire et Cellulaire (IPMC) de Sophia-Antipolis. Ces résultats permettront potentiellement d'établir de nouvelles voies de signalisation jamais décrites dans la relation Cnidaire-Dinoflagellés. Au cours du projet l'ANR inSIDE, l'effet du blanchissement au menthol a également été étudié au niveau lipidique et métabolomique dans le cadre du projet doctoral de Johana Revel à l'Institut de Chimie de Nice (ICN) et contribuera à la meilleure compréhension de ce processus de blanchissement.

Parallèlement, cette première étude a permis une description des mécanismes cellulaires qui interviennent lors du blanchissement en réponse au stress température chez *A. viridis*. Nous avons mis en évidence des mécanismes d'apoptose et d'exocytose lors des temps de stress courts (Figure 15A), puis de la nécrose lors des phases tardives de stress. Les échantillons collectés seront également étudiés en parallèle avec nos partenaires au sein de l'ANR inSIDE comme décrits ci-dessus. D'une façon globale, cette étude montre que la réponse cellulaire au blanchissement dépend du type de stress, de sa durée et de son intensité. Cette première étude a également permis de mettre en évidence la présence d'une régulation de la population de symbiotes à l'état stable. En effet, nous avons montré que même si la densité de symbiotes reste constante dans des conditions stables de symbiose, il existe un processus de régulation et de renouvellement de la population de symbiotes. En effet, nous avons observé la présence de structures d'autophagosomes, de façon constante, dans les tissus d'*A. viridis*. Nous avons également décrit que lors d'un stress nutritif, cette proportion d'autophagosomes était significativement augmentée. Cette observation met en évidence une certaine forme de plasticité d'adaptation de l'hôte au regard d'un stress nutritif, notamment par un mécanisme de digestion *in situ* des symbiotes. De façon remarquable, la densité

de symbiotes totale n'était pas affectée au cours de ce stress nutritionnel, suggérant un mécanisme compensatoire de division des symbiotes permettant le maintien d'une population stable de symbiotes.

Dans notre deuxième étude, l'adaptation du marquage au CFW a permis, par une technique fiable et rapide, d'obtenir un proxy du stade du cycle cellulaire des symbiotes *in hospite*. Ceci a permis d'apporter un certain nombre de réponses aux différents processus cellulaires impliqués à l'état stable et en réponse à différentes conditions de stress, processus observés lors de notre première étude. En effet, ce marquage nous a permis de déterminer la part *in hospite* de symbiotes au stade G1/G0 et S/G2/M. Un troisième profil nous a également permis de définir la proportion de cellules de *Symbiodinium* en symbiophagie. Nous avons ainsi montré qu'à l'état stable, la population de symbiotes *in hospite* était constituée d'une partie de symbiotes au stade G1/G0 de croissance et une autre partie au stade S/G2/M de division cellulaire (Figure 15C). Ces résultats démontrent la part de renouvellement de la population de symbiotes dans des conditions stables de symbiose. Nous avons choisi l'heure de prélèvement en fonction des différentes études effectuées durant ce projet doctoral, à 10:00, et parce qu'il correspond au point de transition entre la période de division et de croissance définit d'après les études suivantes (Fitt, 2000; Kwok and Wong, 2003). Néanmoins, nous envisageons d'effectuer dans un premier temps, un suivi sur une période de 24h afin de déterminer les transitions des phases du cycle cellulaire *in hospite* des symbiotes en conditions stable de symbiose. Dans un deuxième temps, cela nous permettra de reproduire cette étude afin d'établir de façon plus précise la régulation du cycle cellulaire en fonction de l'état symbiotique lors de stress entraînant la rupture de la symbiose.

Avec cette étude, nous avons pu définir des pistes d'études et formuler plusieurs observations. Nous avons déterminé que lors d'un stress nutritionnel, la digestion d'une partie des symbiotes était compensée par une part de symbiotes en division. Egalement, nous avons montré qu'il existait une réduction de la population des symbiotes en phase de division lors des épisodes de rupture de la symbiose. Lors de l'arrêt du stress, la proportion de symbiotes dans chacun des deux stades se répartissait à nouveau dans les proportions observées pré-stress. Il y a donc un contrôle dynamique, de la part de l'hôte et/ou du symbiote, du cycle cellulaire en fonction des conditions physiologiques et environnementales. De façon globale, ces recherches montrent aussi qu'il existe une modification de la propriété de la paroi cellulaire des symbiotes lors d'épisode de stress. La

possibilité de distinguer les différents stades du cycle cellulaire par cytométrie de flux permettra également de préciser les modifications éventuelles des protéines recrutées à la membrane du symbiosome. En collaboration avec le laboratoire PRISM, dans le cadre du projet ANR inSIDE, nous avons entamé l'analyse protéomique des différents échantillons. A terme, cela devrait permettre d'identifier les modifications du métabolisme des symbiotes en fonction du stade du cycle cellulaire.

Ces deux études ont donc été complémentaires dans la compréhension des mécanismes de régulation et d'induction de la réponse cellulaire et subcellulaire lors de différentes phases de la symbiose. Nous avons également observé la présence de nombreux processus cellulaires (apoptose, autophagie, nécrose) remarquables du système immunitaire dans notre modèle biologique. Nous avons pu ainsi établir de nombreux parallèles avec les processus symbiotiques présents dans d'autres organismes modèles tels que les insectes, dont le rôle du système immunitaire est plus largement décrit (Gillespie et al., 1997).

Enfin, les différentes analyses de transcriptomique, protéomique, lipidomique et métabolomique actuellement en cours dans le cadre du projet inSIDE, permettront de préciser au niveau moléculaire, les acteurs impliqués dans les différents processus que nous avons décrit dans ce chapitre.

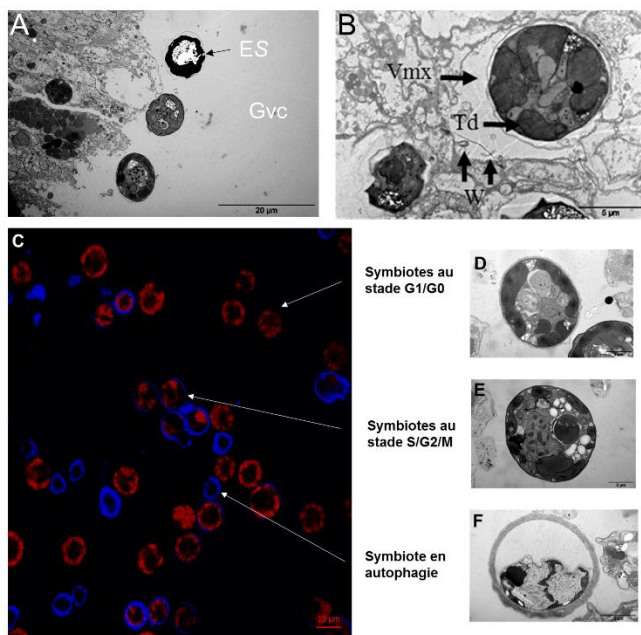


Figure 15: A et B, mécanisme cellulaire majoritaire de diminution de symbiotes lors d'un stress température (A) et menthol (B) chez *A. viridis*. Cavité gastrovasculaire (Gvc), *Symbiodinium* expulsé (ES), matrice vacuolaire (Vmx), désorganisation des thylakoïdes (Td) et membranes autophagiques (W). C-F : Détermination des différents stades du cycle cellulaire des symbiotes, mis en évidence par le marquage CFW. D: phase G1/G0, E: phase S/G2/M et F: symbiote en autophagie

Chapitre 3 : Etude des gènes Niemann-Pick de type C dans la symbiose Cnidaire-Dinoflagellés

3.1. Contexte scientifique et problématique de l'étude

Des gènes cibles potentiellement impliqués dans la symbiose chez *A. viridis* ont été identifiés par la comparaison de l'expression des transcrits chez des individus symbiotiques versus aposymbiotiques (Ganot et al., 2011; Sabourault et al., 2009) et lors d'un stress température et/ou UV (Ganot et al., 2011; Moya et al., 2012). Parmi les gènes les plus différenciellement exprimés, on retrouve les gènes de la *calumenin*, l'*anhydrase carbonique*, *sym32* et *npc2-d*, respectivement impliqués dans le métabolisme du calcium, le transport intracellulaire du carbone inorganique, la reconnaissance cellulaire et dans le métabolisme lipidique. Ces résultats sont cohérents avec l'ensemble des études transcriptomiques réalisées durant la dernière décennie (Tableau 1), et montrent ainsi le rôle de ces processus dans les mécanismes de régulation de la symbiose (Baumgarten et al., 2015; Bellantuono et al., 2012; Grasso et al., 2008; Kuo et al., 2010; Leggat et al., 2003; Lehnert et al., 2014; Rodriguez-Lanetty et al., 2006). Néanmoins, la possibilité de travailler sur les compartiments cellulaires de manière indépendante chez *A. viridis* a permis de mettre en évidence le fait que de nombreux gènes surexprimés en condition symbiotique, étaient également surexprimés dans le compartiment gastrodermique (compartiment symbiotique) par rapport au compartiment épidermique (Ganot et al., 2011). Cette étude a également montré que certains de ces gènes présentaient des duplications spécifiques au groupe des cnidaires. En effet, il a été retrouvé trois copies de la *calumenin* (*Avcalu-a*, *Avcalu-b* et *Avcalu-c*) dont uniquement la copie *Avcalu-a* est régulée en fonction de l'état symbiotique. On distingue également des duplications du gène *Avnpc2* avec la présence de la copie *Avnpc2-d* surexprimée en condition symbiotique dans le gastroderme, alors que la copie *Avnpc2-a* ne paraît pas régulée par l'état symbiotique. Nous avons donc cherché à comprendre ce phénomène, d'abord en caractérisant le répertoire des gènes *npc* chez *A. viridis*, puis en étudiant la régulation de leur expression, à la fois au niveau des transcrits et des protéines.

Chez les vertébrés, les gènes Niemann-Pick de type C (*npc*) codent des protéines exprimées dans les endosomes tardifs et dans les lysosomes et impliquées dans le trafic intra-vésiculaire du cholestérol (Vance, 2010a). Chez l'homme, le cholestérol est transporté dans la cellule via le système endo-lysosomal où il est désestérifié par l'acide lipase, puis pris en charge par la protéine

NPC2 (Storch and Xu, 2009; Tabas, 2002). Il est ensuite transféré de la protéine NPC2 à la protéine transmembranaire NPC1 qui se charge éventuellement de lui faire passer la membrane. NPC2 et NPC1 ont une gamme de substrats assez large et sont impliqués dans le trafic du cholestérol, mais aussi de nombreux autres stérols ou composés lipidiques. Les stérols sont ensuite transportés vers différents compartiments, tels que le réticulum endoplasmique, pour être métabolisés en composés secondaires (Infante et al., 2008a; Infante et al., 2008b) vers les sites de synthèse d'oxystérols, et vers la membrane plasmique (Frolov et al., 2003). Ces protéines permettent l'utilisation des stérols comme composés de structure au niveau de la membrane plasmique, et l'obtention de dérivés de stérols au niveau des mitochondries et du réticulum endoplasmique. Ainsi, les gènes *npc* sont des gènes clés qui régulent l'homéostasie cellulaire du cholestérol (Frolov et al., 2003). Chez l'homme, certaines mutations des gènes *npc1* ou de *npc2* entraînent une rétention du cholestérol dans les lysosomes, perturbant ainsi l'homéostasie intracellulaire des stérols et induisant une maladie neurodégénérative décrite sous le nom de Niemann-Pick Cell de type C (Scott and Ioannou, 2004). Ces gènes *npc* ont été conservés au cours de l'évolution chez de nombreux Métazoaires (Mukherjee and Maxfield, 2004), ce qui suggère une analogie de fonction et de structure. Cependant, les mécanismes d'action de ces deux protéines ne sont pas complètement définis (Storch and Xu, 2009).

De nombreuses études ont démontré que les Cnidaires étaient très riches en lipides, en particulier en stérols (et stéroïdes), en cires estérifiées et en acides gras libres (Harland et al., 1991). Il est important de noter que les Cnidaires ne possèdent pas l'ensemble des enzymes permettant la synthèse *de novo* des stérols à partir d'intermédiaires glycosylés (Baumgarten et al., 2015; Tarrant et al., 2009). Ainsi, la diversité des stérols observée chez l'holobionte provient soit de la digestion des proies capturées par l'hôte, soit de l'activité photosynthétique des symbiotes, ou des deux sources à la fois (Kerr and Baker, 1991b). De plus, les lipides sont en terme quantitatif le deuxième élément transféré du symbiote à l'hôte (Imbs, 2014; Papina et al., 2003). Ceci souligne l'importance du métabolisme lipidique dans l'interaction symbiotique, en particulier dans les cellules du gastroderme. Une étude chez les Scléactiniaux portant sur la comparaison de la composition en lipides chez l'hôte et le symbiote, montre que les stérols produits par les deux partenaires sont différents (Treignier et al., 2009). Cette association pourrait donc permettre un gain en termes de diversité de stérols, et ainsi augmenter la variété de métabolites secondaires disponibles. De plus,

lors d'un stress entraînant la rupture de la symbiose, la composition en lipides est fortement impactée chez l'hôte et le symbiote (Kneeland et al.; Papina et al., 2007; Tchernov et al., 2004). Il a été montré que de nombreuses voies de biosynthèse des dérivés des stérols sont partagées entre l'hôte et les symbiotes suggérant des mécanismes de transport entre les deux partenaires (Baumgarten et al., 2015; Harland et al., 1991a; Sarma et al., 2009). Néanmoins, les mécanismes qui régulent le transport directionnel de ces molécules à travers la membrane pérисymbiotique restent à définir (Davy et al., 2012; Imbs, 2013; Peng et al., 2010). L'étude du trafic vésiculaire des stérols chez les Cnidaires pourrait donc amener d'autres informations sur le dialogue moléculaire impliqué dans le maintien ou la rupture de la symbiose, via l'analyse fonctionnelle des gènes *npc* chez *A. viridis*.

Dans un premier temps, nous avons :

- i) Caractérisé le répertoire des gènes *Avnpc1* et *Avnpc2* chez *A. viridis*,
- ii) Étudié l'expression des différents transcrits identifiés dans chaque compartiment à l'état stable, et la régulation de leur expression chez des individus aposymbiotiques et au cours d'un stress hyperthermal entraînant la rupture de la symbiose.

Puis, à l'aide d'anticorps spécifiques, nous avons :

- iii) Localisé l'expression de la protéine AvNPC2-d et émis une hypothèse sur le rôle et le fonctionnement en tandem des gènes *Avnpc1* et des différentes isoformes du gène *Avnpc2* identifiés, dans le fonctionnement de la symbiose chez *A. viridis*.

Dans une deuxième étude, nous avons déterminé la localisation et la régulation des protéines AvNPC1, AvNPC2-a et AvNPC2-d à l'état stable et lors d'une rupture de symbiose par stress hyperthermal ou blanchissement par le menthol. Nous avons donc :

- iv) Déterminé la localisation et l'expression de ces différentes protéines au niveau cellulaire et subcellulaire dans les différents compartiments, lors de l'état stable et lors de la rupture de la symbiose, ainsi que chez des individus aposymbiotiques,

v) Mis au point un protocole d'enrichissement de fraction de *Symbiodinium* fraîchement isolés possédant ou non la membrane périsymbiotique, afin de valider l'utilisation de AvNPC1 comme un marqueur de la membrane périsymbiotique,

vi) Corréler l'expression de la protéine AvNPC1 avec la dynamique de la modification de composition de la paroi cellulaire des symbiotes

3.2. Approche expérimentale

Afin de caractériser l'ensemble des gènes *npc1* et *npc2* chez *A. viridis*, nous avons recherché dans la banque d'EST préalablement réalisée au laboratoire (Sabourault et al., 2009), l'ensemble des séquences disponibles pour les gènes *npc* par homologie de séquence et de domaines conservés. Nous avons alors pu identifier 4 gènes *Avnpc2* pour lesquels nous avons acquis les séquences entières, par des techniques de biologie moléculaire. Les séquences ont été comparées avec les données disponibles au sein des métazoaires et nous avons pu mettre en évidence des duplications spécifiques des gènes *npc2* chez les anthozoaires. La régulation de l'expression des différents transcrits a été étudiée par PCR-quantitative. Nous avons alors déterminé l'expression spécifique à chaque compartiment et suivi la régulation des transcrits au cours d'un stress hyperthermal pour l'ensemble des 5 gènes caractérisés. Nous avons pu ainsi identifier les gènes *Avnpc1*, *Avnpc2-a* et *Avnpc2-d* comme étant des cibles prioritaires dans l'étude de la régulation des transports de stérols dans la symbiose. L'obtention d'anticorps spécifiques correspondants nous a permis d'étudier la localisation *in hospite* de ces protéines, lors d'un stress hyperthermal et en réponse au blanchissement par le menthol.

Compte tenu de la présence de protéines auto-fluorescentes (GFP dans l'épiderme) et pigments (chlorophylle dans le gastroderme, dû à la présence des symbiotes) au sein des tissus des organismes du groupe de Cnidaires, un travail important de mise au point a été effectué pour valider les protocoles d'immuno-localisation par fluorescence en microscopie confocale. Nous avons choisi un anticorps secondaire possédant une émission non-chevauchante avec les spectres d'auto-fluorescence (collaboration avec la plateforme microscopie de l'Institut de Biologie de Valrose). En effet, la plupart des études publiées précédemment ont utilisé des fluorochromes ayant une gamme d'émission du signal chevauchant l'auto-fluorescence du tissu, rendant les interprétations

peu pertinentes. Nous avons pu démontrer que le fluorochrome AF546 était le seul fluorochrome possédant une gamme d'émission suffisamment spécifique pour extraire le signal caractéristique de la cible visée.

Nous avons ensuite réalisé un suivi quantitatif et qualitatif de la localisation et de l'expression des protéines AvNPC1 et AvNPC2, via des expériences de Western blot (stage de Master 1 de Dounia Chabane) et par immuno-localisation sur coupes de tissus, dans des conditions aposymbiotiques/symbiotiques et sur toute la cinétique de suivi de rupture de symbiose. Concernant les protéines AvNPC1 et AvNPC2-d, localisées au niveau du symbiosome, nous avons développé un marquage sur cellules de symbiotes isolées permettant la visualisation en microscopie confocale. Parmi les cibles préalablement identifiées dans les études de transcriptomique, seule une étude sur le gène *sym32* portait sur la localisation subcellulaire de la protéine à l'interface périsymbiotique (Schwarz and Weis, 2003). En collaboration avec la plateforme du CCMA (Université de Nice Sophia Antipolis), nous avons alors mis au point la localisation par marquage immunogold de la protéine AvNPC1, afin de préciser sa localisation subcellulaire.

Concernant la caractérisation des acteurs moléculaires situés à la membrane périsymbiotique, une seule étude a permis la localisation de 17 protéines (Peng et al., 2010), à partir de l'analyse d'une membrane périsymbiotique purifiée (Kazandjian et al., 2008). Nous avons choisi d'établir un protocole différent, permettant l'établissement de fractions enrichies en symbiotes fraîchement isolées possédant la membrane périsymbiotique, et une fraction totalement débarrassée de cette membrane. En effet, la détermination du protéome de la membrane périsymbiotique à partir d'extraits purifiés de membrane possède, selon nous, deux biais majeurs. Premièrement, elle ne permet pas d'identifier les protéines cytosolubles, présentes dans l'espace périsymbiotique, pourtant déterminantes en ce qui concerne le mécanisme de transport de stérols précédemment décrit. Deuxièmement, il apparaît important de pouvoir déterminer la différence du protéome du symbiote en condition symbiotique et en condition de culture, afin de mettre en évidence les voies de biosynthèse impliquées dans la relation symbiotique. Ce protocole permettra donc, par comparaison des symbiotes avec et sans membrane périsymbiotique, d'obtenir des protéines cibles associées à la membrane périsymbiotique, mais également de déterminer la différence d'expression entre des symbiotes *in hospite* et en culture.

3.3. Résultats

3.3.1. Publication n°3 (publiée dans Molecular Ecology)

Dani, V., Ganot, P., Priouzeau, F., Furla, P. and Sabourault, C. (2014). Are Niemann-Pick type C proteins key players in cnidarian–dinoflagellate endosymbioses? *Mol. Ecol.* **23**, 4527–4540.

Problématique: Quel est le rôle des gènes *Avnpc1* et *Avnpc2* dans l’interaction symbiotique chez *A. viridis* ?

Outils méthodologiques : Déterminer la régulation et la localisation des gènes *Avnpc1* et *Avnpc2* à l’état symbiotique et lors d’une rupture de symbiose.

Principaux résultats : Mise en évidence de duplications spécifiques des gènes *npc2* chez les Cnidaires et de la régulation spécifique de la copie dupliquée *Avnpc2-d*, surexprimé en condition symbiotique et réprimée lors d’un épisode de rupture de symbiose. La protéine NPC2d est exprimée au niveau du symbiosome.

Perspectives : Déterminer la régulation et la localisation des protéines NPC chez *A. viridis*

Are Niemann-Pick type C proteins key players in cnidarian-dinoflagellate endosymbioses?

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Keywords: cnidarian-dinoflagellate interaction, symbiosis, bleaching, sterol transport, NPC1, NPC2

Running title: ROLE OF NPC PROTEINS IN SYMBIOTIC CNIDARIANS

Abstract

The symbiotic interaction between cnidarians, such as corals and sea anemones, and the unicellular algae *Symbiodinium* is regulated by yet poorly understood cellular mechanisms, despite the ecological importance of coral reefs. These mechanisms, including host-symbiont recognition and metabolic exchange, control symbiosis stability under normal conditions, but also lead to symbiosis breakdown (bleaching) during stress. This study describes the repertoire of the sterol-trafficking proteins Niemann-Pick type C (NPC1 and NPC2) in the symbiotic sea anemone *Anemonia viridis*. We found one NPC1 gene instead of two in vertebrates. While only one NPC2 gene is present in most metazoans, this gene has been duplicated in cnidarians and we detected four NPC2 genes in *A. viridis*. However, only one gene (AvNPC2-d) was upregulated in symbiotic sea anemones and displayed higher expression in the gastrodermis (symbiont-containing tissue) than in the epidermis. We performed immunolabeling experiments on tentacle cross sections and demonstrated that the AvNPC2-d protein was closely associated with symbiosomes. In addition, AvNPC1 and AvNPC2-d gene expression was strongly downregulated during stress, especially at the onset of symbiosis breakdown. These data suggest that AvNPC2-d is involved in both the stability and dysfunction of cnidarian-dinoflagellate symbioses.

Introduction

Coral reef ecosystems strongly rely on mutualistic endosymbiosis between cnidarian hosts (such as corals and sea anemones) and photosynthetic dinoflagellates of the *Symbiodinium* genus. Dinoflagellate symbionts are hosted in the gastrodermal tissue layer, in a phagosome-derived vacuole called the symbiosome. Symbionts are therefore separated from the host cytoplasm by a symbiosome membrane of animal origin (Rands et al., 1993; Wakefield and Kempf, 2001). Metabolic exchange is of fundamental importance to cnidarian-dinoflagellate symbiosis, especially in nutrient-poor waters, where up to 90% of symbiont photosynthates are translocated to the animal (Yellowlees et al., 2008b). These photosynthetic products support cnidarian metabolism, growth and reproduction (Steen & Muscatine 1984; Davy *et al.* 2012). In return, the cnidarian host supplies its symbionts with nutrients and the CO₂ required for photosynthesis.

The maintenance of this stable mutualistic association is the result of complex molecular and cellular interactions between the two partners. Processes underlying symbiosis establishment and maintenance have been intensively studied in several Anthozoan species, but some key questions remain unresolved (for review, see Davy *et al.* 2012). This includes mechanisms of recognition and phagocytosis, induction of host-cell immunity and symbiont tolerance, metabolic exchange and nutrient trafficking. Similarly, cellular mechanisms that result in symbiosis collapse and eventually symbiont loss (cnidarian bleaching) have been partially described (reviewed in Weis *et al.* 2008). Transcriptomic studies (microarrays and RNA-Seq) in symbiotic corals and sea anemones have begun to reveal clues about cellular mechanisms involved in the regulation of symbiotic interactions (Grasso et al., 2008; Lehnert et al., 2013; Rodriguez-Lanetty et al., 2006; Sunagawa et al., 2009b). A comparison of gene expression in symbiotic and aposymbiotic specimens of the sea anemone *Anemonia viridis* revealed potential key players in symbiosis maintenance (Ganot et al., 2011), as in symbiosis dysfunction (Moya et al., 2012). The complete separation of tissues from the large tentacles of *A. viridis* also allowed separate study of the epidermis and gastrodermis. Among the genes that were both upregulated in the gastrodermal tissue (containing symbionts) and highly expressed in symbiotic rather than in bleached specimens, we found homologs of Niemann-Pick type C (NPC) proteins (Ganot et al., 2011), which are key regulators of the intracellular sterol-trafficking pathway. Although cnidarians have high fatty acid and sterol contents (Harland *et al.* 1991; Kerr & Baker 1991), they have not been shown to be able to synthesize sterols *de novo* (Tarrant et al., 2009). Sterols are therefore acquired from external

sources, i.e. the diet or from dinoflagellates in the case of symbiotic anthozoans (Treignier et al., 2008). Nonetheless, the molecular mechanisms involved in these sterol translocations from symbionts to the cnidarian host are still unknown.

In vertebrates, NPC genes encode two families of proteins (NPC1 and NPC2), which are located in late endosomes and lysosomes and involved in sterol trafficking as well as in cellular regulation of cholesterol homeostasis (Vance, 2010b). Human NPC1 is a 1256 amino acid protein, which contains thirteen predicted transmembrane domains, three large luminal loops and a C-terminal lysosomal targeting sequence (Infante et al., 2008b; Scott and Ioannou, 2004b). The N-terminal domain of NPC1 projects into the lumen of late endosomes/lysosomes, where it binds cholesterol and oxysterols. In addition, NPC1 has a predicted sterol-sensing domain (SSD) consisting of transmembrane sequences similar to those found in several other proteins involved in cholesterol homeostasis, such as patched proteins (Infante et al., 2008c). In contrast, human NPC2 is a small luminal protein (132 amino acids) that can also be secreted (Vanier and Millat, 2004). NPC2 binds cholesterol that has been released from low-density lipoproteins in the lumen of late endosomes/lysosomes and accelerates the rate of cholesterol transfer to NPC1 (Xu *et al.* 2008; Vance 2010) . NPC2 is able to bind cholesterol as well as a subset of other sterols (Liou et al., 2006). Vertebrate genomes possess one NPC2 gene and two related NPC1 genes (NPC1 and NPC1L1). Whereas NPC1 is widely expressed, NPC1L1 is restricted to intestine and liver and is responsible for dietary cholesterol and biliary cholesterol absorption (Wang and Song, 2012).

In *A. viridis*, we only found one gene for NPC1 but four NPC2-related transcripts. Here we present the complete characterization of the AvNPC1 gene and the four AvNPC2 genes in *A. viridis*, as well as their gene expression profiles in each tissue layer during symbiosis disruption. We demonstrate that several NPC2 gene duplication events occurred independently in diverse cnidarian species. AvNPC1 and AvNPC2-d are mainly expressed in the gastrodermal tissue. We also show that only AvNPC2-d gene expression is regulated by the symbiotic state or during heat-induced symbiosis disruption. Moreover, the AvNPC2-d protein is associated with the symbiosome, as demonstrated by Western-blot and immunolocalization experiments. These data therefore suggest that NPC genes participate in the regulation of cnidarian-dinoflagellate symbiosis.

Materials and methods

Biological material and experimental design

Mediterranean specimens of *A. viridis* were collected in shallow waters at Villefranche-sur-Mer (France). They were maintained in laboratory controlled conditions where they were acclimated for three months before experimentation. Weekly renewed seawater was maintained at $19 \pm 0.5^\circ\text{C}$ in aquaria. Sea anemones were fed once a week with *Artemia* nauplii. Light was provided at a constant saturating irradiance of $180 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ on a 12/12 h light/dark cycle with a metal halide lamp (HQI-TS 400 W, Philips). To generate aposymbiotic specimens, sea anemones were subjected to a hyperthermal stress ($+ 10^\circ\text{C}$) for 20 days. Following bleaching, aposymbiotic specimens were maintained in the dark with the same conditions of temperature, feeding and water renewal. For the hyperthermal stress experiment, three specimens were used as controls and three specimens were shifted abruptly to 29°C and held in this condition for 22 days, with continuous monitoring of seawater temperature. For each specimen, four tentacles were sampled on the following days: d0, d1, d2, d3, d4, d5, d10 and d22. All samplings were done at 10:00 am to avoid variation in gene expression due to the circadian clock (Levy et al., 2011).

Nucleic acid extraction

Nucleic acid extractions were performed as described previously in Ganot *et al.* 2011. Gastrodermal and epidermal tissue layers were separated and total RNA was extracted using Trizol Reagent (Invitrogen) modified by the utilization of bromochloropropane instead of chloroform. A DNase treatment (RQI-DNase, Promega) was performed on RNA samples to avoid genomic DNA contamination. Genomic DNA was extracted from whole tentacles.

Acquisition of complete sequences of NPC genes

Complete transcript sequences were assembled from the first *A. viridis* EST dataset for AvNPC2-a, AvNPC2-b and AvNPC2-d (Sabourault et al., 2009b). These cDNA sequences were verified by specific PCR amplifications and re-sequencing.

Complete cDNA sequences of AvNPC1 and AvNPC2-c were obtained by rapid amplification of cDNA-ends (RACE-PCR) using the 5'/3' RACE Kit (Roche, France).

Specific primers (Table S2) were designed based on the AvNPC1 and AvNPC2-c partial sequences that were identified within *A. viridis* ESTs (Sabourault et al., 2009b). PCR products were cloned into pGEM-T Easy vector and sequenced by the Sanger method (Macrogen Inc., South Korea). To define the genomic structure of all AvNPC2 genes, primers were designed to amplify full length gene sequences. PCR were performed on both cDNA and gDNA from the same individual to compare the genomic structure of all AvNPC2 isoforms.

Analysis of gene expression by real-time quantitative PCR (RT-qPCR)

For both cDNA and gDNA real-time qPCR experiments, specific primers amplifying around 100 bp were designed using the software Primer3 (<http://frodo.wi.mit.edu/primer3/>). All the primer sequences used in this study are listed in Table S2. Amplicon specificity for either *A. viridis* or *Symbiodinium* was tested against epidermal (extracts without symbiont) or *Symbiodinium* culture extracts, respectively (data not shown). Primer efficiencies were determined using standard curve analyses with a 10-fold dilution series of pooled cDNAs from both control and treated samples (data not shown), and ranged from 1.8 to 2. Real-time qPCR products were sequenced (Macrogen Inc., South Korea) and matched the expected product identities.

cDNAs were prepared using SuperScriptII reverse transcriptase (Invitrogen) and a mixture of oligodT and random primers, according to the manufacturer's instructions. Transcript level quantification was performed using the SYBR green fluorescence method and a Light Cycler 480 (Roche). PCR conditions are described in Ganot *et al.* 2011. Each sample was run in triplicate. The dissociation curves showed a single amplification product and no primer dimers.

Several control genes were chosen based on previous microarray results (Ganot et al., 2011; Moya et al., 2012). The expression stability of ten putative control genes was evaluated using the GeNorm software (Vandesompele et al., 2002). A reliable normalization factor was calculated based on the expression level of the most stably expressed control genes (in all conditions). The control genes finally selected in this study are RCC2 (Regulator of Chromosome Condensation protein 2) and COP- γ (Coatomer subunit gamma). Expression levels of target genes were normalized using the GeNorm normalization factor and the results given as expression relative to the d0 value as calibrator.

Symbiodinium density quantification

The relative abundance of *Symbiodinium* vs *A. viridis* cells was quantified using gDNA samples as templates and a real-time qPCR method, described by Ganot *et al.* 2011. The specific nuclear gene used for each partner was ascorbate peroxidase (APX) for *Symbiodinium* and COP- γ for *A. viridis*.

Maintenance of cultured Symbiodinium

Three single-cell derived cell lines of *Symbiodinium* sp. (clade A') were originally isolated from a single specimen of *A. viridis*, and established as clonal *in vitro* cultures at the University of Nice (Marine Symbiosis lab, UMR 7138). The *Symbiodinium* cells were cultured in f/2 medium (Guillard and Ryther, 1962), supplemented with 1% antibiotic-antimycotic solution cocktail (Sigma). Cultures were routinely maintained at 24°C and 90% humidity, under a standard 12h/12h light/dark cycle using an irradiance of 200 $\mu\text{mol quanta m}^{-2} \text{sec}^{-1}$ (JLB ; Solar ultra marin day) in a controlled cabinet (Witeg Labortechnik GmbH ; WiseCube model WGC-450).

Sequence and phylogenetic analyses

Homologous sequences for AvNPC1 and AvNPC2 proteins were identified by iterative BLAST searches using blast analysis tools at the NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), at the Joint Genome Institute (for *Nematostella vectensis* sequences, <http://genome.jgi.doe.gov/>) and at the Okinawa Institute of Science and Technology (for *Acropora digitifera* sequences, <http://marinegenomics.oist.jp>). When needed, sequences were assembled using the CAP3 software (Huang and Madan, 1999). Sequences were then analyzed to find matching Pfam families (Bateman *et al.*, 2002), especially for the presence of a ML domain (MD-2-related lipid-recognition) in NPC2 proteins and a Sterol Sensing Domain (SSD) in NPC1 sequences. Position of the transmembrane domains of NPC1 was predicted by the TMHMM tool (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>). Isoelectric points of deduced AvNCP2 protein sequences were calculated using the ExPASy Compute pI/MW tool (http://web.expasy.org/compute_pi/).

Protein sequences were aligned using the multiple sequence alignment program MAFFT (<http://www.ebi.ac.uk/Tools/msa/mafft/>) and the Blosum62 matrix, and then optimized manually. These alignments were subjected to ProTtest3.0 software (Darriba *et al.*, 2011) to determine the

substitution model that best suited the alignments. These alignments were then used to calculate a 1,000 bootstrap replicate phylogenetic tree (both maximum likelihood and neighbor joining methods) using the phyML3.0 software (Guindon and Gascuel, 2003).

Statistical analyses

Differences in gene expression between epidermal and gastrodermal tissue layers were analyzed using the non-parametric Mann-Whitney U (rank sum). *Symbiodinium* quantification and differential expression of AvNPC genes during the hyperthermal stress were tested using the global non-parametric test of Friedman with Dunn's multiple-comparison post-hoc test. For these analyses, a p-value <0.05 was considered statistically significant. All the analyses described above were conducted using the software GraphPad InStat Version 3 (GraphPad Software, San Diego, CA).

Protein extraction

Gastrodermal (symbiont-containing tissue) and epidermal cell layers were separated as described previously (Richier et al., 2006). Samples were homogenized in an extraction medium containing 50 mM phosphate buffer (pH 7.8) and 10µg/ml protease inhibitor cocktail (Sigma). Homogenates were centrifuged at 1500×g for 3 min to pellet *Symbiodinium* cells. The animal fractions contained in the supernatant were centrifuged again at 4000×g for 3 min to ensure complete symbiont elimination. *Symbiodinium* fractions were washed, centrifuged at 4000×g for 3 min twice to remove animal contaminants and re-suspended in 200µl final extraction medium. Aliquots of these extracts were used for transmission electron microscopy and the remaining part was sonicated (6×10 s for animal fractions and 8×10 s for *Symbiodinium* fractions) and then centrifuged at 13 000×g for 5 min. Supernatants containing the cytosoluble protein extracts were frozen and kept at -80°C until further analyses. Protein concentrations were determined by the Bradford method (Bio-Rad Protein Assay Dye Reagent Concentrate, Sigma).

Transmission electron microscopy

The different *Symbiodinium* fractions were fixed with 2.5 % glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4)/artificial sea water for 2h at room temperature, then washed with 0.1M cacodylate buffer (pH 7.4) and postfixed with 1% osmium tetroxide in cacodylate buffer containing

1% potassium ferrocyanide. After dehydration in acetone, samples were embedded in Epon resin. Ultrathin sections (70-80 nm) were contrasted conventionally using uranyl acetate followed by lead citrate and observed with a JEOL JEM 1400 transmission electron microscope fitted with a CCD camera (Morada, Olympus SIS). At least three independent experiments were examined for each condition.

Antibody production

A custom-made anti-AvNPC2-d antibody was generated against the polypeptide GKISLTLQ. The polypeptide was conjugated to KLH and used to immunize two rabbits. The rabbits were initially immunized with 300 µg of KLH conjugated polypeptide, followed by 5 boosts of 300 µg at 2-week intervals. The rabbit serum exhibiting the highest immunoreactivity against peptide antigen assessed by ELISA was collected on day 128 and was purified by an affinity column containing 5 mg of peptide antigen (AgroBio, France).

Western Blot analysis

Protein samples (5 µg) were separated by SDS-PAGE in a 15% gel and then transferred onto a PVDF membrane using a semi-dry blotting system (Bio-Rad). After overnight blocking (in 5% non-fat dry milk), the membrane was first incubated with the AvNPC2-d antibody (1:2 000 in PBS, 0.1% Tween 20 with 1% skim milk) for 1h at room temperature. Blots were then washed and incubated in a 1:5 000 dilution of HRP-conjugated goat anti-rabbit IgG secondary antibody solution (Bio-Rad). Visualization of the immunoreactive bands was performed using a chemiluminescence substrate (Western Lightning Chemiluminescence reagent Plus, Perkin Elmer LAS, Inc.) and exposure to X-ray film (BioMax Light, Kodak).

Immunofluorescence microscopy

Tentacles of symbiotic *A. viridis* specimens were fixed overnight in 4% paraformaldehyde. Cryosections (12 µm) were incubated in PBS containing 0.1% Triton X-100 (PBT), then blocked for 2 hr in PBT containing 1% BSA. Primary antibody (1:50, rabbit anti-Av NPC2-d antibody) was incubated overnight at 4°C and secondary antibody (anti-rabbit Alexa 546 conjugated (1:500; Thermo Fisher Scientific Inc.) for 2 hr at room temperature. Fluorescence images were acquired using a Zeiss LSM 710 confocal laser-scanning microscope.

Results

NPC2-d – a symbiosis-associated gene – arose from gene duplication in anthozoans

AvNPC2-d, which is homologous to the human Niemann-Pick type C2 gene, is one of the most upregulated genes found in the gastrodermal cells of symbiotic sea anemones (Ganot et al., 2011). A closer investigation of an *A. viridis* cDNA library (Sabourault et al., 2009b) allowed us to find ESTs for at least three other NPC2 paralogs in the *A. viridis* transcriptome (AvNPC2-a, b, c and d). The full length genomic and cDNA sequences of the four genes were cloned and sequenced. Open reading frames (ORFs) of the four genes encoded proteins of 149 to 159 amino acids. The general features of these proteins are listed in Table 1.

Name	gDNA ¹	Protein ²	Mw ³	pI	SP ⁴	ML domain ⁵	EMBL ID ⁶
AvNPC2-a	2178	154	16781.52	5.68	1-19	20-154	HG670305
AvNPC2-b	480	159	17586.63	5.29	1-23	24-157	HG670302
AvNPC2-c	450	149	16216.83	6.24	1-19	20-149	HG670303
AvNPC2-d	450	149	16167.90	7.51	1-19	20-149	HG670304

Table 1 Overview of general features of AvNPC2 proteins ¹ gDNA sequence length (bp); ²protein sequence length (AA); ³molecular weight (Da); ⁴signal peptide position (AA); ⁵ML domain position (AA); ⁶European Nucleotide Archive (EMBL) accession number

A

CLUSTAL format alignment by MAFFT (v7.130b)

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AvNPC2-a  MAKFFLIACMLYVLSL-----AG-AEVVDVDDCSTGGKKGIEKLEIIIPCTQPQQLK 54
HsNPC2    M-RFLAATFLLLALST-----AAQAEPVQFKDC---GSVDGVIEKVNVSFCTQPQQLSK 51
AvNPC2-c  MKFLVLLLCIQTIWLSL-----SE-ARNLSPEDC---GSKVGLVDFDLSPCSQDPCFLKR 51
AvNPC2-d  MKFLVLLLCIQTIWLSL-----SE-ARNLSPEDC---GSKVGLVDFDLSPCSQDPCFLKR 51
AvNPC2-b  M-LLIHACGFVLAFLVLPFSTDAKVVGFADCGSLRPFVEVVVQDLTPCDSDPCEVHV 58
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          :          :          :          :          :          :          :          :          :          :

AvNPC2-a  GSKVQIKVTFVPHNEDLTEATSVVHGIEGGFPVFPPLPNSNCCDSGLTCLKAGQKYVY 114
HsNPC2    GQSYSVNVTFT-SNIQSKSSKAVVHGILMGVFPVFPFPEPDGCK-SGINCPIQKDKTYSY 109
AvNPC2-c  GSNATGTVTFI-PSEEVTSKVVYAVIIGLIPVPLPLPNQNGKGFGLTCLPKSGKQYEL 110
AvNPC2-d  GSNATGTVTFI-PSEEVTSKVVYAVIIGLIPVPLPLPNQNGKGFGLTCLPKSGKQYEL 110
AvNPC2-b  GDNVTAKITFIPEDIVQEGLEFVLAIFASGERLRWLPKPFVCEHDHLKCLKAKEPATA 118
          *  ..  : * *  :          :          :          :          :          :          :          :          :

AvNPC2-a  TSALDVKSEYPAIKVVVWEMQDK-DNNDVFCFKVATQIVS-- 154
HsNPC2    INKLPVKSEYPSIKLVVEWQLQDD-KNQLFCWEIIPVQIVSHL 151
AvNPC2-c  VFSNSVDSRLPAGTFTLKGELKDD-EGNNIYCFEVSLTLQ-- 149
AvNPC2-d  VFSHSIDSTFPAGTFTLKGELKDD-EGNNIYCFEVSLTLQ-- 149
AvNPC2-b  IIQTSIEKIPGNYKIVAQVIMADTVQRMNVFCFRMFVQVTV-- 159
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B

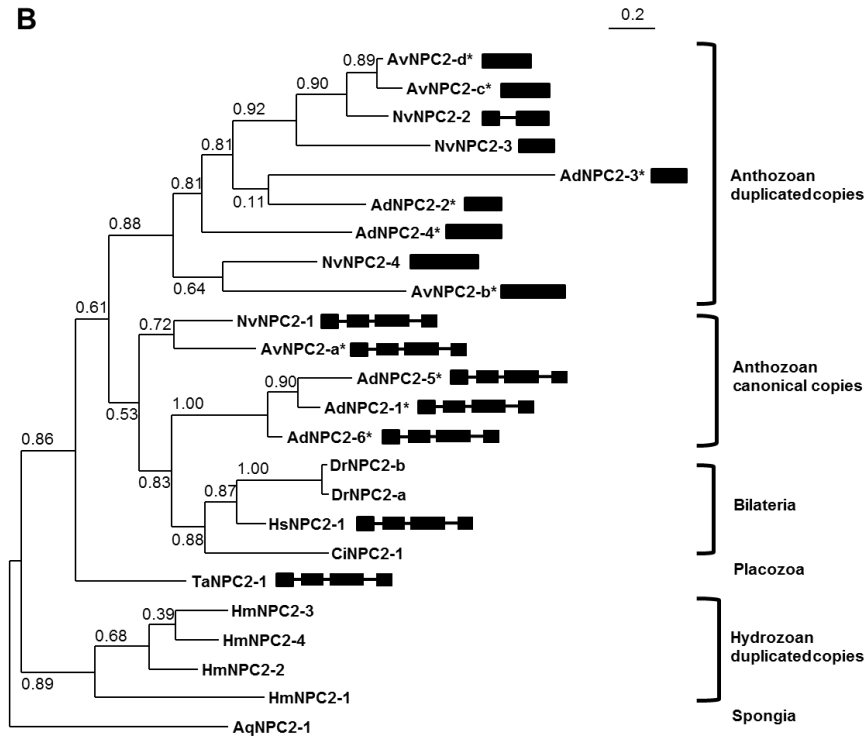


Figure 1 (A) Amino acid sequence alignment of *A. viridis* NPC2 proteins and the human NPC2 protein (MAFFT program). Signal peptides are underlined. (B) Phylogenetic tree of NPC2 proteins from various metazoan taxa. The phylogenetic tree was constructed using the maximum likelihood method and the WAG+I+G substitution model ($I=0.057$ and gamma factor = 5.831). Species that host *Symbiodinium* sp. are highlighted by *. A schematic genomic structure of NPC2 genes is given for *Anemonia viridis*, *Nematostella vectensis*, *Acropora digitifera*, *Homo sapiens* and *Trichoplax adhaerens*. Boxes represent exons. Sequence accession numbers are provided in Supplementary Table S1. Hs: *Homo sapiens*, Dr: *Danio rerio*, Ci: *Ciona intestinalis*, Ad: *Acropora digitifera*, Nv: *Nematostella vectensis*, Av: *Anemonia viridis*, Hm: *Hydra magnipapillata*, Ta: *Trichoplax adhaerens*, Aq: *Amphimedon queenslandica*.

Alignment of the deduced amino acid sequences (Fig. 1A) showed that these proteins exhibited from 62.7% similarity (between AvNPC2-a and AvNPC2-d) to 91.9% similarity between AvNPC2-c and AvNPC2-d, although their theoretical isoelectric point was very different (6.24 and 7.51 for AvNPC2-c and AvNPC2-d, respectively). AvNPC2 proteins all displayed the same organization as the human NPC2, i.e. a peptide signal and the MD-2-related lipid-recognition (ML) domain (Table 1).

Since sequence annotation confirmed that all were homologous to the vertebrate NPC2 gene, we searched for similar sequences in metazoans with a complete genome. NPC2 was also duplicated in the following cnidarian genomes: four copies in *Nematostella vectensis* (non-symbiotic sea anemone) and in *Hydra magnipapillata* (non-symbiotic freshwater hydra), and six genes in *Acropora digitifera* (symbiotic coral). Phylogenetic analyses showed that only one actinarian NPC2 protein clustered with the group of canonical NPC2 proteins found in bilaterians (Fig. 1B). Another cluster was formed by other anthozoan sequences, including the symbiosis-associated isoform AvNPC2-d, suggesting that they arose from at least one duplication event. Moreover, analysis of the genomic organization of *A. viridis* NPC2 sequences confirmed the duplication hypothesis, i.e. AvNPC2-a displayed the same genomic organization as the canonical NPC2 gene, whereas all the extra gene copies were intronless, at least in *A. viridis* (Fig. 1B). However, it seems that the NPC2 canonical gene copy has also been duplicated independently in the coral *A. digitifera*.

Isolation and characterization of the Niemann-Pick C type 1 homolog in A. viridis

Since the NPC2 protein acts in tandem with the NPC1 transmembrane protein to promote intracellular trafficking of sterols in vertebrates, we characterized the *A. viridis* NPC1 homolog. Starting from the *A. viridis* EST collection, we acquired a complete 5009 bp cDNA sequence using RACE-PCR. The AvNPC1 deduced amino acid sequence (1357 residues) contained 13 predicted transmembrane regions and three external loops. Sequence annotation using InterProScan analysis showed that this protein displayed a peptide signal (1-19 AA) and a sterol sensing domain (SSD) at positions 681 to 834, similar to the human NPC1. The AvNPC1 amino acid sequence was aligned with homologous NPC1 sequences that were retrieved from diverse complete metazoan genomes, including cnidarians. Comparison of these sequences revealed that the membrane-spanning regions and the SSD domain have been conserved throughout metazoan evolution (Fig. S1). Phylogenetic

analyses showed that all the cnidarian NPC1 protein sequences clustered together and formed a sister group to the bilaterian ones, for which NPC1 and NPC1L1 arose from a duplication event (Fig. 2).

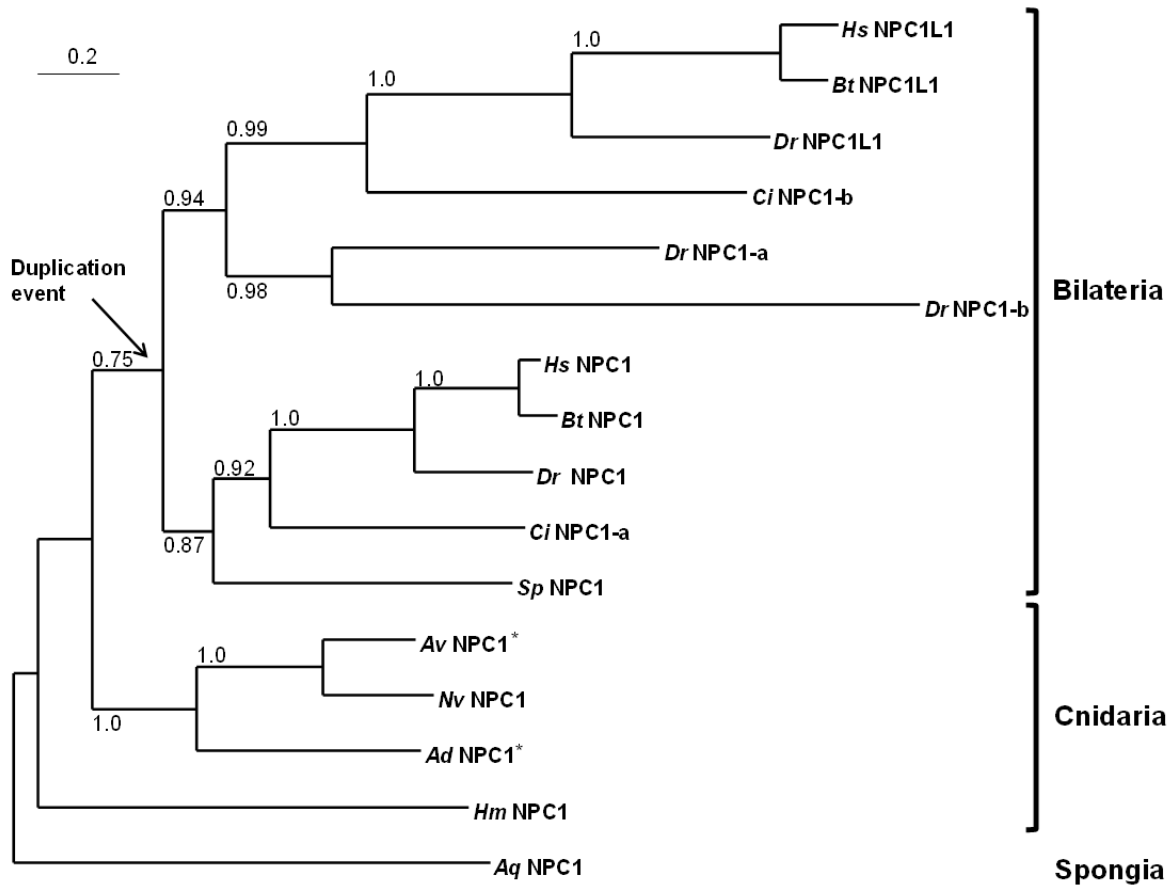


Figure 2: **Phylogenetic tree of NPC1 proteins retrieved from metazoans with complete genomes.** The phylogenetic tree was constructed using the maximum likelihood method and the LG+I+G substitution model ($I=0.1$ and gamma factor = 1.266). Species that host *Symbiodinium* sp. are highlighted by *. Sequence accession numbers are provided in Supplementary Table S1. Hs: *Homo sapiens*, Bt: *Bos taurus*, Dr: *Danio rerio*, Ci: *Ciona intestinalis*, Sp: *Strongylocentrotus purpuratus*, Ad: *Acropora digitifera*, Nv: *Nematostella vectensis*, Av: *Anemonia viridis*, Hm: *Hydra magnipapillata*, Aq: *Amphimedon queenslandica*.

Tissue-specific expression of A. viridis NPC transcripts

To investigate the spatial expression patterns of NPC transcripts, epidermal and gastrodermal tissues were separated from tentacles of symbiotic (control conditions) sea anemones. Transcript levels, which were quantified using qRT-PCR, showed that AvNPC2-b and AvNPC2-c were expressed less than AvNPC2-a and AvNPC2-d in total tentacle extracts (Fig. 3). For example, AvNPC2-b was 10-fold less expressed than AvNPC-a and 4.4-fold less expressed than AvNPC2-d. When comparing tissue specificity in control sea anemones, we noticed that the expression of AvNPC1, AvNPC2-a, and AvNPC2-d genes was significantly higher in gastrodermal cells than in the epidermis ($p < 0.05$). Interestingly, the AvNPC2-d gene exhibited the highest gastrodermal/epidermal expression ratio (7.2-fold, Fig. 3).

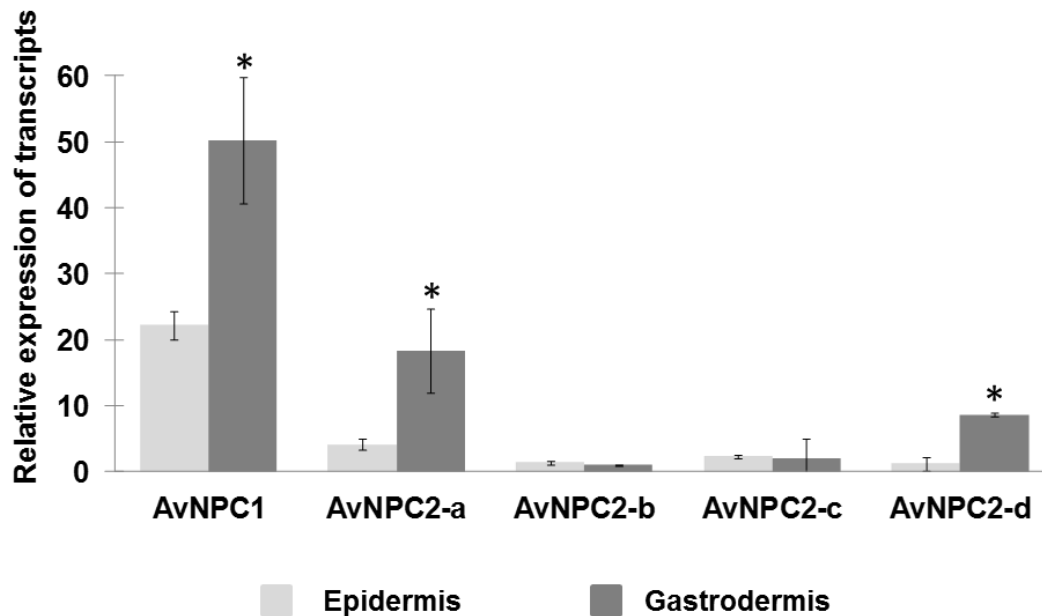


Figure 3: **Relative gene expression of AvNPC1, AvNPC2-a, AvNPC2-b, AvNPC2-c and AvNPC2-d transcripts in the epidermis and gastrodermis of symbiotic sea anemones.** Results are normalized to the expression of the control genes RCC2 and COP- γ . Statistical analyses using the Mann-Whitney U test revealed significant differential gene expression between epidermal and gastrodermal tissue layers in AvNPC1, AvNPC2-a and AvNPC2-d ($p = 0.03$). Error bars represent standard errors of the means (\pm SEM).

We then used the same approach to monitor the expression pattern of these genes in sea anemones subjected to a heat-induced bleaching. First, to verify that the hyperthermal stress (+10°C) caused *A. viridis* symbiosis breakdown via loss of dinoflagellates (bleaching), symbiont densities of the stressed specimens (29°C) were compared to those under the reference treatment (19°C). The dinoflagellate density did not significantly change during the 22 days in reference specimens (Fig. S2). In contrast, exposure to 29°C temperatures induced a significant decrease in symbiont density in stressed specimens, leading to a loss of 60% of symbionts after 22 days (Fig. S2).

Hyperthermal stress did not have a significant effect on the expression of AvNPC2-b and AvNPC2-c genes in both tissues (epidermis and gastrodermis, Fig. S3). AvNPC2-a expression was modulated in the epidermis, where we measured a significantly higher expression level at day 10 compared to day 1 (*post hoc* test of Dunn, $p < 0.05$, Fig. 4). AvNPC2-d gene expression was strongly downregulated in the gastrodermis at day 1 (7.1-fold, $p < 0.05$), remained low for 4 days, and increased back to its basal level after 10 days (Fig. 4). AvNPC1 also displayed slightly reduced expression only in the gastrodermis during the first days of stress ($p < 0.05$, Fig. 4). The significant reduction in AvNPC2-d in gastrodermal cells after 1 day of hyperthermal stress is thus consistent with the hypothesis that AvNPC2-d may regulate symbiotic interactions.

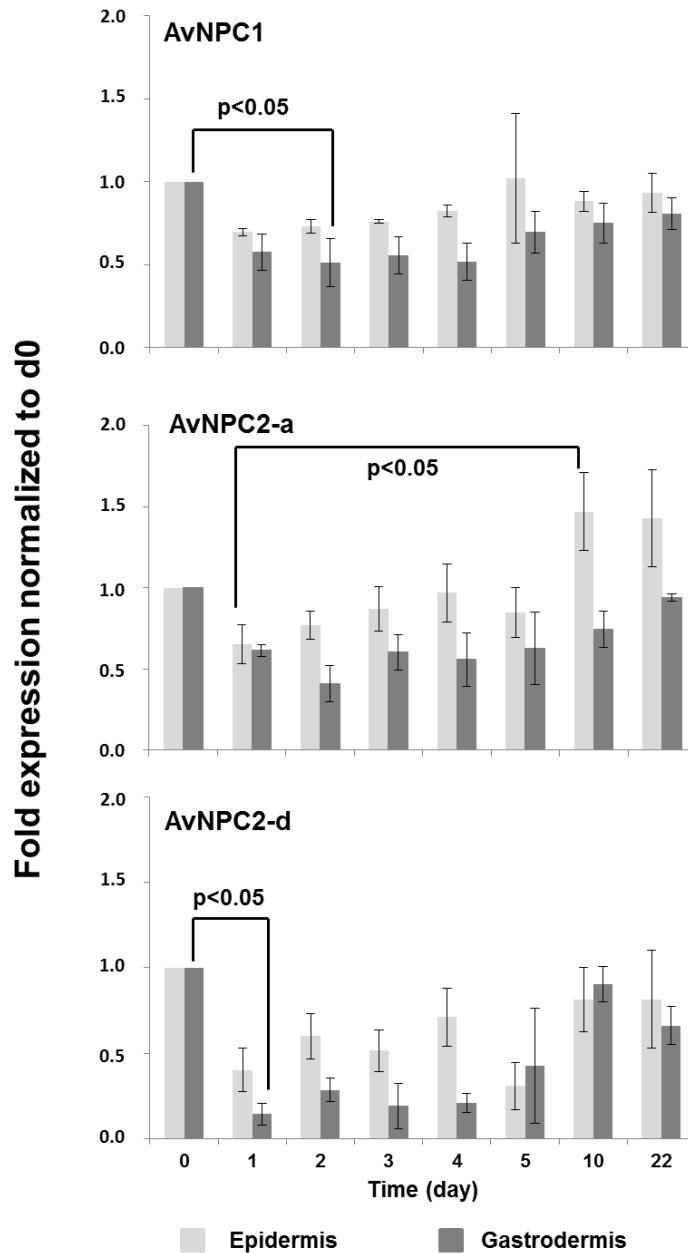


Figure 4: Relative gene expression of AvNPC1, AvNPC2-a and AvNPC2-d transcripts in the epidermis and gastrodermis tissue layers of sea anemones in response to hyperthermal stress (+10°C). Gene expression, normalized to RCC2 and COP-γ, is given relative to day 0. Statistical analyses were performed using the Friedman global test followed by the post-hoc test of Dunn. Error bars represent standard errors of the means (±SEM).

The AvNPC2-d protein is associated with symbiosomes

The NPC2 protein localizes in late endosomes/lysosomes in vertebrates. As the cnidarian symbiosome is derived from phagosomes (Davy et al., 2012c), we hypothesized that one or more of the four AvNPC2 proteins could localize to the symbiosome lumen. In order to test this hypothesis, we developed an anti-AvNPC2-d antibody and we first tested its reactivity against symbiotic and bleached specimens by Western blotting (Fig. 5A). This antibody recognized a single band of approximately 20 kDa only in samples containing symbionts (tentacle and gastrodermis), but not in the aposymbiotic extract (bleached). We further screened different tissue fractions (Fig. 5B): freshly isolated *Symbiodinium* (with the symbiosome membrane, FIS), tentacle animal fraction (TAn) and gastrodermis (G) experimentally-deprived of symbionts, animal epidermis (w/o symbionts), and cultured *Symbiodinium* (from *A. viridis*). Only the sample containing symbiosomes (symbionts surrounded by the symbiosome membrane, lane FIS) showed the presence of AvNPC2-d. Furthermore, this specific labeling could be eliminated by pre-adsorption of the antibody with the peptide antigen (Fig. 5C).

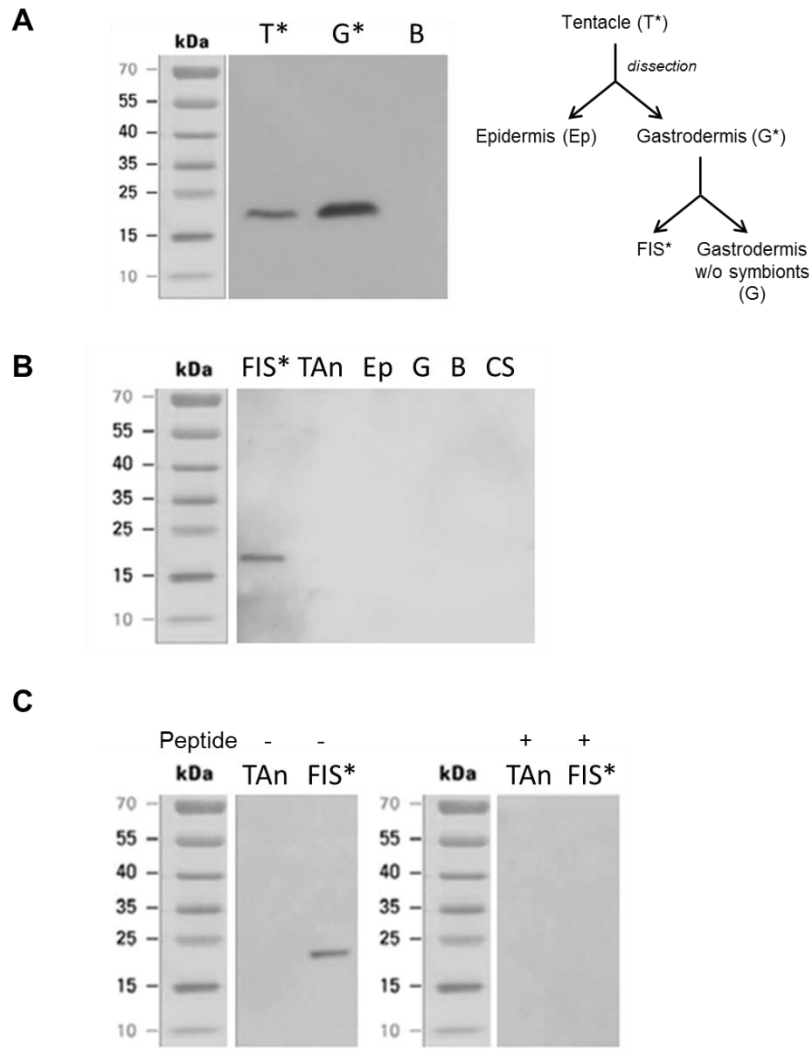


Figure 5: **Western blot analyses with anti-AvNPC2-d antibody.** (A) *A. viridis* tissue protein extracts (20 μ g) from symbiotic (lanes T* and G*) or aposymbiotic specimens (lane B) were probed with the rabbit anti-AvNPC2-d antibody. Lane T, total tentacle extract (epidermis and gastrodermis containing symbionts); Lane G, gastrodermis fraction containing symbionts; Lane B, total tentacle protein fraction extracted from an aposymbiotic (bleached) specimen of *A. viridis*. * indicates the presence of symbionts in the protein extract. (B) Animal and/or dinoflagellate fraction protein extracts (5 μ g). Lane FIS, freshly isolated *Symbiodinium* (symbiosomes) extracted from tentacles. Lane TAn, total tentacle animal fraction (epidermis and gastrodermis) deprived of symbionts. Lane Ep, tentacle epidermis fraction. Lane G, tentacle gastrodermis fraction deprived of symbionts. Lane B, total animal protein fraction extracted from an aposymbiotic specimen of *A. viridis*. Lane CS, protein extracted from cultured *Symbiodinium*. * indicates the presence of symbionts in the protein extract. (C) Competition experiment with the peptide antigen GKISLTQ (200 ng/ml). Lanes TAn, total tentacle animal fraction (epidermis and gastrodermis) deprived of symbionts (20 μ g); Lanes FIS, freshly isolated *Symbiodinium* (symbiosomes) extracted from tentacles (5 μ g). On the right panel, the antibody was pre-absorbed with the peptide antigen (peptide +). * indicates the presence of symbionts in the protein extract.

Transmission electronic microscopy confirmed the presence of a perisymbiotic membrane only in samples containing *Symbiodinium*. These pictures showed that symbionts were surrounded by multilayered symbiosome membranes when observed *in hospite* within the gastrodermis (Fig. 6A), in isolated symbiotic gastrodermal cells (Fig. 6B) and in a freshly isolated *Symbiodinium* preparation (FIS, Fig. 6C). On the contrary, the symbiosome membrane was absent from cultured *Symbiodinium* (Fig. 6D).

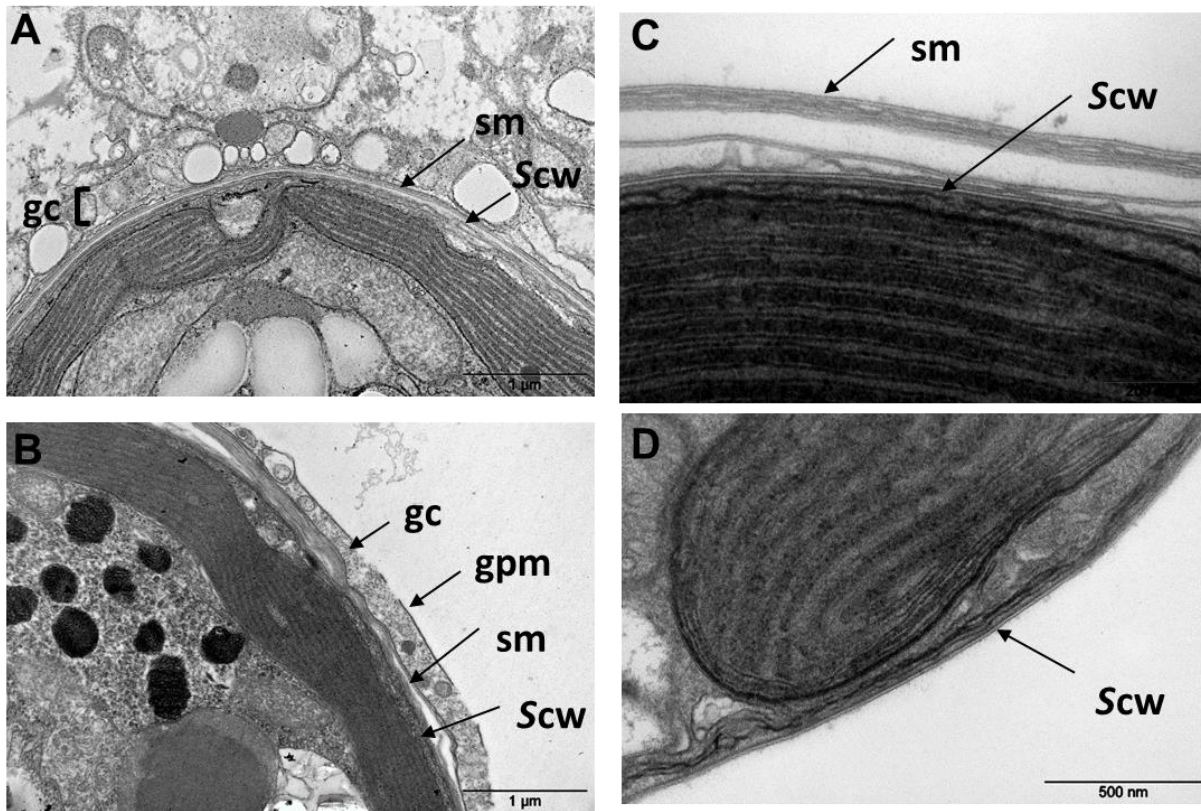


Figure 6: Transmission electron micrographs of *in hospite* and free-living *Symbiodinium* cells. Gastrodermal tissue (A), isolated symbiotic gastrodermal cells (B) and freshly isolated *Symbiodinium* (C): from the outer to the inner membrane, we observed the plasma membrane of gastrodermal cells (gpm), multilayered symbiosome membranes (sm) and the *Symbiodinium* cell wall (Scw); gc, gastrodermal cell cytoplasm. (D), Cultured *Symbiodinium* with a single *Symbiodinium* cell wall (Scw).

Since AvNPC2-d was present in isolated symbiotic gastrodermal cells and in FIS fractions, but not in cultured *Symbiodinium*, we proposed that AvNPC2-d was dependent on symbiosome maintenance. To confirm this hypothesis, we used the anti-AvNPC2-d antibody in fluorescent immunolocalization experiments. Incubations without antibodies, without primary antibody or with preimmune serum allowed the detection of basal autofluorescence of the tissue (endogenous GFPs and symbiont chlorophylls, Fig. S4). Images of tentacle cross sections (Fig. 7A) clearly indicated that AvNPC2-d was only present in the gastrodermis of symbiotic sea anemones and was surrounding the symbionts, showing that AvNPC2-d was associated with the symbiosome.

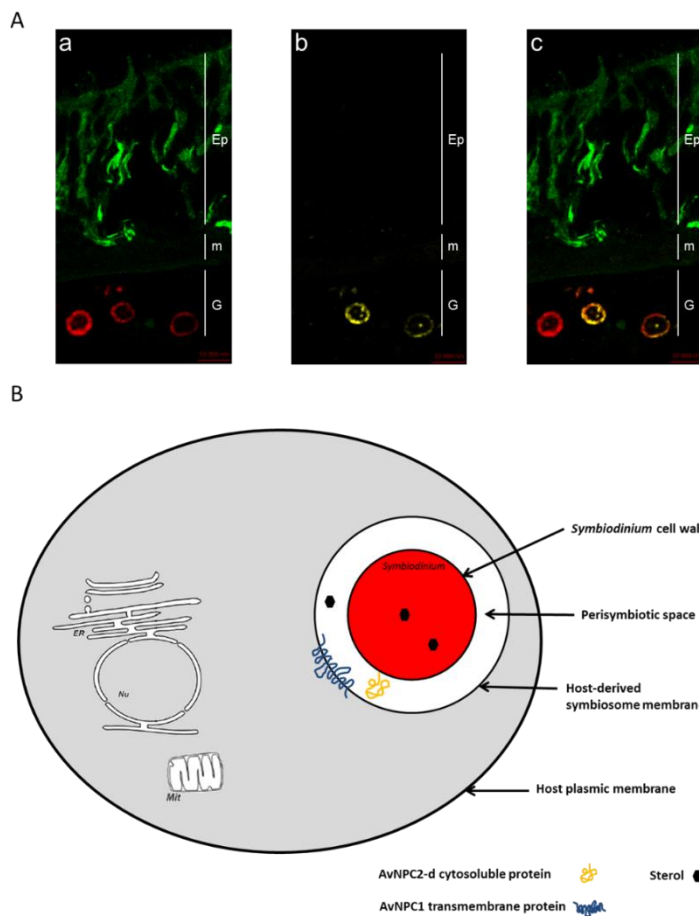


Figure 7: Immunolabeling showing the AvNPC2-d protein (yellow) surrounding dinoflagellate symbionts (red) within the gastrodermis of a tentacle cross section. **a**, basal autofluorescence of the tissue (endogenous cnidarian GFPs in green and dinoflagellate chlorophylls in red). **b**, specific labeling of AvNPC2-d. **c**, merged images of **a** and **b**. Ep, epidermis layer; m, mesoglea; G, gastrodermis layer. **(B)** Model of the subcellular location of AvNPC proteins and their role in endosomal/lysosomal transport of symbiosis-derived sterols in a symbiotic gastrodermal cell.

Discussion

Cnidarians express at least four NPC2 genes

Here, we described the NPC repertoire in several anthozoans (*A. viridis*, *N. vectensis* and *A. digitifera*). Searches in sequence databases identified several NPC2 paralogs in cnidarians, while there was only one gene copy in the placozoan *Trichoplax adhaerens*, or sponges and chordates (Fig. 1B). No NPC genes (both NPC1 and NPC2) have been identified in unicellular eukaryotes or plants. Searches in endosymbiotic *Symbiodinium* sequence databases (ESTs, NGS datasets and the *Symbiodinium minutum* genome), as well as PCRs on DNA extracted from cultured symbionts (data not shown), confirmed that all of these NPC sequences were of *A. viridis* origin. Phylogenetic analyses identified one canonical NPC2 gene (AvNPC2-a), whereas the other gene copies (AvNPC2b to d) have been independently duplicated in anthozoans and hydrozoans (Fig.1B). Interestingly, canonical NPC2 anthozoan genes have the same genomic organization as their chordate orthologs, whereas the other anthozoan duplicated copies are intronless (Fig. 1B). This suggests that these duplicated genes have been generated by retropositions of an NPC2 mRNA intermediate. The well-accepted principle is that after duplication each copy can evolve independently, leading to functional novelties (Conant and Wolfe, 2008).

AvNPC2-d mRNA and protein expression patterns correlate with cnidarian adaptation to dinoflagellate endosymbionts

The AvNPC2-d duplicate gene is one of the most significantly upregulated genes found in the symbiotic sea anemone *A. viridis* (Ganot et al., 2011). Similar results were found in *Aiptasia pulchella* and *Aiptasia pallida* (Kuo et al., 2010; Lehnert et al., 2013). Here, we demonstrated that AvNPC2-d was more expressed in the gastrodermis, i.e. the tissue hosting dinoflagellate symbionts, than in the epidermis (Fig. 3). Moreover, during a bleaching event, AvNPC2-d gene expression is drastically downregulated at the onset of symbiosis breakdown, especially in the gastrodermis (Fig. 4). Together, these findings indicate that the AvNPC2-d protein is involved in the symbiotic interaction.

Since NPC2 is an endosomal protein in vertebrates, we hypothesize that it may be localized in the phagosome derived symbiosome of *A. viridis* (Fig. 7B). In total protein extracts, Western blot experiments using a specific anti-AvNPC2-d antibody recognized an immunoreactive band of

approximately 20 kDa (Fig. 5), which was markedly reduced after antibody pre-adsorption with the peptide antigen (Fig. 5C). This result is compatible with the theoretical AvNPC2-d molecular weight (16.2 kDa) and post-translational modifications, such as glycosylations described in human NPC2 (Liou et al., 2006). We further screened protein extracts from epidermis (non-symbiotic tissue), gastrodermis without symbionts (removed by centrifugation), symbiosomes (symbionts surrounded by the perisymbiotic membrane), whole tentacles from non-symbiotic specimens (bleached sea anemones), and cultured symbionts. Interestingly, we found an immunoreactive band only in samples containing symbiosomes (Fig. 5A, 5B). Immunolocalization experiments on tentacle sections using the anti-AvNPC2-d antibody clearly demonstrated that the AvNPC2-d protein colocalized with the symbiont periphery within the gastrodermis (Fig. 7A). These results strongly support a role for AvNPC2-d in symbiosis homeostasis.

In addition to NPC2 genes, there are several other examples of genes involved in the symbiotic interaction that were also duplicated in cnidarian genomes. In each tested case, one of the duplicated copies was upregulated in the symbiotic (as opposed to the aposymbiotic) condition and showed preferential gastrodermal expression. These include genes involved in recognition and innate immunity, such as scavenger receptors (Ganot et al., 2011; Rodriguez-Lanetty et al., 2006), C-type lectins (Grasso et al., 2008; Sunagawa et al., 2009b), C3 complement (Ganot et al., 2011; Sunagawa et al., 2009b), dermatopontin (Ganot et al., 2011; Moya et al., 2012), and Sym32 (Ganot et al., 2011; Moya et al., 2012; Reynolds et al., 2000), as well as genes involved in metabolic exchange and trafficking, such as carbonic anhydrases (Ganot et al., 2011; Grasso et al., 2008; Moya et al., 2012; Weis and Reynolds, 1999), and Rhbg solute transporters (Ganot et al., 2011; Sabourault et al., 2012). The AvNPC2-d duplicate may therefore be considered a novel key player in symbiosis maintenance.

Indeed, new duplicates are a main source of genome innovation and may evolve under positive selection. This process, which is referred to as neofunctionalization of new genes, results in rapid changes in proteins encoded by new genes, further leading to a gain of function. An alternative hypothesis is the occurrence of subfunctionalization, and especially specialization, where paralogs have evolved particular adaptations to different tissues or different developmental stages through a neutral mutation process (Rastogi & Liberles 2005). Many recent examples of adaptive gene duplications to various environmental conditions have been described in different species (reviewed in Kondrashov 2012). Interestingly, extensive NPC2 duplications also occurred

in the Hexapoda lineage. For example, there are eight NPC2 genes in *Drosophila melanogaster*. NPC2a is the *Drosophila* ortholog of the vertebrate NPC2 gene and displays the broadest expression pattern, whereas the other NPC2 gene copies are either not detectably expressed or expressed in restricted locations (Huang et al., 2007). Thus, both in insects and in cnidarians, NPC2 genes were duplicated and maintained (*A. digitifera* and *A. viridis* diverged several hundred million years ago) suggesting neo- or subfunctionalization, likely in sterol trafficking.

Putative biological functions of NPC proteins in symbiotic cnidarians

NPC1 and NPC2 are two cholesterol-binding proteins that act sequentially in the same pathway, transferring unesterified cholesterol out of the late endosomes/lysosomes in vertebrates. Mutations in either the NPC1 or the NPC2 gene result in the fatal inherited Niemann-Pick type C disease (Vance, 2010b). Proper cholesterol metabolism and homeostasis regulation is critical for cell membrane fluidity, cell signaling and steroid hormone biosynthesis in both vertebrates and invertebrates (Chang et al., 2006). Cnidarians are steroid-rich organisms and vertebrate-type sex steroids have been identified in their tissues (Tarrant et al., 2009). Their role in the normal physiology of cnidarians is not clear, but data suggest that some compounds may be produced for chemical defense (Qi et al., 2008). However, cnidarians have not been shown to synthesize sterols, as the genomes available so far lack some of the genes encoding critical enzymes required for *de novo* synthesis (Tarrant et al., 2009). Cnidarians must therefore obtain cholesterol directly from dietary intake or from dinoflagellate sterols that are then converted to cholesterol (Withers et al., 1982). This suggests that cnidarians may have genetic mechanisms that regulate the uptake, trafficking, and conversion of dietary cholesterol in cells. Recent studies suggested that sterols, such as polyunsaturated fatty acids, may first be synthesized by dinoflagellate symbionts and transferred to the animal, where they can be further modified (Treignier *et al.* 2008). NPC proteins may therefore be involved in the sterol acquisition from the symbionts to the cnidarian host. Interestingly, insects, that also possess several NPC2 duplicates, are unable to synthesize cholesterol and depend on sterols in their diet for their proper development and viability. Functional studies in *Drosophila* demonstrated that the regulation of cholesterol uptake and/or trafficking by NPC1, NPC2a and NPC2b is crucial for ecdysteroid biosynthesis (Ryusuke et al., 2011). NPC2 is also involved in chemical communication (lipid semiochemicals), as recently shown in worker ants (Ishida et al., 2014).

Recent discoveries also point towards an additional although completely different role for NPC1, which is required for flavovirus entry (White and Schornberg, 2012). Indeed, NPC1 is able to bind cell surface viral glycoproteins and participates in the delivery of the viral genome into host cells (Carette *et al.* 2011). In addition, *D. melanogaster* NPC2 proteins bind bacterial cell wall components and may function in immune signaling pathways (Shi *et al.*, 2012). Similar results have been found in vertebrates (Schrantz *et al.*, 2007). These observations may give rise to new working hypotheses about the recognition and phagocytosis of dinoflagellate symbionts. For example, *Symbiodinium* cells exhibit a complex surface glycome on their cell walls (Logan *et al.*, 2010a), which could be recognized by host NPC proteins. As vertebrate NPC proteins are expressed in endosomes, cnidarian NPCs may also participate in the maturation of phagosomes and their conservation as symbiosomes. Such a mechanism may be part of the process enabling dinoflagellates to persist in symbiosomes (for review, see Davy 2012).

Conclusion

Overall, the AvNPC2-d expression pattern suggests that this gene is involved in cnidarian symbiosis maintenance. One possibility is that the AvNPC1/AvNPC2-d team simply participates in sterol transfer from dinoflagellate symbionts to host cells, or activates key signaling pathways by regulating sterol trafficking. A more exciting scenario is supported by the emerging picture which suggests that NPC proteins are also involved in pathogen recognition and entry. As such, AvNPC2-d may be specialized for binding the *Symbiodinium* glycolipid surface and may participate in symbiont selection and/or symbiont persistence. The role of NPC proteins in symbiotic cnidarian physiology remains to be investigated in closer detail. This includes localization of AvNPC1 and the four AvNPC2 proteins within the whole body, as well as at the cellular scale, which is currently under way. Moreover, the comparison of NPC expression patterns within aposymbiotic specimens and non-symbiotic species (e.g. *N. vectensis*) will confirm these findings. Identification of AvNPC1 and AvNPC2 endogenous ligands will also help to understand how these proteins may regulate symbiotic interactions.

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Authors' contributions

CS designed the study; VD, FP and CS performed the experiments; VD, PG and CS performed data analyses; VD and CS wrote the manuscript; VD, PG, FP, PF and CS edited the manuscript and all authors approved the manuscript.

Data Accessibility

The GenBank accession numbers for the cloned NPC sequences are as follows: gDNA Av NPC2-a, HG670305; gDNA Av NPC2-b, HG670302; gDNA Av NPC2-c, HG670303; gDNA Av NPC2-d, HG670304; cDNA, AvNPC1, HG670297; cDNA Av NPC2-a, HG670298; cDNA Av NPC2-b, HG670299; cDNA Av NPC2-c, HG670300; cDNA Av NPC2-d, HG670301.

Microarray data have been deposited into the NCBI's Gene Expression Omnibus (GEO) database and are accessible through platform record GPL10546 and GEO SuperSeries accession number GSE22375 and GSE28577. *A. viridis* EST sequences have been deposited into GenBank dbEST ([GenBank:FK719875–FK759813]).

Supplementary data

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AvNPC2-a   MAKFFLIACMLYVLSL-----AG-AEVVDFDDCSTGGKKGGEIEKLEIIPCPTQPCQLKK 54
HsNPC2     M-RFLAATFLLLALST-----AAQAEPVQFKDC---GSVDGVIKEVNVSPCPTQPCQLSK 51
AvNPC2-c   MKFLVLLLLCLQIIWSL-----SE-ARNLSFEDC---GSKVGKLVSFDLSPCSQDPCFLKR 51
AvNPC2-d   MKFLVLLLLCLQIIWSL-----SE-ARKLSFKDC---GSKVGKLVSFDLSPCSQDPCI IKR 51
AvNPC2-b   M--LLIHACGFVVLAFLVLPPISTDAKVVGFACGSLIRPVGEVVQVDLTPCDSDFCVFHV 58
           *  :.      :      : * . : * . *      * : . . : * * : * * :
           :

AvNPC2-a   GSKVQIKVTFVPHNEDLTEATSVVHGEIGGFVFPFPLPNSNCKKDSGLTCPLKAGQKYVY 114
HsNPC2     GQSYSVNVTFI-SNIQSKSSKAVVHGILMGVFPVFPPIPEPDGCK-SGINCPIQKDKTYSY 109
AvNPC2-c   GSNATGTVTFI-PSEEVTSKVVYAYVIIIGLIPVPLPLPNQNGCKGFLTCPLKSGKQYEL 110
AvNPC2-d   GSNATGTVTFI-PSEEVTSKVVYMYAIIIGFIPVPLPLPNTDGCKGYGLTCPLKSGKPDEL 110
AvNPC2-b   GDNVTAKITFIPEDIVQEGLEFLVLAIFASGERLRWPLKNPFVCEDHNLKCPLKAKEPATA 118
           *..  . : * * .      : * : : * : . : * * : : :

AvNPC2-a   TSALDVKSEYPAIKVVVKWEMQDK-DNNDVFCFKVATQIVS-- 154
HsNPC2     LNKLPVKSEYPSIKLVVEWQLQDD-KNQSLFCWEIPVQIVSHL 151
AvNPC2-c   VFSNSVDSRLPAGTVTLKGELKDQ-EGNNIYCFEVSLTLQ--- 149
AvNPC2-d   VFSHSIDSTFPAGTVTLKGELKDQ-EENNIFCGKISLTLQ--- 149
AvNPC2-b   IIQTSIEKIPGNYKIVAQVYMADTVQRNYVFCFRMPVQVTY-- 159
           :..  . : . : : * . : : * . : :

```

Figure S1: Amino acid sequence alignment of the *A. viridis* NPC1 protein, human NPC1 and NPC1L1 protein (MAFFT program). Signal peptides are underlined and the SSD domain is highlighted in gray.

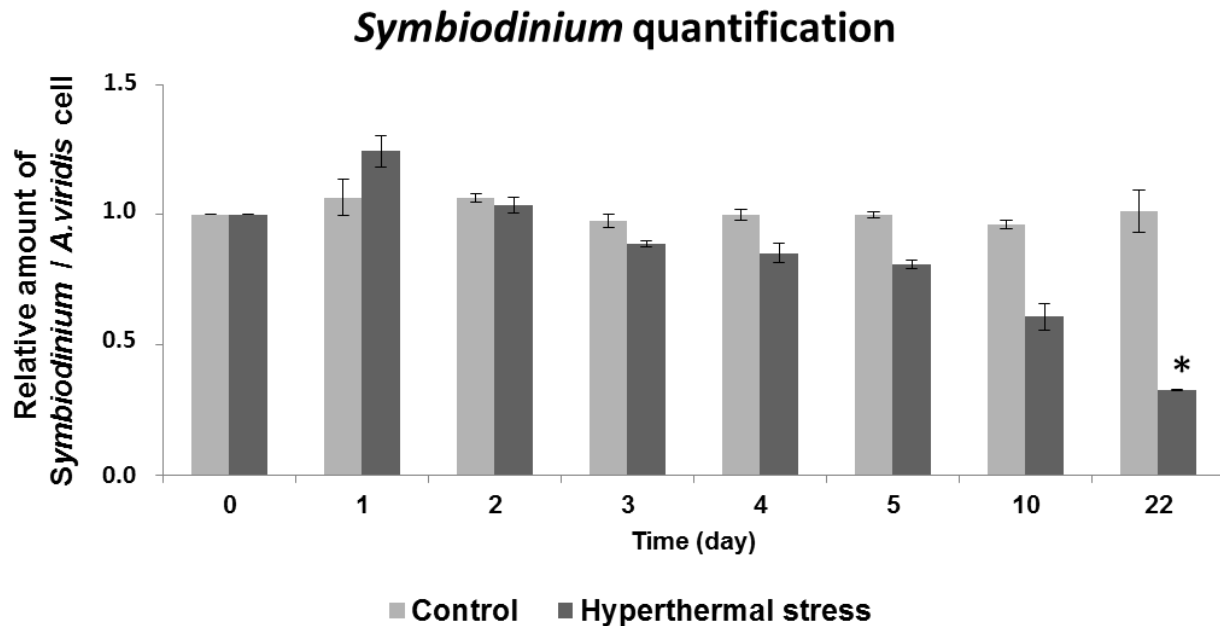


Figure S2: *Symbiodinium* quantification by real-time quantitative PCR measurement of the relative nuclear gene copy number (symbiont to host nuclei) during hyperthermal stress. Histogram shows the average value from *Symbiodinium* ascorbate peroxidase (APX) vs. *A. viridis* COPγ. Error bars represent standard errors (±SEM). Decrease in *Symbiodinium* density during hyperthermal stress was considered very significant at d22, according to the Friedman global test (p=0.45).

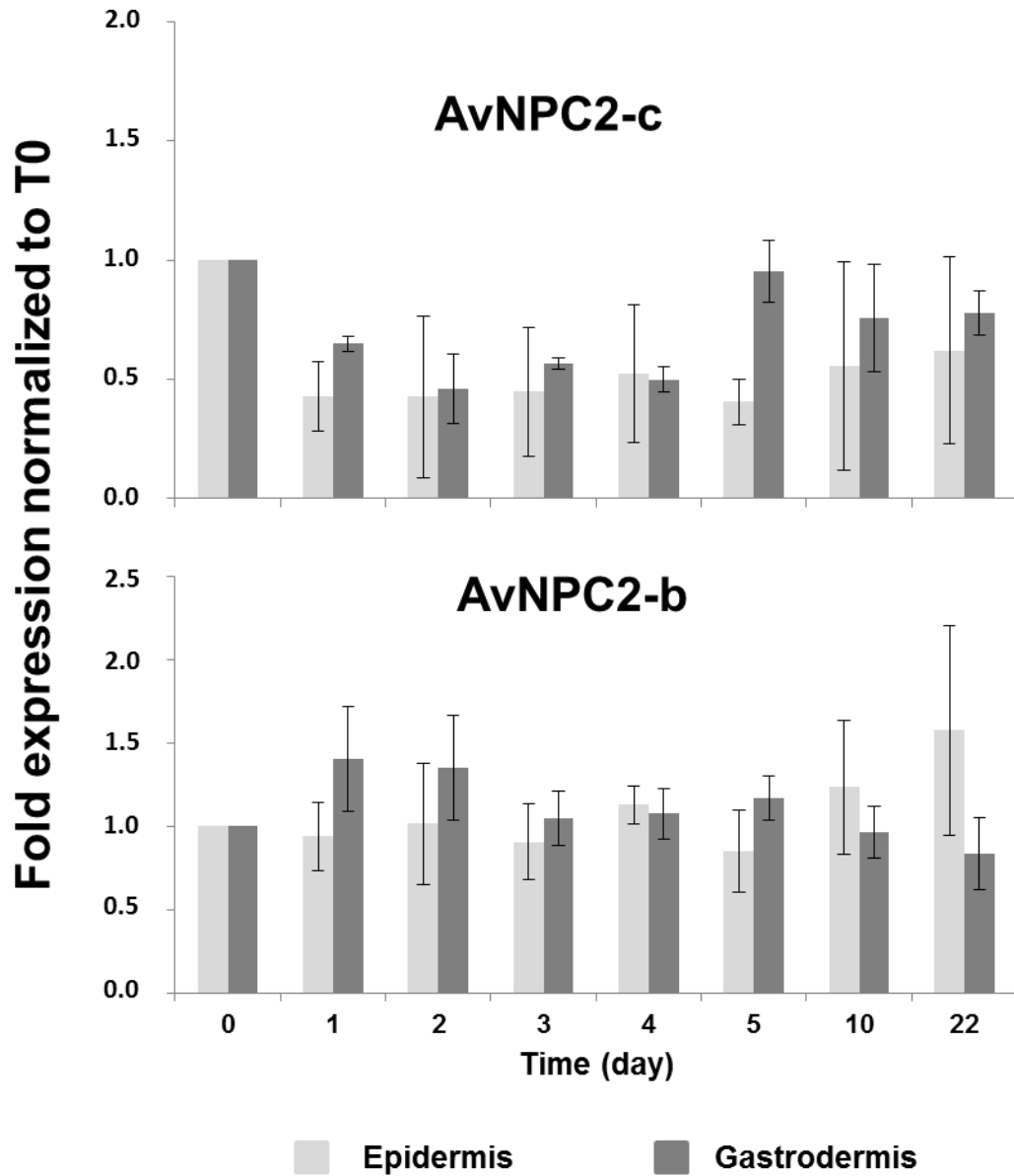


Figure S3: Relative gene expression of AvNPC2-b and AvNPC2-c transcripts in the epidermis and gastrodermis tissue layer of sea anemones in response to hyperthermal stress (+10°C). Gene expression, normalized to RCC2 and COP- γ , is given relative to day 0. Error bars represent standard errors (\pm SEM).

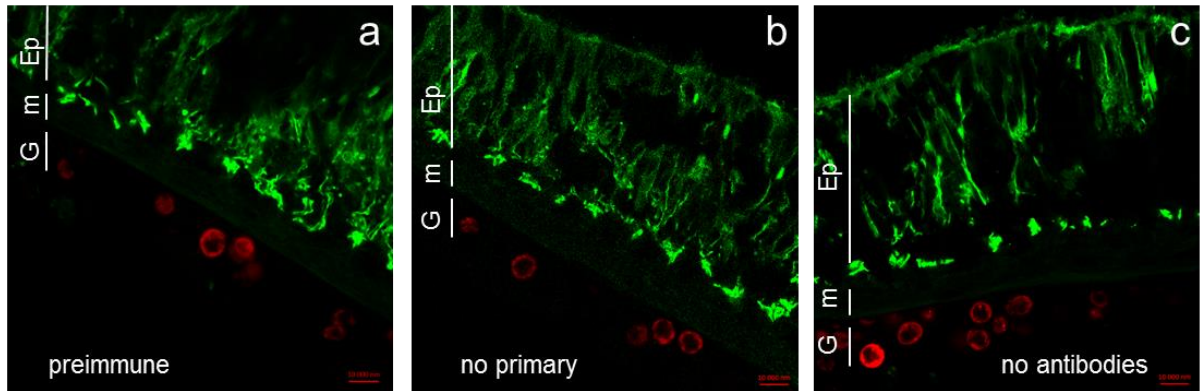


Figure S4: Immunolocalization control analyses. Tissue sections were incubated with the preimmune serum (a, dilution 1:50), without primary antibody (b) or without antibodies (c), a condition that shows the basal autofluorescence of the tissue (endogenous cnidarian GFPs in green and dinoflagellate chlorophylls in red).

3.3.2. **Publication n°4** (en préparation)

Dani, V, Priouzeau, F, Pagnotta, S, Mertz, M, Lacas-Gervais, S, Sabourault, C. NPC1 and NPC2d are expressed at the symbiosome and play a role in the symbiotic interaction between *Anemonia viridis* and *Symbiodinium* spp (en préparation).

Problématique: Quel est le rôle des protéines NPC1 et NPC2 dans l'interaction symbiotique chez *A. viridis* ?

Outils méthodologiques : Déterminer la régulation et la localisation des protéines NPC1 et NPC2 (NPC2a et NPC2d) à l'état symbiotique et lors d'une rupture de symbiose.

Principaux résultats : Localisation des protéines NPC1 et NPC2-d au voisinage des symbiotes. Développement de la protéine NPC1 comme un marqueur de membrane perisymbiotique. Mise en évidence d'une subfonctionnalisation de la protéine NPC2-d par rapport à l'expression de la protéine canonique NPC2-a. Développement de NPC2-d comme un marqueur précoce de blanchissement. Etablissement d'un protocole d'enrichissement des symbiotes isolés possédant ou non la membrane pérисymbiotique.

Perspectives : Déterminer la fonction des protéines NPC1 et NPC2-d par des expériences d'affinité protéine/ligand. Utiliser le protocole d'enrichissement en symbiotes isolés possédant la membrane perisymbiotique pour étudier le protéome de la membrane pérисymbiotique.

NPC1 and NPC2d are expressed at the symbiosome and play a role in the symbiotic interaction between *Anemonia viridis* and *Symbiodinium* spp

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Keywords: cnidarian-dinoflagellate interaction, symbiosis, symbiosome membrane, NPC

Running title: NPC PROTEIN IN CNIDARIA SYMBIOSIS

Abstract

The symbiotic interaction between cnidarians (*e.g.* corals and sea anemones) and the unicellular dinoflagellate *Symbiodinium spp* is triggered by both host-symbiont recognition and metabolic exchanges between the two partners. This intimate partnership control symbiosis stability under normal conditions, but also lead to symbiosis breakdown (bleaching) during stress. At the center of this molecular communication, the perisymbiotic membrane that derived from internalization by the host by a phagocytosis process, still needed to be fully characterized. In this study, we determined the localization and regulation of the sterol-trafficking proteins Niemann-Pick type C (NPC1 and NPC2) in the symbiotic sea anemone *Anemonia viridis* at a protein level. We first (i), establish that AvNPC1 is a biomarker of the perisymbiotic membrane by both immunolocalization on different cnidarian species and immune-gold labelling on *A. viridis* (ii), we developed a protocol to obtain enriched fraction of symbiosome with or without perisymbiotic membrane by applying mechanical stress (iii), we demonstrate that there is a subfunctionalization of the AvNPC2 protein with the canonical copy AvNPC2-a expressed only in the epidermal tissue layer and AvNPC2-d closely associated to symbiosome and (iv), we show that the duplicated copy AvNPC2-d, whose protein expression is drastically reduced in both hyperthermal and menthol induced bleaching, is an invaluable biomarker of bleaching.

Introduction

Despite the ecological importance of coral reefs, the mechanism that modulate the intimate association between the cnidarian host, such as coral and sea anemones, and the unicellular dinoflagellate from the genus *Symbiodinium*, are regulated by yet poorly understood mechanisms of recognition and molecular communication (Davy et al., 2012). The first step in the onset of the symbiosis is the recognition of host-specific *Symbiodinium* by the host (Baker, 2003). This interaction is triggered by glycan motifs expressed at the cell surface of the *Symbiodinium* (Logan et al., 2010b) that allow specific recognition by the host that possess pattern recognition receptors expressed at the cell surface (review in Davy et al., 2012). In this mechanism of symbiont selection, the glycan-lectin interaction has been a specific field of investigation (Jimbo et al., 2000; Jimbo et al., 2005; Jimbo et al., 2013; Kvennefors et al., 2008; Kvennefors et al., 2010; Vidal-Dupiol et al., 2009). The symbionts are then internalized by a phagocytosis process in the gastrodermal tissue layer of the host (Muscatine et al., 1975). The symbiosis maintenance of *in hospite* symbionts then required an arrest of the phagosomal maturation that lead to the conversion of the phagosomes into symbiosomes (Hohman et al., 1982). They are then surrounded by a host origin perisymbiotic membrane that derived from this vesicular endocytosis internalization process (Wakefield and Kempf, 2001b). In stable condition, this multilayer membrane is at the center of trophic exchanges and molecular communication between the two mutualistic partners. The characterization of the symbiosome membranes is primary to decipher the mechanisms implicated in first, the establishment and maintenance of the symbiosis and further metabolic exchanges between the two partners. In this mechanism, the subcellular localization of a fascilin domain protein Sym32, implicated in cell-cell and cell-extracellular matrix interaction, is the only one that has been clearly demonstrated to be associated to the symbiosome membrane by immune-gold labelling (Schwarz and Weis, 2003). As well, Chen and coworkers describe the repertoires of Rab genes involved in the persistence and/or degradation of symbiont *in hospite* (Chen et al., 2003; Chen et al., 2004; Chen et al., 2005; Hong et al., 2009a).

Recently, Peng and coworkers (Peng et al., 2010b) were the first to characterize the symbiotic interface by purification of the perisymbiotic membrane (Kazandjian et al., 2008). This study reveal 17 protein implicated in cell recognition (GPCR-like receptors), cytoskeletal remodelling (Actin), transport (ABC transporter), stress response (HSP), etc ... However, to date,

no transcriptomic or proteomics studies confirmed the localization of neither Sym 32 nor Rab protein at the symbiotic interface, emphasising the lack of a powerful technic to screen the global symbiosome membrane proteome without cross-contamination by host cytoplasmic components.

Meanwhile, multiplication of transcriptomic studies and genome sequencing of symbiotic cnidarian allowed the identification of several key genes putatively implicated in symbiosis initialization and maintenance, metabolic exchanges and nutrients trafficking (Desalvo et al., 2008; DeSalvo et al., 2010; Grasso et al., 2008; Kuo et al., 2004; Kuo et al., 2010; Lehnert et al., 2014; Rodriguez-Lanetty et al., 2006; Sunagawa et al., 2009; Ganot et al., 2011; Moya et al., 2012; Sabourault et al., 2009; Shinzato et al., 2011). In a major parts of these studies, the Niemann-Pick genes (NPC) were pinpointed and recently, several studies have targeted NPC genes as valuable key players in the symbiotic association (Baumgarten et al., 2015; (Baumgarten et al., 2015; Dani et al., 2014; Ganot et al., 2011; Lehnert et al., 2014) and reveal that the cnidarian specific duplicated gene; the endosomal sterol transporter AvNPC2-d; could be involved in the metabolic exchanges between the two partners in interaction with the transmembrane protein AvNPC1 (Dani et al., 2014b).

In this study, we localized at a cellular and subcellular level, the protein AvNPC1, AvNPC2-a and AvNPC2-d in stable symbiotic conditions and during hyperthermal stress. We also characterize NPC protein regulation and localization in the menthol bleaching stress, recently used as a novel fast-bleaching method to obtain “healthy” aposymbiotic specimens (Wang et al., 2012) by inducing rapid autophagy response of the host (Dani et al., submitted). We show that AvNPC1 was clearly localized at the perisymbiotic membrane of *in hospite* symbionts. We then use this marker to validate a simple and speed technical way, adapted from Trautman and coworkers (Trautman et al., 2002) to totally remove symbiosome membranes from freshly isolated *Symbiodinium* from *Anemonia viridis*. We then show a subfunctionalization of the AvNPC2 gene. The canonical AvNPC2-a was epidermal specific and the duplicated AvNPC2-d protein was specifically expressed at the symbiosome and clearly correlated with symbiont presence. These result promote AvNPC2-d as an invaluable bleaching marker in the cnidarian-dinoflagellate association.

Materials and methods

Biological material and experimental design

Mediterranean specimens of *A. viridis* were collected in shallow waters at Villefranche-sur-Mer (France). They were maintained in laboratory controlled conditions where they were acclimated for three months before experimentation. Weekly renewed seawater was maintained at $19 \pm 0.5^\circ\text{C}$ in aquaria. Sea anemones were fed once a week with *Artemia*. Light was provided at a constant saturating irradiance of $180 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ on a 12/12 h light/dark cycle with a metal halide lamp (HQI-TS 400 W, Philips). All stressed specimens were starved for one week before experimentation and throughout the stress experiments (days 0 to 22). Normal feeding was reintroduced at day 23. Control specimens was fed during all the experimentation.

Specimens of symbiotic *A. viridis* were subjected to a hyperthermal stress ($+10^\circ\text{C}$) for 22 days. Animals were shifted abruptly to 29°C and held in this condition for 22 days, with continuous monitoring of seawater temperature. Following bleaching, aposymbiotic specimens were maintained in the dark with the standard conditions of temperature, feeding and water renewal.

For the menthol treatment, specimens of symbiotic *A. viridis* were incubated in 0,58mM menthol for 15 days using an exposure/resting cycle of 8h/16h. Following bleaching, aposymbiotic specimens were maintained in the dark in the standard conditions of temperature, feeding and water renewal.

Starved specimens were maintained under control conditions of temperature and water renewal but starved from day 0 to day 22, then fed normally until the end of the experiment (day 59).

Control specimens were maintained in standard conditions and sampled as were the stressed specimens. For each specimen, tentacles were sampled at the following days: d0, d5, d22 and d59. All samplings were performed at 10:00 am to avoid variation due to circadian clock (Levy et al., 2011).

To avoid contamination of the protein and RNA samples used for other purpose, all specimens were starved throughout the stress experiment (days 0 to 22), excepted for control specimens that were maintained under standard laboratory conditions. Specimens of *A. digitifera* (FR14-068-02767-I) and *S. pistillata* (FR15-068-03752-I) were imported from the United-States

and maintained in optimal conditions two weeks in Aquarium recifal, Antibes, FRANCE before experimentation.

***Symbiodinium* density quantification**

For each time point, two tentacles per individual were sampled and weighed using a precision balance (Precisia, Model 202A, Sweden). Samples were incubated for 90 min in 1M NaOH solution at 37°C in a drying chamber (Jouan, EB28, France) to allow animal tissue digestion and *Symbiodinium* isolation (Zamoum & Furla 2012). An aliquot of this homogenate (40 µl) was diluted and used to determine the *Symbiodinium* cell density using a Neubauer-Hauser hemocytometer (VWR, Fontenay-sous-Bois, France) under a light microscope (Leica, Wetzlar, Germany). Ten replicates were quantified and results were weight normalized. *Symbiodinium* quantifications were tested using repeated ANOVA measurements with the Tukey multiple comparison post-hoc test. For these analyses, a p-value of <0.05 was considered statistically significant. All analyses described above were conducted using GraphPad InStat Version 3 software (GraphPad Software, San Diego, CA).

Maintenance of cultured *Symbiodinium*

Three single-cell derived cell lines of *Symbiodinium* sp. (clade A') were originally isolated from a single specimen of *A. viridis*, and established as clonal in vitro cultures at the University of Nice (Marine Symbiosis lab, UMR 7138). The *Symbiodinium* cells were cultured in f/2 medium (Guillard and Ryther, 1962), supplemented with 1% antibiotic-antimycotic solution cocktail (Sigma). Cultures were routinely maintained at 24°C and 90% humidity, under a standard 12h/12h light/dark cycle using an irradiance of 200 µmol quanta m⁻² sec⁻¹ (JLB ; Solar ultra marin day) in a controlled cabinet (Witeg Labortechnik GmbH ; WiseCube model WGC-450).

Protein extraction

Gastrodermal (symbiont-containing tissue) and epidermal cell layers were separated as described previously (Richier et al., 2006). Samples were homogenized in an extraction medium containing 50 mM phosphate buffer (pH 7.8) and 10µg/ml protease inhibitor cocktail (Sigma). Homogenates were centrifuged at 1500×g for 3 min to pellet *Symbiodinium* cells. The animal fractions contained in the supernatant were centrifuged again at 4000×g for 3 min to ensure

complete symbiont elimination. *Symbiodinium* fractions were washed, centrifuged at 4000×g for 3 min twice to remove animal contaminants and re-suspended in 200µl final extraction medium. Aliquots of these extracts were used for transmission electron microscopy and the remaining part was sonicated (6×10 s for animal fractions and 8×10 s for *Symbiodinium* fractions) and then centrifuged at 13 000×g for 5 min. Supernatants containing the cytosoluble protein extracts were frozen and kept at -80°C until further analyses. Protein concentrations were determined by the Bradford method (Bio-Rad Protein Assay Dye Reagent Concentrate, Sigma).

Antibody production

Custom-made anti-AvNPC1, anti-AvNPC2-a and anti-AvNPC2-d were generated against, respectively, the polypeptide CLIGSNYSAIQ, EVVDFDDG, GKISLTLQ (AgroBio, La Ferté Saint-Aubin, France). The polypeptide was conjugated to KLH and used to immunize two rabbits for each antibody. The rabbits were initially immunized with 300 µg of KLH-conjugated polypeptide, followed by five boosts of 300 µg at 2-week intervals. The rabbit serum exhibiting the highest immunoreactivity against peptide antigen assessed by ELISA was collected on day 128 and was purified by an affinity column containing 5 mg of peptide antigen (AgroBio).

Western Blot analysis

Protein samples, 5 µg for compartment samples (Freshly isolated *Symbiodinium*: FIS*, Cultured *Symbiodinium*: CS, Epidermis: Ep and Gastrodermis: G) and 20 µg for total sample (Total animal fraction: Tan and Total protein fraction of a bleached specimens: B) were separated by SDS-PAGE in a 15% gel for NPC2 and 10% for NPC1. They were transferred onto a PVDF membrane 0.2 µm for NPC2 and Nitrocellulose 0.45 µm for NPC1 using a semi-dry blotting system (Bio-Rad). After overnight blocking (in 5% non-fat dry milk), the membrane was first incubated with the anti-AvNPC2-d antibody (1:2 000 in PBS, 0.1% Tween 20 with 5% skim milk), anti-AvNPC1 antibody (1:500 in PBS, 0.1% Tween 20 with 5% skim milk) or anti-AvNPC2-a antibody (1:5 000 in PBS, 0.1% Tween 20 with 5% skim milk) for 1h at room temperature. Blots were then washed and incubated in a 1:5 000 dilution of HRP-conjugated goat anti-rabbit IgG secondary antibody solution (Bio-Rad). Visualization of the immune-reactive bands was performed using a chemiluminescence substrate (Western Lightning Chemiluminescence reagent Plus, Perkin Elmer LAS, Inc.) and exposure to X-ray film (BioMax Light, Kodak).

Western blot quantification

For each protein, we determine the relative expression on the three specimens submitted to each stress (starvation or bleaching) on the day 0, day5, day 22 and day 59 of the kinetic. We calculate the pixel quantity in each band in a fixed area on Image J and the relative expression of each band were compared to day 0. The mean of the relative expression for each protein were compared during the bleaching experiment and between both stresses. We also controlled that no change in the relative expression of these proteins occurred in the control experiment.

Immunofluorescence microscopy on *A.viridis* tentacle

Tentacles of symbiotic *A.viridis* specimens were fixed overnight in 4% paraformaldehyde at 4°C. Cryosections (12 µm) were incubated in PBS containing 0.1% Triton X-100 (PBT) for permabilization, then blocked for 2 hours in PBS containing 1% BSA. Primary antibody (1:50, rabbit anti-AvNPC2-d antibody; 1:100, rabbit anti-AvNPC1 antibody, 1:100, rabbit anti-AvNPC2-a antibody) was incubated overnight at 4°C and secondary antibody (anti-rabbit Alexa 546 conjugated (1:500; Thermo Fisher Scientific Inc.) for 2 hr at room temperature. Fluorescence images were acquired using a Zeiss LSM 710 confocal laser-scanning microscope. The confocal microscope was a Zeiss LSM 710 on an inverted Axio Observer.Z1 stand (Carl Zeiss Microscopy GmbH, Jena, Germany), using objectives Plan Apo 63X oil 1.4 NA. The LASERs used were LASER diode 405 nm and/or Argon LASER (458, 488, 514 nm) and/or HeNe 633 nm. The microscope was equipped with an automated xy stage for mosaic acquisitions (Märzhäuser, Wetzlar, Germany), and z-acquisitions were done using the microscope z-drive.

Immunofluorescence microscopy on freshly isolated *Symbiodinium*

To obtain freshly isolated *Symbiodinium* from *A.viridis*, gastrodermal (symbiont-containing tissue layer) and epidermal cell layers were separated as described previously (Richier et al., 2006). Gastrodermal cell layer were homogenized in filtered sea water (0, 2 µm) and centrifuged at 500 x g for 3 min to pellet *Symbiodinium* cells. A 500 µl suspension of endodermal cell (>5 x 10⁵ cells/ml) was drawn gently back and forth through a 23 gauge metal hypodermic needle fitted to a 5 ml syringe. Three plunges of the syringe were required to remove gastrodermal tissue and to obtain intact symbiosome with the perisymbiotic membrane. A further 67 plunges were required to obtain *Symbiodinium* free of perisymbiotic membrane without damaging the *Symbiodinium* cell integrity.

Three additional washing step were required to remove cell and membrane waste after syringe step. At each step, a fraction were directly fixed for transmission electron microscopy and the other fraction were conserved in PBS to immunolocalization.

For corals, *Symbiodinium* cell are separated from the tissue and skeleton using gentle brushing applied on the specimens. The sample is then centrifuge first at 100 x g for 30 seconds to pellet skeleton waste and the supernatant containing both tissue and *Symbiodinium* cells were centrifuged at 500 x g for 3 minutes three times to pellet *Symbiodinium* in SWF.

To confirm the localization of AvNPC1 and AvNPC2-d on the symbiosome, samples were incubated in PBS containing 0.1% Triton X-100 (PBT), then blocked for 2 hours in PBT containing 1% BSA. Primary antibody (1:50, rabbit anti-Av NPC2-d antibody; 1:100, rabbit anti-Av NPC1 antibody, 1:100, rabbit anti-Av NPC2-a antibody), then incubated overnight at 4°C and secondary antibody (anti-rabbit Alexa 546 conjugated (1:500; Thermo Fisher Scientific Inc.) for 2 hours at room temperature. Samples were wash 3 times in PBS and were trapped onto the polycarbonate filter (Isopore, 0.4 μ m HTBP, HTBP01300, Millipore, Billerica, MA, USA) by gentle vacuuming. The filter was put onto a slide glass, seal with Fluoromount™ Aqueous Mounting Medium (F4680 Sigma) to prevent dehydration and oxidation, covered with a cover slip, and kept in the dark. Fluorescence images were acquired using a Zeiss LSM 710 confocal laser-scanning microscope.

Immunogold microscopy on A. viridis freshly isolated Symbiodinium

Immunocytochemistry was performed on FIS with intact perisymbiotic membrane from a healthy specimen of *A. viridis* and fixed in 1% paraformaldehyde and 1% glutaraldehyde fixative in PBS for 1.5 h. Samples were rinsed three times, 10 min each time, in PBS and then dehydrated for 15 min in each concentration of a methanol (MeOH) series (15%, 30%, 50%, 85%, 95%, 100%, 100%). Tentacles were infiltrated with LR White resin on a rotating table in a series of MeOH dilutions (1:3 LR White: MeOH overnight, 1:1 over-night, 100% LR White for 3 h), and then placed in gelatin capsules in fresh LR White. LR White was allowed to polymerize at 52 °C for 2 days. Ultra-thin, gold–silver sections were cut with a diamond knife and placed onto Formvar-coated nickel grids. These grids were processed for immunocytochemistry as follows: they were immersed in blocking solution (PBS 5% BSA) for 15 min, incubated in a 1:1000 dilution of sym32 anti- serum or a 1:1000 dilution of preimmune serum in PBS for 1.5 h, rinsed 3 times, 10 min each

time, in PBS/BSA 0.1% Tween 20, incubated in a 1:75 dilution of EM grade goat anti-rabbit IgG–15-nm colloidal gold (Ted Pella) in PBS for 1 h, rinsed as above, rinsed in ePure water for 5 min, and then allowed to dry. Grids were stained in 2% uranyl acetate for 5 min, rinsed by dipping in water 10 times each in three changes of water, then immediately stained in 0.4% lead acetate for 3 min, with water rinses as above, and then air dried. Between 5 and 10 grids of each type of anemone were viewed under 60 kV using a CM-12 Phillips transmission electron microscope.

Correlation between cellulosic contents and AvNPC1 in hospite *Symbiodinium*

FIS extracted from *A. viridis* were stained sequentially with the anti-AvNPC1 antibody (1:100) and secondary antibody (AF546 1:500), then rinsed three times in PBS and twice in SWF. Samples were then stained with CFW at a final concentration of 20 µg/ml in SWF, rinsed three times in SWF and then trapped onto the polycarbonate filter (Isopore, 0.4 µm HTBP, HTBP01300, Millipore, Billerica, MA, USA) by gentle vacuuming. The filter was put onto a slide glass, seal with Fluoromount™ Aqueous Mounting Medium (F4680 Sigma) to prevent dehydration and oxidation, covered with a cover slip, and kept in the dark. Fluorescence images were acquired using a Zeiss LSM 710 confocal laser-scanning microscope.

Statistical analyses

Symbiodinium quantification using Anova one-way and differential expression of AvNPC proteins during the both starved and bleaching stresses were tested using the global non-parametric test of Kruskal-Wallis with Dunn's multiple-comparison post-hoc test. For these analyses, a p-value <0.05 was considered statistically significant. All the analyses described above were conducted using the software GraphPad InStat Version 3 (GraphPad Software, San Diego, CA).

Results

The transmembrane protein AvNPC1 is a symbiosome marker of symbiotic active symbionts.

We show that AvNPC1 was expressed both in the epidermal and the gastrodermal tissue layer in symbiotic and bleached specimens (Fig. 1A). Furthermore, the protein was expressed in the FIS sample, indicating an association with the symbiosome.

The result observed in Western Blot was confirm by localization in tissue section of

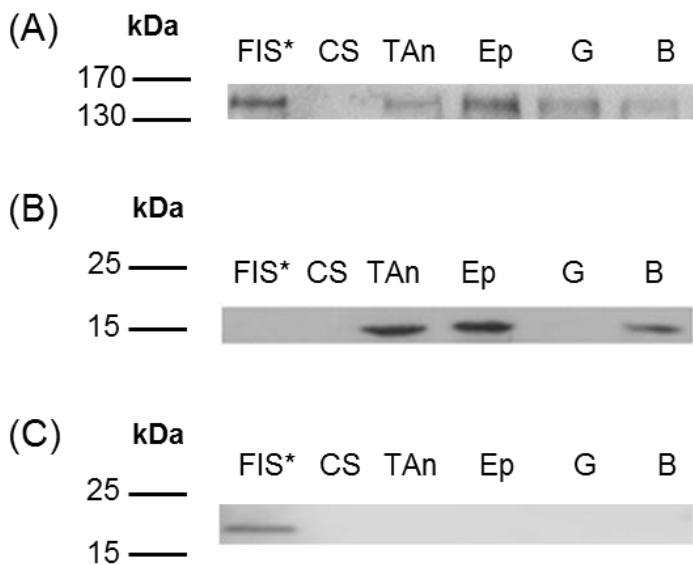


Figure 1: **Western blot analyses of AvNPC protein in different sample of *A. viridis*.** **A)** Anti-AvNPC1 antibody (1:500), **B)** anti-AvNPC2-a antibody (1:5000) and **C)** anti-AvNPC2-d antibody (1:2000). FIS* (Freshly isolated *Symbiodinium* with perisymbiotic membrane, 5 μ g), CS (Cultured *Symbiodinium*, 5 μ g), TAn (Total animal fraction, 20 μ g), Ep (Epidermal fraction, 5 μ g), G (Gastrodermal fraction, 5 μ g) and B (Total fraction of a bleached specimen, 20 μ g). For all experiments, secondary antibody Goat anti-Rabbit were used at a dilution of 1:500

A. viridis tentacle (Fig. 2A). We show that AvNPC1 was expressed in vesicles in the epidermis and in the gastrodermis but also that AvNPC1 was strongly expressed associated with symbiosomes. The localization of the AvNPC1 protein on the symbiosome membrane was also confirm by positive immunolabelling on FIS extracted from *A. viridis* tentacle (Fig. 3A). To test if AvNPC1 could be a symbiosome marker on coral species, we conducted immunolocalization on FIS extracted from *Acropora digitifera* and *Stylophora pistillata*.

The result show a positive staining on the symbiosome membranes (Fig. 3B, C) and a blast against the AdNPC1 and SpNPC1 sequences confirm the possibility of a cross-reactivity of the AvNPC1 antibody (data not shown).

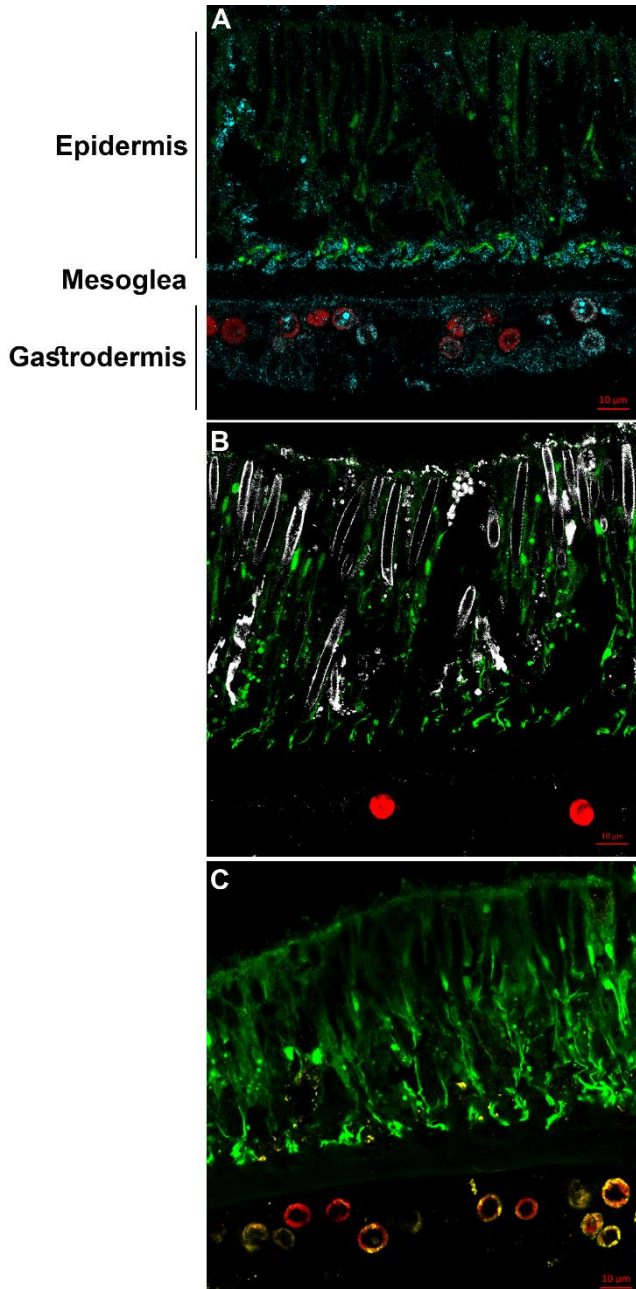


Figure 2: **Immunolocalization in tissue cryosection of *A. viridis* tentacle of AvNPC proteins** A) AvNPC1 protein in blue, B) AvNPC2-a protein in white and C) AvNPC2-d protein in yellow. In green, the autofluorescence of natural GFP expressed by the host, in red the natural autofluorescence of the chlorophyll pigments expressed by the *Symbiodinium*

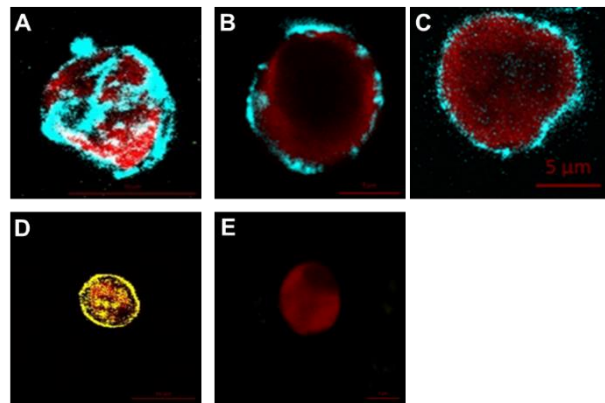


Figure 3: **Immuno-labeling of NPC protein on FIS extracted from *A. viridis* (A,D,E), *A. digitifera* (B) and *S. pistillata*.** A) AvNPC1 protein in blue, B) NPC1 protein reveal with AvNPC1 antibody, C) NPC1 protein reveal with AvNPC1 antibody, D) AvNPC2-d protein in yellow and E) AvNPC2-a protein in white. The red represent the natural autofluorescence of chlorophyll pigments of the *Symbiodinium*.

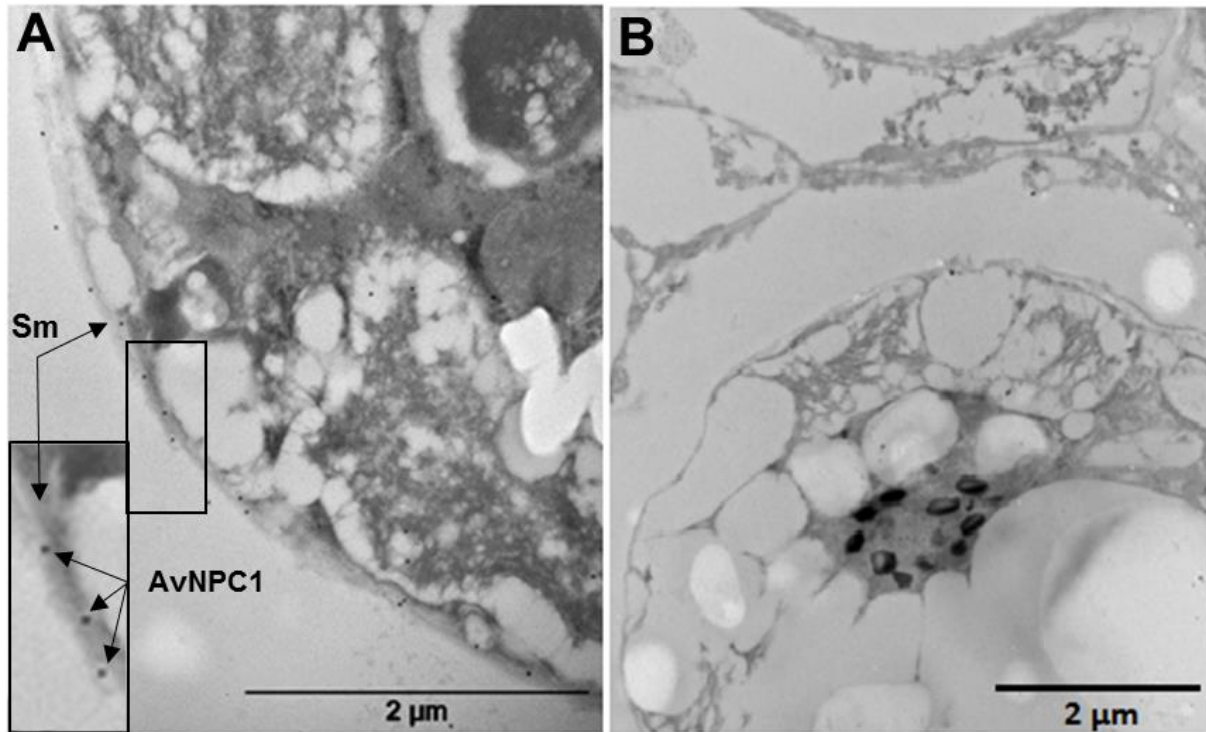


Figure 4: Transmission electron micrographs of immunogold-labeled sections of freshly isolated *Symbiodinium* from the symbiotic *A. viridis*. (A) Illustration of gold spheres labeled against AvNPC1 associated with the perisymbiotic membrane. (B) Secondary antibody alone

Furthermore, localization of the AvNPC1 protein on FIS extracted from *A. viridis* at an ultrastructural level by immunogold labelling, show that the AvNPC1 protein was clearly localize at the perisymbiotic membrane (Fig. 4A).

Using CalcoFluor White staining properties (stain cellulosic contents), we further show that the AvNPC1 protein was localized only on symbionts containing high cellulosic contents therefore absent on symbionts depleted of cellulosic contents (Fig. 5).

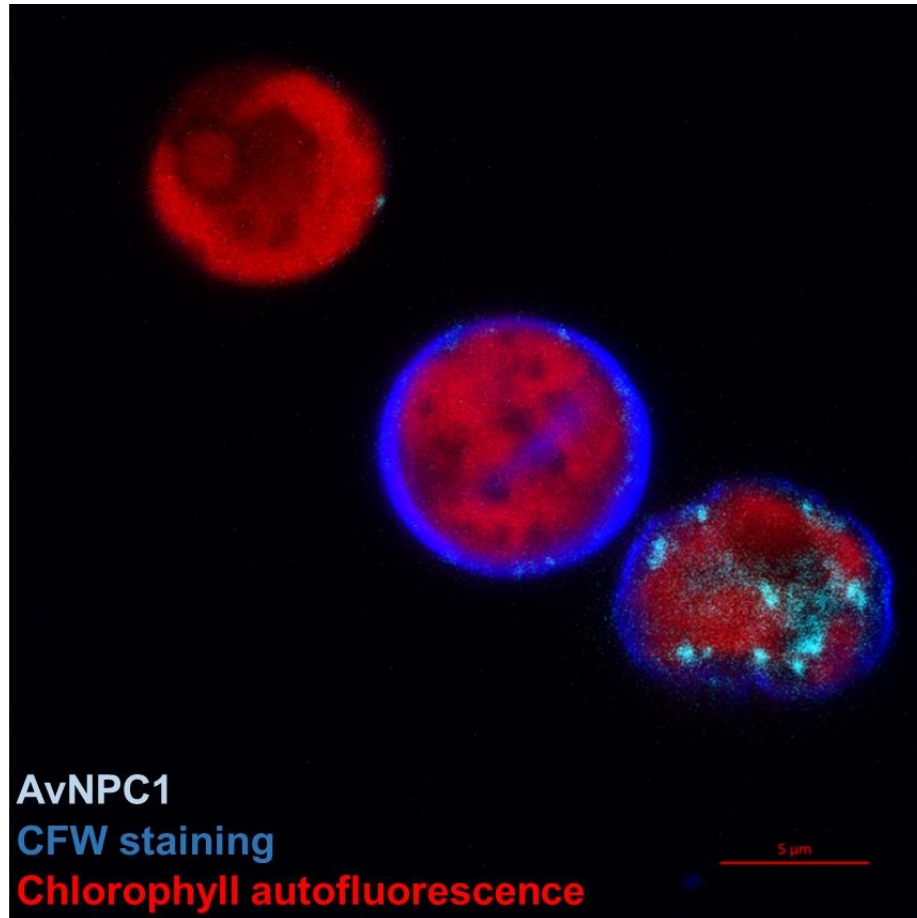


Figure 5: **Double-labelling AvNPC1/CFW on FIS extracted from *A. viridis* tentacle.** In hospite symbionts with low cellulosic contents are not surrounded by AvNPC1. On contrary, *in hospite* symbiont with high cellulosic contents are partially surrounded by AvNPC1 transmembrane protein. In red the natural autofluorescence of the chlorophyll pigments expressed by the *Symbiodinium*, in dark blue the CFW staining cellulosic components and in light blue the AvNPC1 protein.

Elimination of the perisymbiotic membrane

By drawing back and forth gastrodermal cell three times back and forth a 23 gauge metal hypodermic needle (Fig 6), we were able to obtain an enriched fraction of isolated *Symbiodinium* cells with intact symbiosome membranes (Fig 7A-B) as revealed by AvNPC1 staining and TEM visualization.

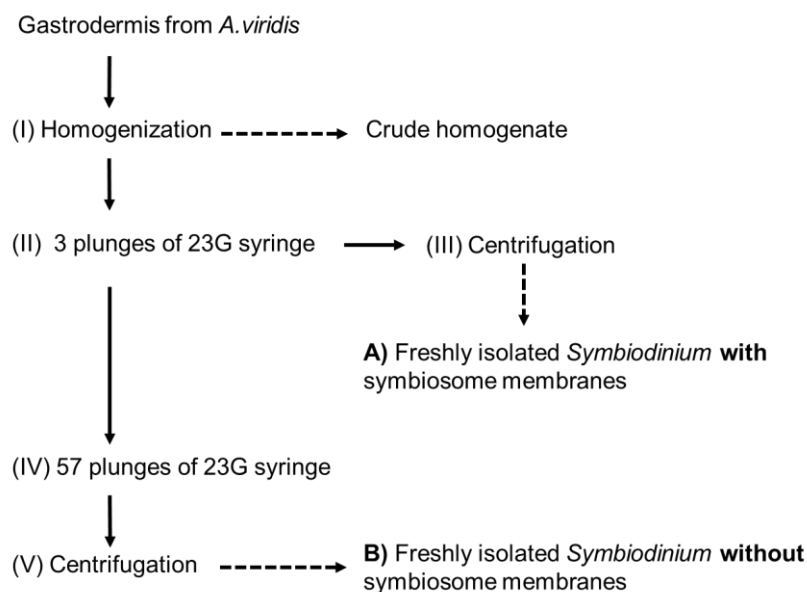


Figure 6 : **Protocol design of fraction enrichment** in A) Freshly isolated *Symbiodinium* with symbiosome membranes and B) Freshly isolated *Symbiodinium* without symbiosome membranes

To almost totally eliminate the perisymbiotic membrane without affecting *Symbiodinium* integrity, 67 additional plunges back and forth in the needle were necessary (Fig 7C-D). This last sample harbour the same membrane profile that cultured *Symbiodinium* (Fig 7E-F), with only the *Symbiodinium* cell wall. In intermediate samples (42p and 60p), the perisymbiotic membrane was increasingly damaged (data not shown). We show that the presence of AvNPC1 staining on symbiosome was correlated with the integrity of the symbiosome membrane (Fig. 7B and D).

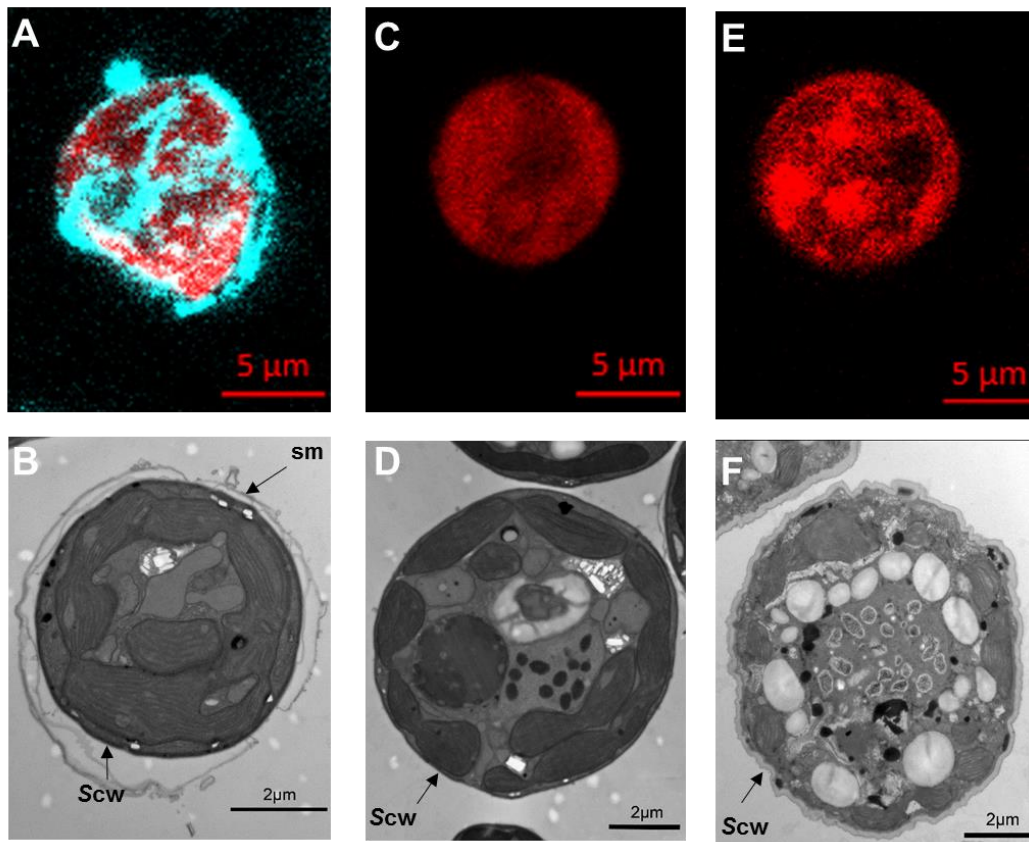


Figure 7 **Elimination of the perisymbiotic membrane on FIS extracted from *A. viridis***

(A) Immunolabelling of FIS submitted to 3 AR by AvNPC1 in blue and (B) profile of corresponding *Symbiodinium* showing presence of the perisymbiotic membrane (C) Immunolabelling of FIS submitted to 70 AR by AvNPC1 and (D) profile of corresponding *Symbiodinium* showing absence of the perisymbiotic membrane (E) Immunolabelling of cultured *Symbiodinium* and (F) profile of corresponding *Symbiodinium* showing absence of perisymbiotic membrane

Then, 58% of the *Symbiodinium* extracted and passed three times in the needle were stained by AvNPC1, whereas only 45% and 17% of *Symbiodinium* were stained in sample passed seven and forty-two times in the needle respectively (Fig 8). However, these three last samples were all statistically different from cultured *Symbiodinium* ($p < 0.0001$) stained by AvNPC1 at 2%. On the contrary, the *Symbiodinium* freshly extracted passed sixty and seventy times in needle, stained respectively at 12% and 8%, are closely related to culture *Symbiodinium* as regard to AvNPC1 staining. Focusing on membrane(s) profiles surrounding the different *in hospite* *Symbiodinium* by

electronic transmission microscopy and AvNPC1 symbiosome specific staining, the three needle passage treatment allow us to obtain a fraction of enriched freshly isolated *Symbiodinium* with intact symbiosome and the seventy needle passage treatment was used to obtain a fraction with intact *Symbiodinium* without the perisymbiotic membrane.

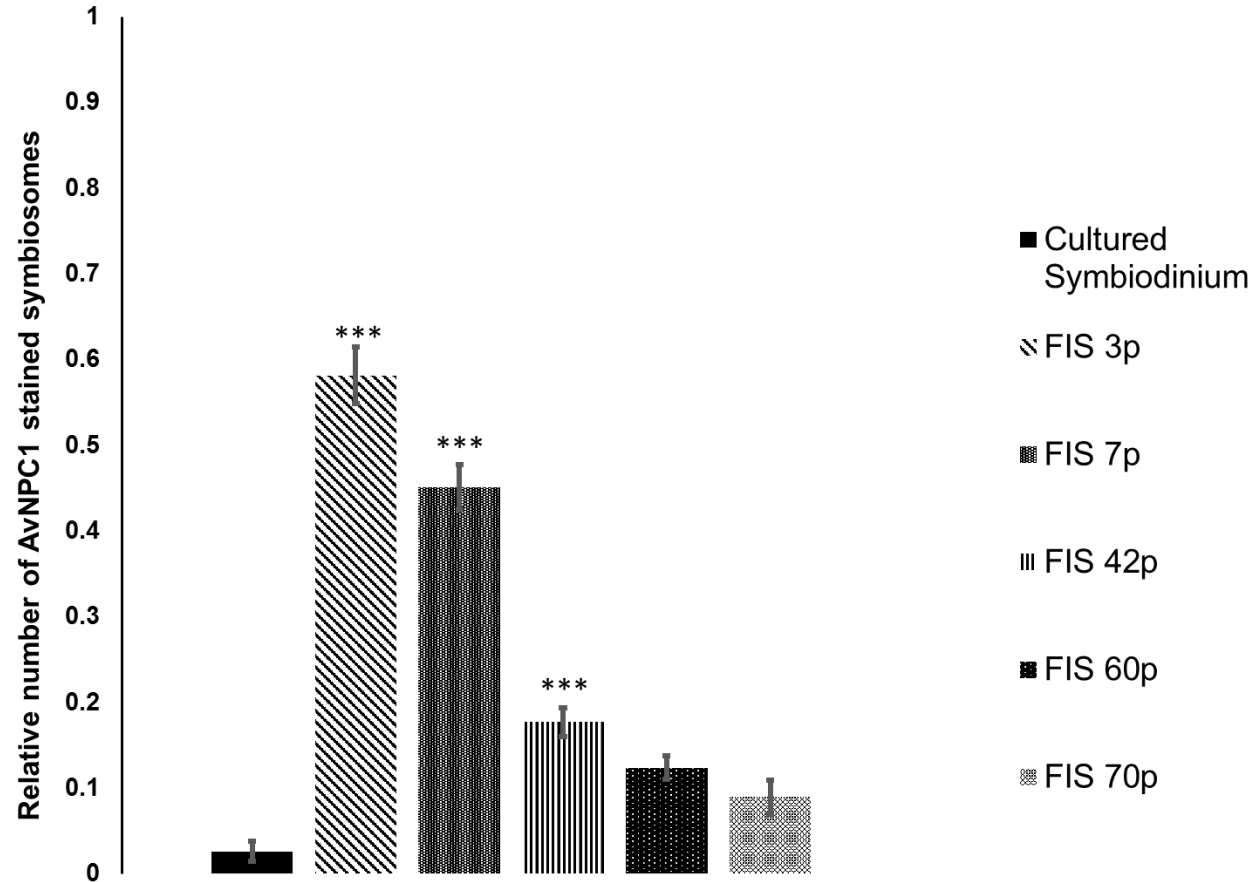


Figure 8: **Relative number of AvNPC1 stained symbiosome submitted to increasing mechanical stress.** Samples statistically different from cultured *Symbiodinium* were assessed by One way ANOVA statistical test and considered different for $p < 0.0001$ (represented by ***on the graph).

Subfunctionalization of the AvNPC2 proteins

We show that the canonical protein AvNPC2-a was expressed only in the epidermis tissue layer both in symbiotic and aposymbiotic specimens (Fig. 1B). Immuno-localization on an *A. viridis* tentacle of a symbiotic specimen show that AvNPC2-a was effectively expressed in the epidermis, closely associated with both cilia on the external part of the cell in contact with seawater and associated with cnidocyte structures (Fig. 2B). As expected by previous result, this protein was not associated with the symbiosome (Fig 3E). Otherwise, the cnidarian specific duplicated protein AvNPC2-d was uniquely expressed in the FIS protein sample (Fig. 1C), indicating that this protein was specifically associated with the symbiosome. This result was confirm by *in hospite* immuno-localization (Fig 2B) and on FIS (Fig. 3D). For all protein, absence of band in the cultured *Symbiodinium* sample ensure that these proteins are from *A. viridis* origin.

Regulation of AvNPC1, AvNPC2-a and AvNPC2-d proteins during starvation and bleaching experiment

During starvation, we observed that AvNPC1, AvNPC2-a and AvNPC2-d were not regulated and expression levels maintained constant during all the experiment (Fig. 9).

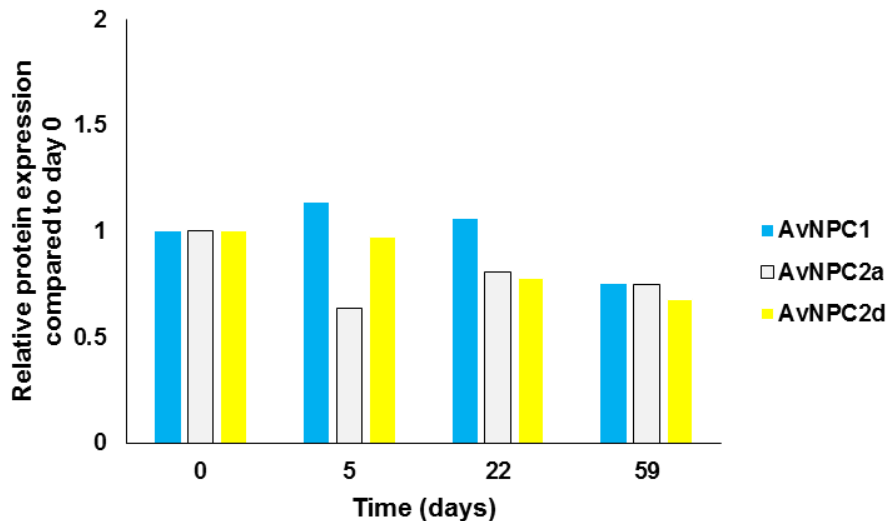


Figure 9 **Relative expression of AvNPC1 and AvNPC2 protein in control specimen from day 0 to day 59.** No modification of the protein expression of neither AvNPC1 nor AvNPC2a and AvNPC2d were observed during the experiment. A statistical difference of $p < 0.05$ were represented by *.

Moreover, no modification on the localization of the proteins (Fig. S1) and on the quantity of *in hospite Symbiodinium* were observed during all the experiment (Fig.11).

During the hyperthermal bleaching experiment, we show differential regulation between AvNPC1, AvNPC2-a and AvNPC2-d. Concerning AvNPC1, we show that this protein was not regulated during the bleaching process (Fig. 10A). Furthermore, this protein was always associated with the symbiosome of the remaining resident symbionts during the stress experiment (Fig. S2 A-C). The epidermal AvNPC2-a protein was also not statistically regulated during the hyperthermal stress (Fig. 10A), even if a straight diminution of his expression was notice at the end of the hyperthermal stress (day 22) and at the end of the experiment (day 59).

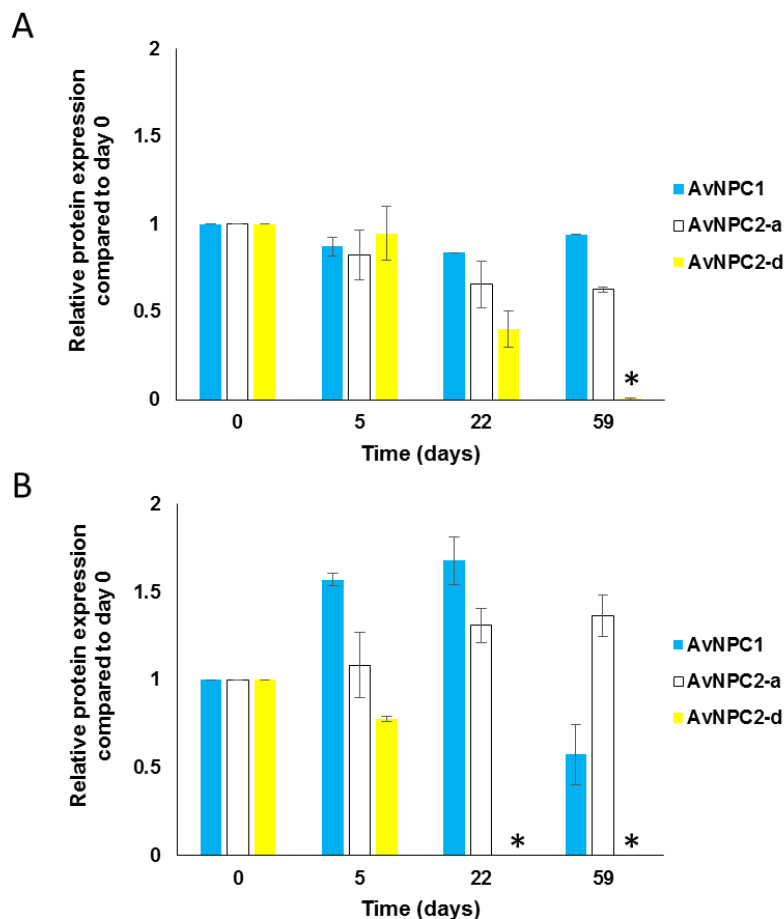


Figure 10: **Relative expression of AvNPC1 and AvNPC2 protein in hyperthermal stress (A) and menthol stress (B).** Error bars represent SD. Statistical difference between the relative expression at d0 and during the kinetic were test using the global non-parametric test of Kruskal-Wallis with Dunn's multiple-comparison post-hoc test and considered significant for $p < 0.05$ represented by *

Remarkably, we found that this protein was specifically localized on cilia, at each time of the kinetic, with no expression on cnidocyte structures (Fig. S2 D, E, F). Concerning the symbiosome specific AvNPC2-d protein, we show that this protein was equally expressed at day 0 and day 5 and down-regulated at day 22 (Fig. 10A). Moreover, AvNPC2-d was statistically strongly down-regulated at day 59, in accordance with bleaching threshold (Fig. 11). We still found some spot of AvNPC2-d on tissue immune-localization corresponding to nearly digest *in hospite Symbiodinium* (Fig. S2I). Nevertheless, the concordance between symbiont presence and AvNPC2-d expression was clearly visualized during the kinetic.

Concerning the menthol stress, we found a different regulation of AvNPC1 and AvNPC2 proteins during the bleaching experiment. First, we show an up-regulation of AvNPC1 during the stress (day5) and even 1 week post stress (day 22) (Fig 10B). On contrary, AvNPC1 expression return to its basal level at day 59. AvNPC1 was localized both in gastrodermis and epidermis, and surrounding symbiont during all stress experiment (Fig. S3A, B). At day 59, all symbiont were expelled or digested in gastrodermal tissue and AvNPC1 were localized in both tissues layer in vesicles like structure (Fig. S3C). We thus show that AvNPC1 expression was regulated by the menthol stress but not by the thermal stress in *A. viridis* specimens.

Concerning AvNPC2-a, we found that the expression of this protein was increasing during the all experiment. Besides, we found strong localization of this protein closely associated with the cnidocytes, the predation organites, during all stress experiment (Fig. S3D-F). This result could be a relocalization of the expression to heterotrophic pathway that occur in response to symbiosis loss. For AvNPC2-d, we found the same type of down-regulation as the hyperthermal stress experiment but in a stronger manner. Indeed, the protein wasn't expressed anymore until day 22 (Fig. 10A). Concerning the presence of AvNPC2-d, this protein, as in hyperthermal stress, was strongly correlated with the symbiont presence and totally absent until the specimens were bleached (less than 50 percent of the initial population) (Fig 11). This strongly suggest that AvNPC2-d is a marker of the bleaching threshold.

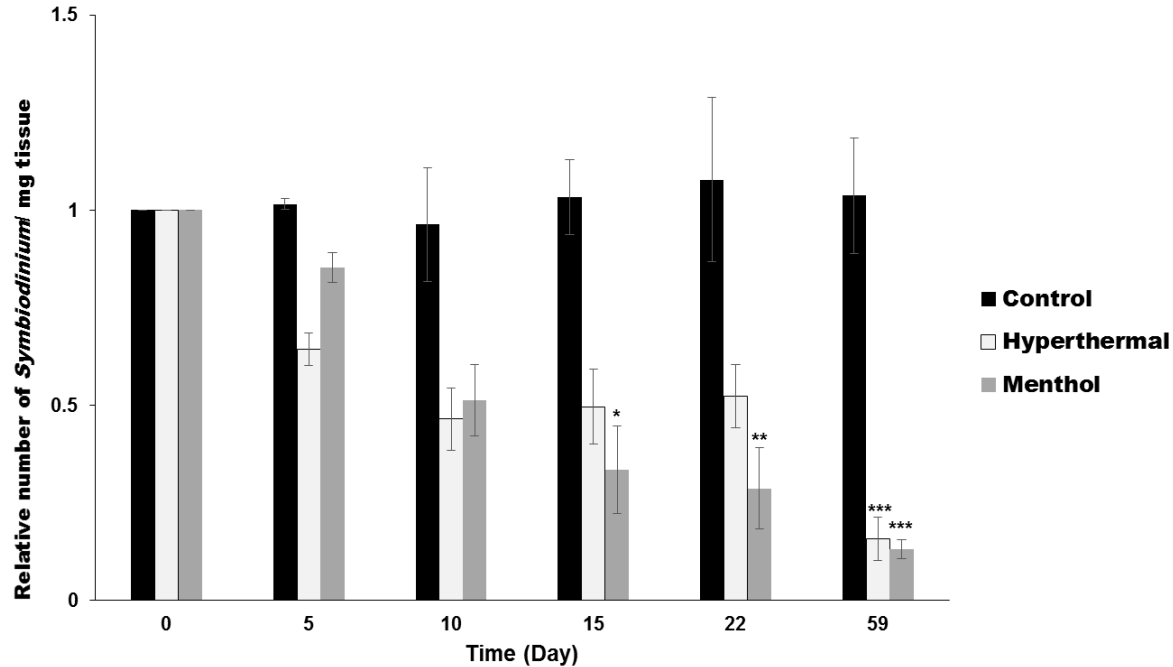


Figure 11. **Relative number of Symbiodinium cell per mg of tissue in control, hyperthermal and menthol stressed specimens from day 0 to day 59.** Error bars represent standard error (\pm SEM). A statistical difference of $p < 0.05$ were represented by *, $p < 0.01$ by ** and $p < 0.001$ by ***.

Discussion

In a previous study, we have shown that AvNPC1, a transmembrane protein was present in a unique gene in cnidarian species but that AvNPC2, a cytosoluble protein, was duplicated in cnidarian species with at least three surnumerary copies (Dani et al., 2014). At a transcriptional level, we shown that only the expression of the gastrodermal duplicated copy AvNPC2-d was drastically reduce at the induction of the stress leading to bleaching. In this study, we describe both protein localization and regulation of AvNPC1, AvNPC2-a and AvNPC2-d during hyperthermal stress and menthol stress inducing both bleaching in *A. viridis*.

The transmembrane protein AvNPC1 is a marker of the perisymbiotic membrane

In this study, we show that the AvNPC1 protein was distributed among both tissue layer in symbiotic and apo-symbiotic specimens (Fig 1A, Fig 2A). Moreover, this protein was neither regulated by both hyperthermal and menthol bleaching stress nor relocalized (Fig 8B, S2A-C, S3A-

C). These results are consistent with transcriptional regulation of *Avnpc1* gene previously obtained (Dani et al., 2014b). At a transcriptional level, NPC1 has been shown to be negatively regulated during night period in *Acropora millepora* compared to light period (Bertucci et al., 2015a). Most notably, we have demonstrated by immune-gold labelling in *A. viridis* and by immuno-fluo labeling in both *A. viridis*, *A. digitifera* and *S. pistillata* (Fig 3A, B,C) that this protein was colocalized at the perisymbiotic membrane (Fig 4A). The AvNPC1 protein could then constitute an invaluable marker of the perisymbiotic membrane in both coral and sea anemones. Even if NPC1 have been more studied for his putative role in cholesterol-binding pathway in the human disease Niemann-Pick type C (Infante et al., 2008b; Infante et al., 2008d; Scott and Ioannou, 2004a; Vance, 2010a), an emergent role involved NPC1 in binding cell surface viral glycoproteins in Ebola virus (Carette et al., 2011b; Côté et al., 2011; Dahlmann et al., 2015; Miller et al., 2012) and flavovirus entry (White and Schornberg, 2012). The localization of this protein on the perisymbiotic membrane could then serve to both recognition of the symbiont cell surface glycome (Logan et al., 2010) during acquisition but could also actively participate to phytosterol transfer from the symbiont, in duo with AvNPC2-d in normal symbiosis conditions (Dani et al., 2014).

To date, only two study attempt to characterize symbiotic interface in Cnidarian-Dinoflagellate association (Peng et al., 2010b; Stochaj and Grossman, 1997) mainly because at least only one study proposed a technical way to purified the perisymbiotic membrane (Kazandjian et al., 2008). Furthermore, comparison with cultured *Symbiodinium* induce a bias provoked by the culture condition selection. We further use the perisymbiotic marker capacity of AvNPC1 to immunolabel the presence or absence of the perisymbiotic membrane in freshly extracted *Symbiodinium* (FIS) subjected to different mechanical stress to discard the perisymbiotic membrane (Fig 7). We show that the method consisting in three back and forth in the needle is sufficient to discard gastrodermal cell components and to obtain an enriched fraction of FIS with the perisymbiotic membrane (Fig 7A). This technic is then a good way to discard gastrodermal host specific proteins that has been a major flow by cross-contamination in previous perisymbiotic membrane proteome identification in cnidarian (Peng et al., 2010a) and in other model like peribacteroid membrane from *Pisum sativum* (Saalbach et al., 2002). Furthermore, seventy back and forth in the needle was the best condition to obtain a 91% fraction of symbiont without the perisymbiotic membrane but with a preserved cell integrity (Fig 7B). These two conditions are actually under-sequencing (work in progress) and could lead to direct identification of the

perisymbiotic membrane proteome by subtraction of specific symbiont-less membrane condition to enriched symbiont-membrane condition. Furthermore, comparison of symbiont membrane-less condition and cultured *Symbiodinium* condition could reveal the proteome modification of specific *Symbiodinium* proteins expressed in symbiotic conditions.

Furthermore, we show in a previous study that in *A. viridis*, *in hospite* symbionts are composed of a heterogeneous population in both G1/G0 active metabolic phase and S/G2/M division phase (Dani et al., en préparation). In the present study, we observed that AvNPC1 expression was correlated with high cellulosic contents in *Symbiodinium* cell wall (Fig. 4). Consistent with a previous result that observed that *npc1* expression was upregulated during light period in *Acropora millepora* (Bertucci et al., 2015a), our study showed that AvNPC1 was differentially expressed depending on the physiological state of *in hospite* *Symbiodinium*. We then hypothesize that the perisymbiotic membrane could be actively remodeled during the life cycle of the symbiont. As shown by many works on coral (Chen et al., 2003; Chen et al., 2004; Chen et al., 2005) and other symbiotic systems like rhizobia-leguminous (Catalano et al., 2004), the symbiosome proteome is remodeled under an active process depending on symbiont cell-cycle state and symbiotic conditions. The capacity to study separately these different *in hospite* symbionts population could lead to enhance comprehension on symbiosis maintenance and regulation.

Sub-functionalization of AvNPC2 proteins

A previous study has shown cnidarian specific gene duplication of targeted genes putatively implicated in symbiosis pathway (Ganot et al., 2011). This study also demonstrated that the following symbiosis related genes, NPC2, Calumenin, Sym32 and C3 complement families were also upregulated in symbiotic condition. In a previous study (Dani et al., 2014), we focused on Avnpc1 and Avnpc2 genes and show that *npc2* genes present at least 3 supernumerary copies in cnidarians by a retro-transcription of the RNA copy of the canonical Avnpc2-a copy. These results were further confirmed by the transcriptomic study (Lehnert et al., 2014) and genome investigation on *Aiptasia* (Baumgarten et al., 2015) showing the organization of *npc2* genes in two clusters probably issued from a tandem duplication. In our study, all the paralogs were overexpressed in the symbiotic compartment. We show that at a protein level, the canonical protein AvNPC2-a was expressed uniquely in the epidermal tissues layer (Fig 1B and 2B) and that the duplicated copy

AvNPC2-d was only expressed in the gastrodermal tissue layer and more precisely, associated with the symbiosome (Fig 1C, 2C, 3C). This constitute a unique example of a gene duplication that present a sub-functionalization in response to symbiosis establishment in Cnidarian-Dinoflagellate association. In other models, this mechanism as also been involved in symbiotic genes as LCO receptor genes (De Mita et al., 2014) and GTPases (Yuksel and Memon, 2009) in *Rhizobium* spp.. Gene duplication could also induce a gain of diversity in molecular exchanges at the symbiotic interface, as shown by amino acids transporters duplication in the pea aphid (Duncan et al., 2014). Duplication of genes is a common mechanism for gene preservation mostly by rapid subfunctionalization (Lynch and Force, 2000; Rastogi and Liberles, 2005) that further lead to neofunctionalization (He and Zhang, 2005). This mechanism as occurred especially for NPC2 in *Drosophila melanogaster* with the presence of eight different NPC2 genes that displays different expression pattern and levels of expression (Huang et al., 2007; Shi et al., 2012). To further conclude to neo-functionalization, the determination of the potential ligand of both AvNPC2-a and AvNPC2-d; currently under investigation; would allow us to answer this critical question.

The symbiotic AvNPC2-d protein is a biomarker of bleaching

In this study, we also follow the regulation of AvNPC1 and AvNPC2 protein during both control and bleaching stresses in *A. viridis*. Similarly as transcriptomic regulation (Dani et al., 2014b), AvNPC1 and AvNPC2-a protein weren't regulated by hyperthermal stress (Fig. 9B-C). We also notice no re-localization during this stress (Fig. S2A-F). Concerning the menthol bleaching experiment, we show a slight increase of AvNPC1 during stress (day5 and day 22) (Fig. 9B) and a continuous increase of AvNPC2-a (Fig. 9C), with a specific expression, localized on cnidocyte structure (Fig. S3D-F). This relocalization could be the result of switch from autotrophy to heterotrophy as previously mentioned in a transcriptomic study (Ganot et al., 2011). The most interesting regulation concerned the AvNPC2-d protein that show a drastic down-regulation during both stress (Fig. 9D) and a strong correlation with symbiont presence (Fig. 10, Fig. S2G-I and Fig. S3G-I). Even if the diminution of the expression was more abrupt in the menthol bleaching experiment than in the hyperthermal stress, the loss of detection of the AvNPC2-d protein was correlated with the shiftline of diminution of initial symbiont population under the 50% bleaching line (Fig 10) that occurs at day 15 in the menthol bleaching experiment and at day 59 in the hyperthermal stress experiment. As occurrence of bleaching events is increasing in frequencies

(Hoegh-Guldberg, 1999; Hoegh-Guldberg et al., 2007) and leading to massive extinction of coral reefs species (Pandolfi et al., 2011), it is therefore crucial to develop molecular tools to monitor and prevent massive bleaching events. But to date, the only marker of coral bleaching is the ocular observation of the bleaching himself (Glynn, 1993; Weis and Reynolds, 1999). To date, Sym32 protein expression was found to be correlated with symbiont density in cadmium bleaching marker (Mitchelmore et al., 2002), and GFP gene expression designed as a toolkit to monitor host heat stress with a reef-building coral (Smith-Keune and Dove, 2007). The AvNPC2-d is then a new invaluable biomarker of bleaching state in the cnidarian-dinoflagellate symbiosis because it response in a binary manner, e.g. absent when symbiont density has cross the shifting baseline of bleaching.

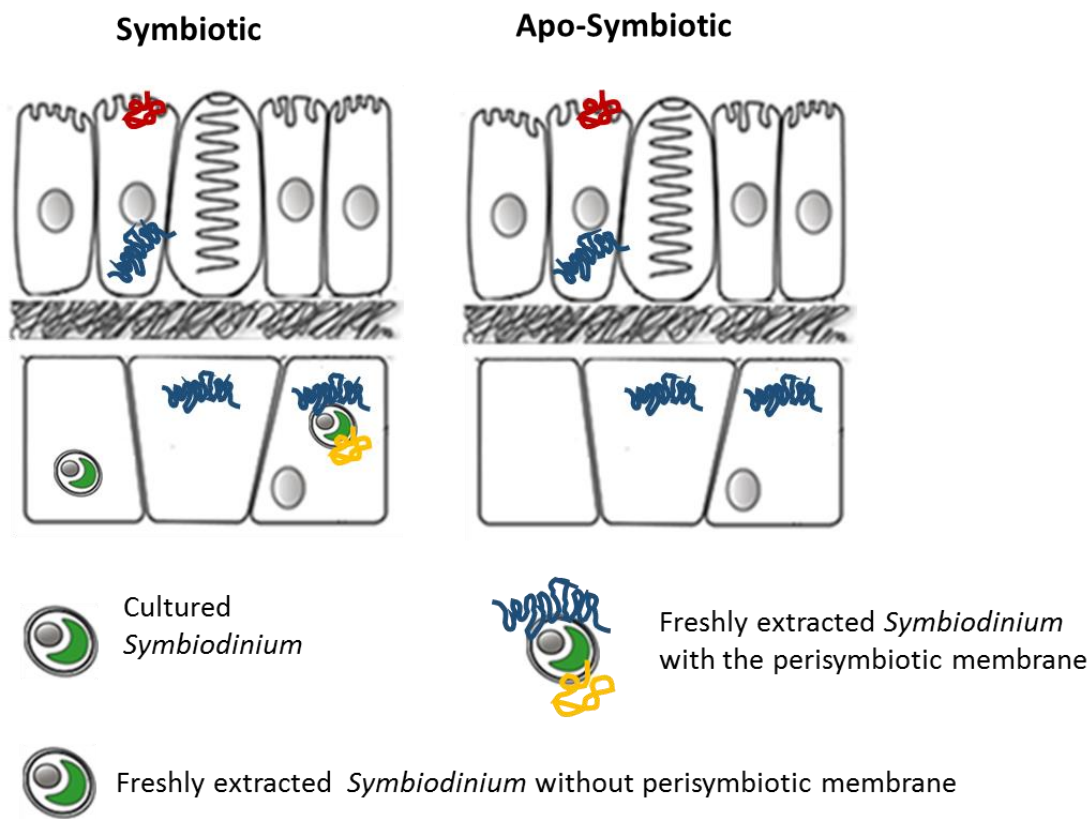


Figure 12: Model of the subcellular location of AvNPC proteins in the tissue layer of symbiotic and apo-symbiotic specimens, in cultured *Symbiodinium* and in both freshly isolated *Symbiodinium* with and without the perisymbiotic membrane.

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Supplementary figures

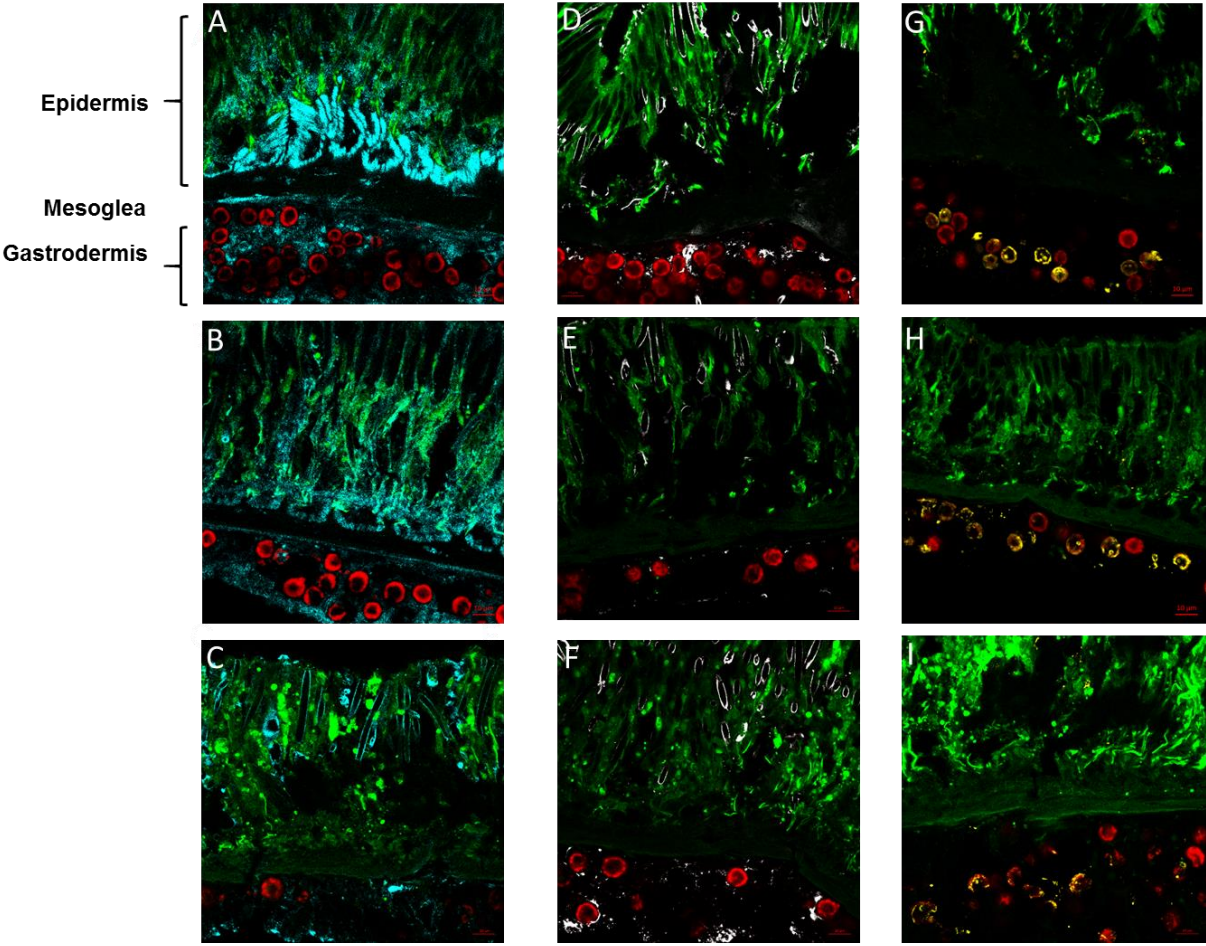


Figure S1: **Immunolocalization of AvNPC1 and AvNPC2 protein in the control condition in tissue section of *A. viridis*** **A-B-C)** AvNPC1 localization in blue at day 5, day 22 and day 59, **D-E-F)** AvNPC2-a localization in white at day 5, day 22 and day 59, **G-H-I)** AvNPC2-D localization in yellow at day 5, day 22 and day 59. In green, the autofluorescence of natural GFP expressed by the host, in red the natural autofluorescence of the chlorophyll pigments expressed by the *Symbiodinium*.

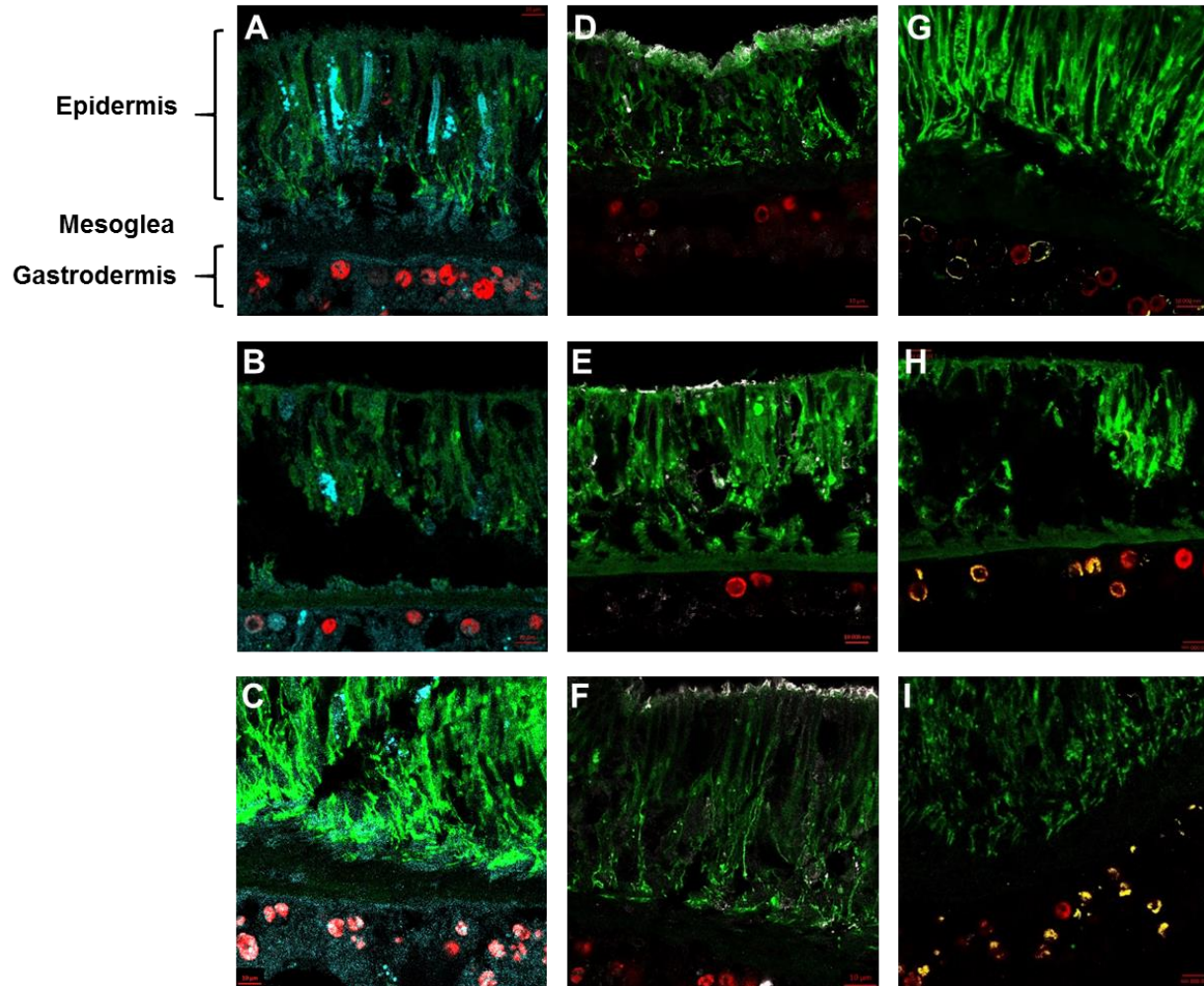


Figure S2: **Immunolocalization of AvNPC1 and AvNPC2 protein in the hyperthermal bleaching experiment in tissue section of *A. viridis*** **A-B-C)** AvNPC1 localization in blue at day 5, day 22 and day 59, **D-E-F)** AvNPC2-a localization in white at day 5, day 22 and day 59, **G-H-I)** AvNPC2-D localization in yellow at day 5, day 22 and day 59. In green, the autofluorescence of natural GFP expressed by the host, in red the natural autofluorescence of the chlorophyll pigments expressed by the *Symbiodinium*.

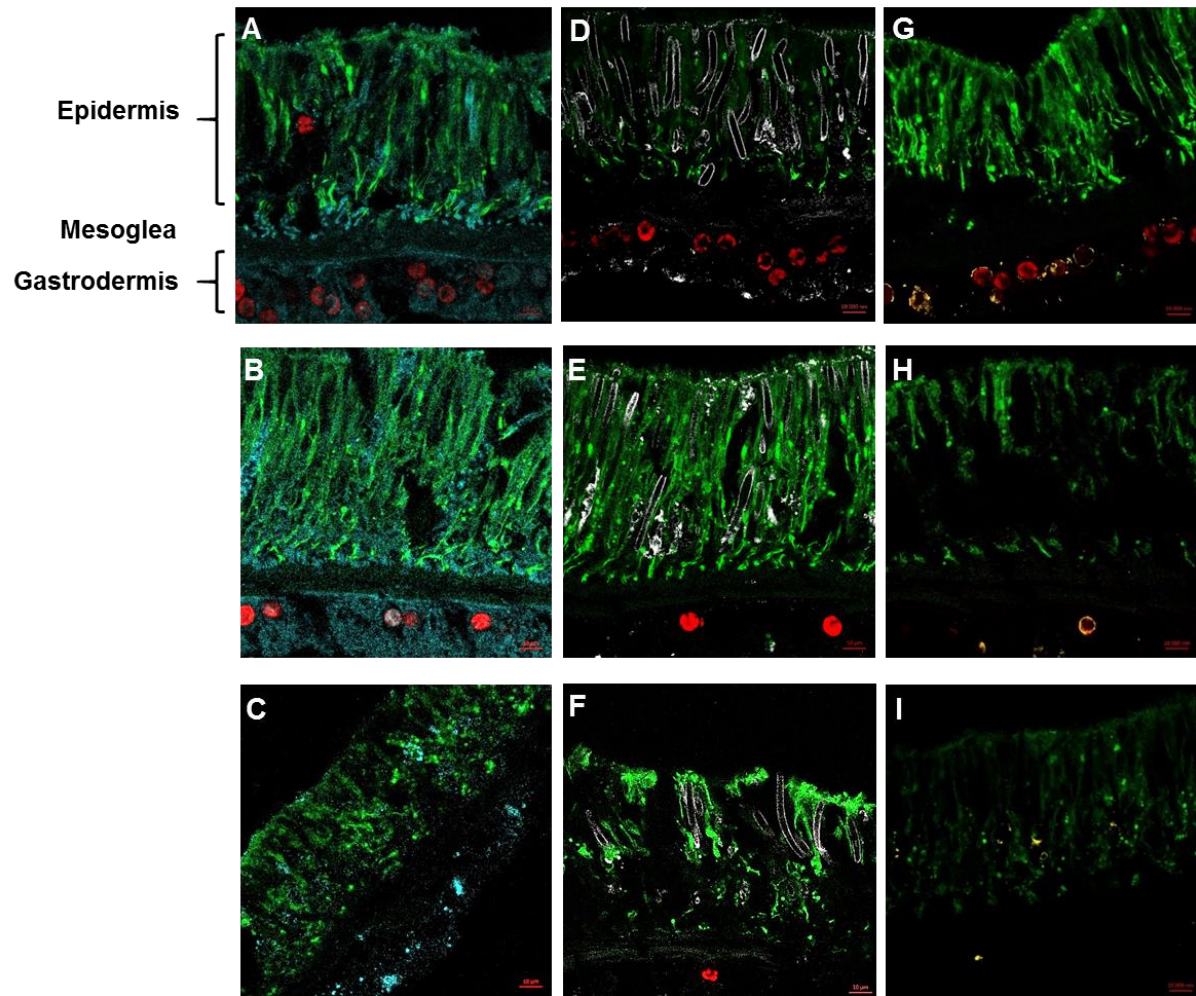


Figure S3: **Immunolocalization of AvNPC1 and AvNPC2 protein in the menthol bleaching experiment in tissue section of *A. viridis*** **A-B-C)** AvNPC1 localization in blue at day 5, day 22 and day 59, **D-E-F)** AvNPC2-a localization in white at day 5, day 22 and day 59, **G-H-I)** AvNPC2-D localization in yellow at day 5, day 22 and day 59. In green, the autofluorescence of natural GFP expressed by the host, in red the natural autofluorescence of the chlorophyll pigments expressed by the *Symbiodinium*.

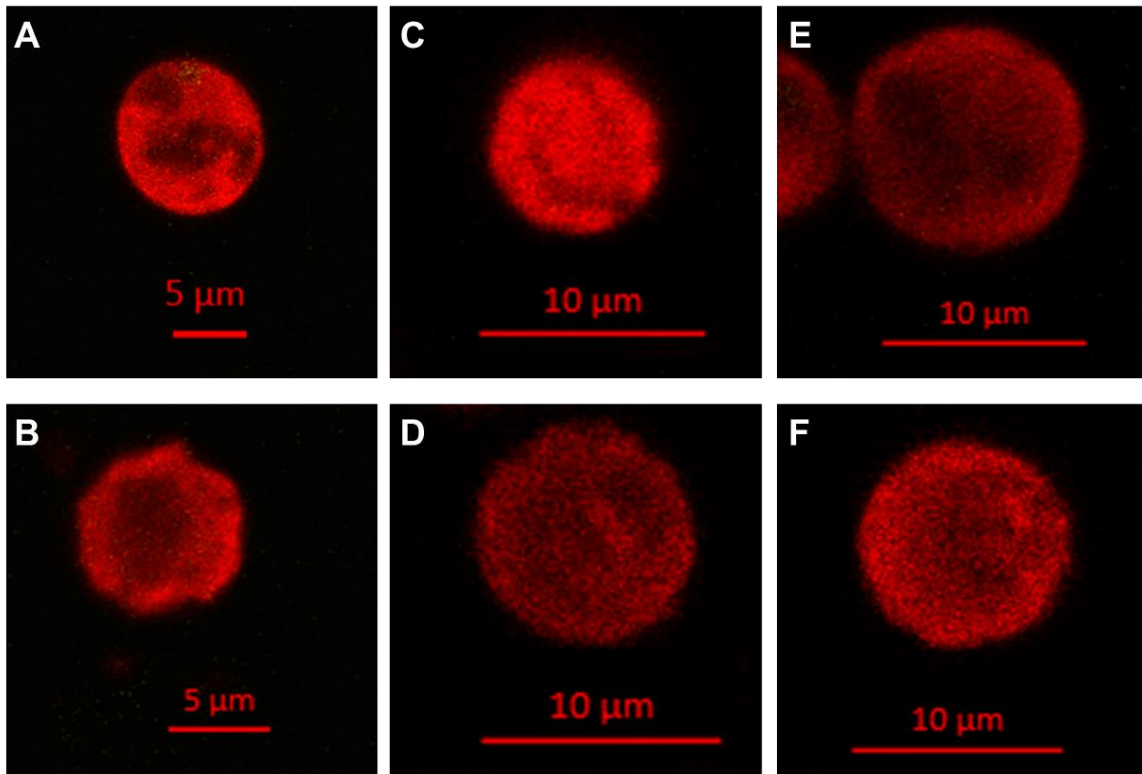


Figure S4: **Negative control.** Anti-AvNPC1 antibody alone (A, C, E) and secondary antibody AF546 alone (B, D, F) immunolabelling against FIS extracted from *A.viridis* (A, B), *A.digitifera* (C, D) and *S.pistillata* (E, F). In red, the autofluorescence of chlorophyll pigments in the *Symbiodinium* cell.

3.4. Discussions et Perspectives

Ces deux études complémentaires nous ont permis de localiser et de caractériser la régulation de l'expression de l'ensemble du répertoire de NPC1 et NPC2, au niveau des transcrits et des protéines chez *A. viridis*.

Ainsi, nous avons mis en évidence la présence d'une seule copie du gène *npc1* chez les anthozoaires. *Avnpc1* est exprimé aussi bien dans l'épiderme que dans le gastroderme et est peu régulé par la condition symbiotique (Figure 17A). Néanmoins, nous avons montré que la protéine AvNPC1 est exprimée au niveau de la membrane perisymbiotique chez *A. viridis* et chez deux espèces de corail (Figure 17B). Nous avons également localisé la protéine AvNPC1 au niveau de la membrane perisymbiotique chez *A. viridis* par marquage immunogold en microscopie électronique. La protéine NPC1 est ainsi le premier marqueur de membrane perisymbiotique démontré chez plusieurs espèces de Cnidaires. Nous avons alors utilisé ce marqueur de membrane perisymbiotique afin d'établir un protocole nous permettant d'obtenir une fraction enrichie en symbiotes possédant la membrane perisymbiotique et une fraction totalement débarrassée de la membrane perisymbiotique (Figure 17B). Ces différentes conditions ont ensuite été analysées par protéomique shotgun et les résultats préliminaires obtenus font l'objet du chapitre 4. De plus, le co-marquage AvNPC1/CFW nous a permis de mettre en évidence qu'il y avait vraisemblablement des modifications dans le recrutement des protéines exprimées à la membrane perisymbiotique en fonction du contenu cellulosique de la paroi cellulaire des symbiotes *in hospite*. Ces résultats suggèrent une dynamique du protéome de la membrane perisymbiotique selon l'état du cycle cellulaire des symbiotes. Dès lors, l'identification des acteurs impliqués dans les différentes phases permettront d'apporter des informations supplémentaires sur le contrôle de l'hôte/symbiote *in hospite*.

Concernant NPC2, nous avons démontré la présence de duplications spécifiques au groupe des Cnidaires par rapport au répertoire présent chez les vertébrés (un seul gène *npc2*). En effet, on note trois copies supplémentaires chez *A. viridis*, issues vraisemblablement d'un mécanisme de rétrotransposition de la copie canonique *Avnpc2-a* et au moins trois copies surnuméraires chez l'ensemble des Cnidaires étudiés.

Parmi les gènes issus de duplications, l'expression très faible des isoformes *Avnpc2-b* et *Avnpc2-c* semble suggérer que ceux-ci sont soit des pseudogènes soit qu'ils sont exprimés à des stades différents de l'adulte.

Encart 1 : Etudes préliminaires chez *Nematostella vectensis*, anémone de mer non symbiotique

Une étude préliminaire que nous avons menée chez l'anémone de mer non symbiotique *Nematostella vectensis*, dans le cadre du stage de Master 2 de Aurélie Dolciami, en collaboration avec l'équipe Embryogenèse, Régénération & Vieillessement de l'Institut de Recherche sur le Cancer et le Vieillessement (IRCAN) à Nice, montre que les gènes *npc2* de *N. vectensis* sont différentiellement exprimés en fonction du stade de développement (œuf, larve, planula, adulte).

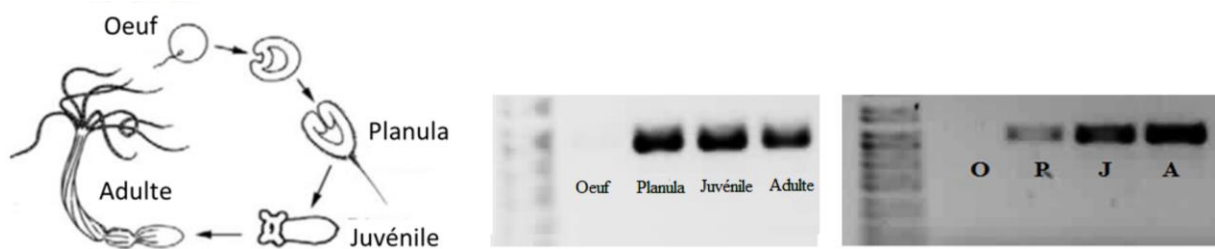


Figure 16: Schéma représentant les différents stades de développement de *Nematostella vectensis*. Au centre et à droite, profils d'expression des gènes *npc2(1)* et *npc2(3)* chez *N. vectensis* en fonction des stades de développement.

Même si des études plus approfondies, notamment par localisation de l'expression par hybridation in situ et immuno-localisation sont nécessaires, ces résultats suggèrent un rôle des gènes *npc* dans le développement. Ce type de régulation a déjà été mis en évidence chez *Drosophila melanogaster* (Huang et al. 2007), dont les gènes *npc2* présentent de nombreuses duplications (Wang et al. 2011; Shi et al. 2012).

Au contraire, au niveau transcriptomique, seul le gène dupliqué *Avnpc2-d* est surexprimé en condition symbiotique et son expression est réprimée lors du stress entraînant la rupture de la symbiose (Figure 17A). Au niveau des protéines, nous avons montré une sub-fonctionnalisation. En effet, la protéine AvNPC2-a est exprimée majoritairement au niveau de l'épiderme alors que la protéine AvNPC2-d est exprimée uniquement au niveau du symbiosome. De plus, dans les deux stress induisant la rupture de la symbiose, l'expression de cette protéine est fortement diminuée et absente chez les individus aposymbiotiques. De façon remarquable, l'expression de la protéine AvNPC2-d n'est plus détectable au-delà de 50% de diminution du stock initial de symbiotes dans les tentacules, pour tous les traitements étudiés. Même si des études complémentaires sont nécessaires, notamment sur une plus grande diversité d'espèces, la protéine AvNPC2-d pourrait donc constituer un marqueur de stress indiquant le point critique de blanchissement, lors de la rupture de l'association symbiotique. Il s'agira donc de déterminer par la suite si ce marqueur peut être généralisé à l'ensemble des cnidaires symbiotiques et permettre ainsi de mettre à disposition des institutions de contrôle, un nouveau marqueur de l'état de santé des coraux.

Afin de poursuivre la caractérisation de la protéine AvNPC2-d, nous avons produit cette protéine en système hétérologue chez des cellules d'insecte (Sf9). Grâce au travail mené par Johana Revel et l'ICN au sein du projet ANR inSIDE, nous sommes actuellement en possession de plusieurs stérols purifiés à partir des tissus d'*A. viridis*. Ces stérols présentent la particularité d'être trouvés en quantités différentes chez des individus symbiotiques par rapport à des individus aposymbiotiques et leur concentration varie également au cours d'un stress. Il s'agira donc de tester leur spécificité d'interaction avec la protéine AvNPC2-d, afin de préciser son rôle fonctionnel dans la symbiose. L'analyse du génome et du transcriptome d'*Aiptasia* a récemment montré que 6 copies de *npc2* existent dans le génome de cette anémone de mer symbiotique (Baumgarten et al., 2015). Ces gènes sont organisés en deux clusters, l'un possédant des introns et la capacité de transporter le cholestérol (*npc2a* et *npc2f*). L'autre cluster, composé de quatre gènes *npc2* (*npc2b*, *c*, *d* et *e*) qui possèdent un ou aucun intron. Ceci suggère d'une part une duplication en tandem des gènes *npc2*. De plus, ils possèdent une mutation dans un domaine impliqué dans la fixation des substrats, qui pourrait donc modifier la capacité de liaison avec le cholestérol (Lehnert et al., 2014). Ces gènes seraient alors impliqués dans le transport des stérols d'origine photosynthétiques et dans la reconnaissance des symbiotes par l'hôte (Baumgarten et al., 2015; Dani et al., 2014; Lehnert et al., 2014).

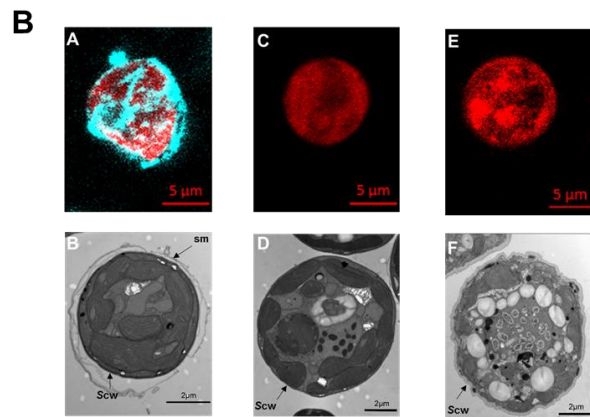
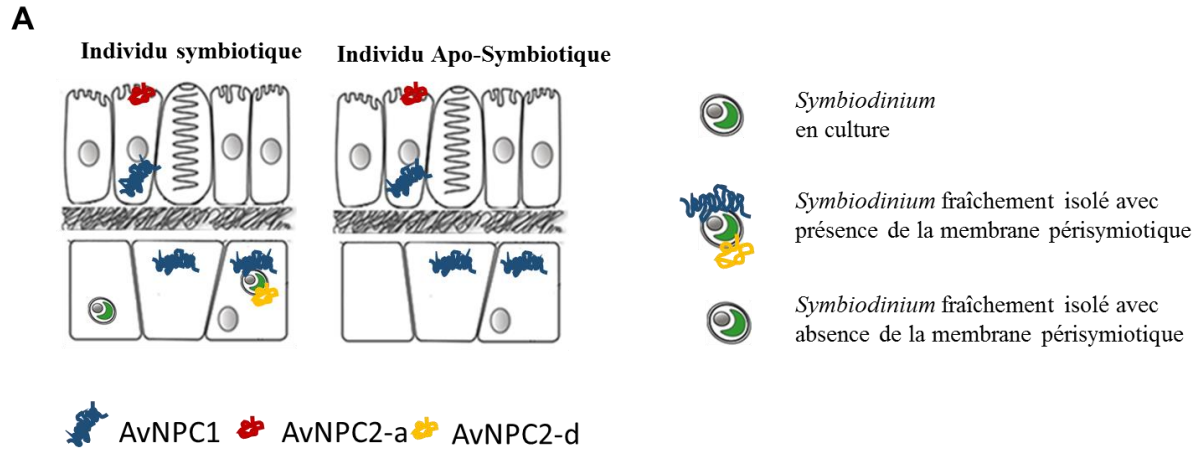


Figure 17: (A) Schéma global de la localisation et la régulation des protéines NPC en fonction de la condition symbiotique chez *Anemonia viridis*. (B) Localisation de la protéine AvNPC1 en immuno-localisation sur des cellules de *Symbiodinium* fraîchement isolées possédant (B-A) ou ne possédant pas (B-C) la membrane pérismyotique, et chez des *Symbiodinium* en culture (B-E). Visualisation en microscopie électronique à transmission des cellules de *Symbiodinium* correspondantes (B-B,B-D,B-F).

Chapitre 4 : Caractérisation de l'interface hôte/symbiote

4.1. Contexte scientifique et problématique de l'étude

Dans les différentes endosymbioses intracellulaires qui font l'objet d'études à l'heure actuelle, telles que la symbiose entre bactéries et insectes (Douglas, 1998), la symbiose plante légumineuse-bactéries *Rhizobium* fixatrices d'azote (Harrison, 2005), la symbiose Cnidaire-Dinoflagellés (Davy et al., 2012a), ainsi que les systèmes parasitaires intracellulaires comme *Toxoplasma gondii* (Sabin and Olitsky, 1937), le processus d'internalisation du symbiote diffère selon les modèles. Néanmoins, la caractéristique commune qui dérive de l'internalisation et du maintien du symbiote dans les cellules de l'hôte est la présence d'une membrane symbiotique à l'interface hôte / symbiote. Cette membrane constitue alors l'interface d'échange entre les deux partenaires. Le symbiosome, défini par Neckelmann en 1983 chez l'hydre, constitue le complexe comprenant les membranes situées autour du symbiote et le symbiote lui-même (Neckelmann and Muscatine, 1983). Chez les Cnidaires symbiotiques, Rands et ses collaborateurs ont montré qu'il s'agissait d'une unique membrane d'origine animale qui dérive de l'internalisation du symbiote par un mécanisme de phagocytose (Rands et al., 1993a). Cependant, en microscopie électronique, la membrane apparaît comme un complexe composé de plusieurs membranes. La mise au point d'un marqueur spécifique de la membrane de l'hôte et de la membrane du symbiote par Wakefield en 2001, a permis de confirmer que ce complexe est composé d'une membrane unique extérieure d'origine animale, et d'une succession d'une ou plusieurs membranes internes (Wakefield and Kempf, 2001b) qui dérivent des phases successives du cycle cellulaire du symbiote (Wakefield et al., 2000a).

Par inhibition de la maturation du phagosome vers le processus de dégradation lysosomale, la membrane du phagosome mature alors en membrane de symbiosome (Hill and Hill, 2012). Cette membrane pérисymbiotique est au centre de l'ensemble des processus permettant la mise en place et le maintien de l'association symbiotique. En effet, de nombreuses études ont montré la présence de motifs glycosylés exprimés à la surface des symbiotes (Logan et al., 2010b). Parallèlement, Weis et ses collaborateurs ont montré la présence de la protéine Sym32, impliquée dans la reconnaissance cellule/cellule, à la membrane pérисymbiotique par des expériences de localisation par immunofluorescence et par marquage immuno-gold (Schwarz and Weis, 2003). Il apparaît donc que la membrane pérисymbiotique a conservé de nombreux acteurs moléculaires impliqués dans la

reconnaissance des symbiotes lors de l'internalisation par le phagosome. Une série d'études menées sur *Aiptasia pulchella* ont également montré que la mise en place du symbiosome était corrélée avec l'expression des protéines Rab4. Cette protéine, exprimée au niveau du phagosome précoce, permettrait la migration du complexe phagosome / symbiote dans les cellules de l'hôte (Hong et al., 2009b). Quant aux protéines Rab5, exprimées au niveau des phagosomes tardifs, elles seraient impliquées dans l'inhibition de la maturation des phagosomes (Chen et al., 2004). Le maintien du symbiosome dans des conditions stables de symbiose semble corrélé à la co-localisation de Rab11 et Rab3 au niveau du symbiosome (Chen et al., 2005; Hong et al., 2009).

L'internalisation du symbiote engendre également un remodelage du cytosquelette. Dans le modèle de parasitisme par *Toxoplasma*, qui appartient au groupe des Apicomplexes, groupe frère des Dinoflagellés, le complexe parasitophore semble intimement lié au réticulum endoplasmique, rendant sa purification très compliquée (Martin et al., 2007; Morrissette and Sibley, 2002). Dans le modèle symbiotique Légumineuse/*Rhizobium*, le cytosquelette est également intimement lié avec l'organisation du symbiosome (Whitehead et al., 1998). Chez *A. viridis*, nous avons pu montrer par microscopie électronique de surface (SEM) (Dani et al., soumise à publication), que le symbiote était enchâssé dans une matrice tubulaire. Ces résultats sont également cohérents avec la présence d'actine d'origine animale et Dinoflagellé, retrouvée par l'équipe de Peng dans l'étude portant sur la caractérisation du protéome du symbiosome chez *Aiptasia pulchella* (Peng et al., 2010a).

Dans des conditions stables de symbiose, cette membrane est à l'interface de nombreux échanges bidirectionnels entre les deux partenaires de la symbiose. Il a été montré que jusqu'à 90% des apports trophiques de l'hôte étaient issus des photosynthétats transmis par le symbiote (Muscatine, 1990). Plusieurs études ont ainsi pu montrer la présence de transports directionnels à travers cette membrane (Bachar et al., 2007; Patton et al., 1977; Whitehead and Douglas, 2003) et notamment des lipides (Dunn et al., 2012; Imbs et al., 2014). En effet, l'étude de Peng a révélé la présence d'une ATP synthase, de transporteurs ABC et de la protéine enolase, impliquée dans la glycolyse (Peng et al., 2010a). Récemment, nous avons mis en évidence la présence à la membrane pérисymbiotique des protéines AvNPC1 et AvNPC2, impliquées dans le transport de stérols, par localisation par immunofluorescence et marquage immunogold (Dani et al., en préparation; Dani et al., 2014a). Cependant, très peu d'acteurs moléculaires impliqués dans les échanges métaboliques ont pu être identifiés. Basé sur des études de transcriptomique, de nombreux acteurs

moléculaires potentiellement impliqués dans la relation symbiotique, ont été possiblement associés à la membrane périsymbiotique ou dans l'espace périsymbiotique, de par leur fonction. C'est le cas par exemple des anhydrases carboniques impliquées dans le transfert de carbone inorganique, de la caténine delta impliquée dans l'adhésion cellulaire (Ganot et al., 2011). Néanmoins, aucune étude n'a pu formellement prouver leur association au symbiosome. Ainsi, malgré une connaissance grandissante concernant les processus d'échanges métaboliques et les acteurs impliqués dans la symbiose Cnidaire-Dinoflagellés, les acteurs moléculaires localisés à l'interface symbiotique, matérialisé par la membrane périsymbiotique et l'espace périsymbiotique, restent à définir dans leur immense majorité (Davy et al., 2012a).

4.2. Approche expérimentale

Les études en microscopie électronique et protéomique ont montré que le symbiosome était intimement lié avec le cytosquelette de la cellule hôte. Cette organisation entraîne une difficulté technique pour isoler et purifier la membrane périsymbiotique. Récemment, par stress mécanique et purification sur gradient de Percoll, l'équipe du Dr Quinnell a mis au point un protocole de purification des membranes du symbiosome (Kazandjian et al., 2008). La pureté de l'extrait de membrane du symbiosome a ensuite été vérifiée par incubation dans le fluorochrome FM 1-43 (FM® 1-43FX, fixable analog of FM® 1-43 membrane stain). Ce marqueur se fixe aux membranes constituées de bicouches lipidiques, permettant de mettre en évidence d'une part la présence de la membrane et l'intégrité de la cellule du symbiote à une concentration optimale de 30 μM . En revanche, l'étude montre qu'une concentration de 50 μM entraîne un marquage des cellules du gastroderme et du cytoplasme de la cellule hôte. Cette technique de séparation mécanique des cellules du gastroderme et des symbiotes est issue d'une publication plus ancienne, parue en 2002 (Trautman et al., 2002). L'étude conduite chez *Zoanthus robustus* (Hexacoralliaire) a démontré que le passage de cellules de gastroderme cinq à sept fois à travers une seringue équipée d'une aiguille de 23 gauge permettait de récupérer des symbiotes dont 73% possédaient la membrane périsymbiotique. L'extrait était également constitué de 6% de cellules endodermiques avec le symbiote et 21% de cellules de symbiote sans membrane. Dans cette étude, la détermination de la présence ou absence de la membrane a été mise en évidence par le marqueur fluorescent MDY-64 (Yeast Vacuole Membrane Marker MDY-64), connu pour se fixer sur les phospholipides, mais

dont la spécificité n'a pas été démontrée. Ce protocole est alors celui utilisé par Peng dans son étude du protéome des membranes du symbiosome chez *Aiptasia pallida* (Peng et al., 2010a). Il apparaît donc plusieurs freins majeurs à la détermination du protéome de la membrane périsymbiotique. Premièrement, la possibilité d'obtenir des extraits purifiés de membrane périsymbiotique semble indissociable d'une contamination avec le cytosquelette de la cellule hôte et donc de protéines cytoplasmiques de la cellule hôte. De plus, l'étude de la membrane périsymbiotique uniquement ne permettrait pas de déterminer les protéines situées dans l'espace périsymbiotique. Enfin, le marquage de la membrane périsymbiotique par des sondes non spécifiques, ne paraît pas pouvoir déterminer avec précision la présence ou l'absence de cette membrane.

Dans ce contexte, nous avons choisi un modèle expérimental différent. En effet, nous avons mis au point un protocole d'enrichissement de fractions de symbiotes possédant ou non la membrane périsymbiotique par stress mécanique, via le passage de cellules de gastoderme isolées à travers une seringue équipée d'une aiguille de 23 gauge. La présence de la membrane est ensuite vérifiée grâce à un marquage par l'anticorps anti-AvNPC1, protéine dont nous avons démontré la localisation à la membrane périsymbiotique dans notre précédente étude (Dani et al., en préparation), développée dans le chapitre 3. Nous avons ainsi pu montrer que 3 passages successifs des cellules de gastroderme isolées dans la seringue de 23 gauge permettaient d'une part de séparer les symbiotes du gastroderme et d'obtenir des fractions enrichies à 60% de symbiotes possédant la membrane périsymbiotique et que 67 passages supplémentaires permettaient d'obtenir une fraction de symbiotes débarrassés à 90% de la membrane périsymbiotique. La comparaison de ces deux conditions par la caractérisation des protéines différenciellement exprimées, permettra d'obtenir la composition d'une part des protéines membranaires localisées à la membrane périsymbiotique, mais également des protéines présentes dans l'espace périsymbiotique. La comparaison avec des échantillons d'épiderme et de gastroderme purifiés permettra également d'affiner nos résultats. Par ailleurs, nous disposons de culture de symbiotes au laboratoire. Ainsi, la comparaison entre le protéome des symbiotes isolés à partir des cellules du gastroderme avec les *Symbiodinium* en culture, pourra nous conduire à définir les modifications du protéome des symbiotes en condition symbiotique. Les extractions et les échantillons protéiques de ces différentes conditions ont été préparés au laboratoire puis séquencés par protéomique shotgun via la technologie de FASP, suivie d'une analyse par NanoLC/Q-exactive développée au laboratoire PRISM (Lille1).

4.3. Résultats

4.3.1. Publication n°5 (soumise)

Johana Revel*, **Vincent Dani***, Fabrice Priouzeau, Mohamed Mehiri, Lionel Massi, Laure Capron, Maxence Wisztorski, Dounia Mouajjah, Kévin Lebrigand, Virginie Magnone, Sophie Pagnotta, Sandra Lacas-Gervais, Marjolijn Mertz, Pascal Barbry, Michel Salzet, Cécile Sabourault The symbiotic interface in the *Symbiodinium-Anemonia* interaction: Insight from proteomics and metabolomics studies. *Symbiosis* (soumise) IF=1.438

Problématique: Quels sont les acteurs moléculaires exprimés à l'interface de communication entre les deux partenaires, caractérisé par la membrane périsymbiotique chez *A. viridis* ?

Outils méthodologiques : Par comparaison des symbiotes pourvus ou non de leur membrane périsymbiotique, nous avons établi le protéome du symbiosome par analyse protéomique shotgun.

Principaux résultats : Identification de 202 protéines associées à la membrane périsymbiotique, protéines impliquées principalement dans la reconnaissance des symbiotes, le remodelage du cytosquelette, les échanges métaboliques et la réponse au stress.

Perspectives : Détermination des modifications éventuelles du protéome de la membrane périsymbiotique en fonction du stade du cycle cellulaire des symbiotes et de l'état symbiotique.

Proteomics and metabolomics provide insight into the

***Symbiodinium-Anemonia* symbiotic interaction**

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Running title: cnidarian-dinoflagellate host-symbiont interface

Abstract

The sea anemone *Anemonia viridis* hosts its photosynthetic dinoflagellate symbionts (*Symbiodinium* spp.) within gastrodermal cells. Symbionts are mainly acquired by a phagocytosis process and are maintained in a symbiotic entity, which is referred to as the symbiosome. The host-derived perisymbiont membrane, or symbiosome membrane, is central to the metabolic exchanges between the two partners. In order to investigate the host-symbiont interface, we used shotgun proteomics and metabolomics to identify proteins and metabolites involved in the symbiotic interaction. Symbiosomes with or without perisymbiont membrane were prepared and we identified 202 proteins that were overexpressed in intact symbiosomes, including proteins involved in recognition, cytoskeletal remodeling, metabolic exchanges and stress response. Characterization and distribution of metabolites differentially expressed between healthy symbiotic and bleached sea anemones allowed the identification of metabolites potentially involved in the interaction, such as betaines and phospholipids. These results indicated that the recruitment of proteins at the symbiosome membrane is a complex and dynamic process. Proteomics and metabolomics results demonstrated that both host and symbionts seemed to contribute to shape the host-symbiont interface. Moreover, the symbiosome membrane is involved in many cellular and molecular processes that underlie the symbiotic interaction.

Introduction

Cnidarian–dinoflagellate endosymbioses have a key role in marine biodiversity as they form both the trophic and structural foundation of coral reef ecosystems, as well as diverse temperate habitats. This association is defined as a holobiont, composed of a cnidarian host, photosynthetic endosymbiotic dinoflagellates of the *Symbiodinium* genus, and a complex bacterial flora. Cnidarians host their unicellular symbionts within the gastrodermal cell layer. The symbiotic association is centered around nutrient exchange. The dinoflagellate symbionts fix large quantities of inorganic carbon and translocate most of their photosynthates to support the host metabolism, growth and reproduction. In return, the dinoflagellates have access to a stable and protected habitat, as well as essential nutrients, such as carbon, nitrogen and phosphorus, in part by recycling host waste products (Davy et al., 2012a). The dinoflagellates may be maternally transmitted or acquired from the surrounding seawater, mainly by phagocytosis (Muscatine et al. 1975). Symbionts somehow arrest the host phagosomal maturation and reside in a vesicular symbiotic compartment defined as the symbiosome (Davy et al., 2012a; Hohman et al., 1982). Indeed, Chen and coworkers demonstrated that *Symbiodinium* survive in symbiosomes by manipulating endosomal trafficking (Chen et al., 2003; Chen et al., 2004), a mechanism that has also been described in many intracellular parasitic associations, such as interactions between Mycobacteria or Apicomplexans and animal cells (Koul et al., 2004; Schwarz et al., 2008a). The symbiosome membrane complex consists of a series of membranes of algal origin and an outer host-derived membrane (Peng et al., 2010a; Wakefield and Kempf, 2001b).

However, the survival of the cnidarian-dinoflagellate association is threatened by global climate change and multiple stressors, including elevated temperature and ocean acidification, leading to symbiosis breakdown and cnidarian bleaching. Bleaching corresponds to a loss of color resulting from the loss of *Symbiodinium* cells and of their photosynthetic pigments (Weis, 2008). Bleached individuals have a drastically reduced survival rate and fitness, as well as an increased susceptibility to diseases (Hoegh-Guldberg et al., 2007). Despite the importance of cnidarian-dinoflagellate associations, especially coral reefs, and the increasing threats that these ecosystems face due to climate change and other environmental perturbations, the molecular and cellular mechanisms that underlie cnidarian-dinoflagellate symbioses remain poorly understood (Davy et

al., 2012a). This includes cellular mechanisms of host-symbiont recognition and metabolic interactions.

Combination of omics technologies, including genomics, transcriptomics, proteomics and metabolomics, started to fill this gap and enhanced our knowledge of the mechanisms that underlie the symbiotic interaction (Davy et al., 2012a; Gordon and Leggat, 2010; Meyer and Weis, 2012). Targeted metabolite analyses were performed to investigate the nutritional role of *Symbiodinium*, as well as the production of defense compounds, such as the UV protecting mycosporine-like amino acids (MAAs, Gordon and Leggat 2010). Many transcriptomic studies unraveled genes involved in symbiont recognition and engulfment, as well as symbiosis maintenance (Davy et al., 2012a; Meyer and Weis, 2012). For example, several studies strongly suggested the putative role of host membrane as signaling and interacting/entry platforms for *Symbiodinium* (Meyer and Weis, 2012). Previous investigations in the sea anemone *Anemonia viridis* suggested the involvement of host membrane proteins, such as Sym32, NPC1 and NPC2-d, and lipid compounds in the symbiotic interaction with *Symbiodinium* clade temperate A (Dani et al., 2014a; Ganot et al., 2011; Moya et al., 2012). However, the symbiosome molecular components and functions are still poorly understood.

The aim of this study was to focus on the host-symbiont interface to appreciate its role in the symbiotic interaction in *A. viridis*. We combined proteomics and metabolomics to characterize the symbiosome membrane proteome, and to identify new mediators of this interaction (lipids and secondary metabolites). In addition, MALDI mass spectrometry imaging was performed to follow the distribution of metabolites on tentacle cross sections from healthy and stressed sea anemones.

Methods

A. viridis culture conditions

Mediterranean *A. viridis* (Forskål, 1775) specimens were collected during the autumn season in 19°C shallow waters at Villefranche-sur-Mer (France). The only *Symbiodinium* clade hosted by *A. viridis* is the clade “temperate A”, which is mainly acquired by vertical transmission. Sea anemones were maintained in laboratory-controlled conditions where they were acclimated for one month before experimentation. Seawater was maintained at $19 \pm 0.5^\circ\text{C}$ in aquaria and renewed weekly. Sea anemones were fed brown shrimp once a week. Light was provided at a constant irradiance of $180 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ on a 12/12 h light/dark cycle with a metal halide lamp (HQI-TS 400 W, Philips). To avoid contamination of the protein and RNA samples, all specimens were starved throughout the stress experiment (days 0 to 22), excepted for normal specimens that were maintained under standard laboratory conditions. At the end of the stress experiment (day 22), bleached specimens were maintained under control temperature, feeding and water renewal conditions.

For the menthol treatment, symbiotic *A. viridis* specimens (n=3) were incubated in 0.58 mM menthol for 22 days under a 8/16 h exposure/resting cycle and normal temperature, with daily seawater renewal, according to Dani et al. (Dani et al., soumise à publication) . For each specimen, tentacles were sampled and processed immediately at the following days: d0, d1, d5, d10, d15, d22 and d59. All samplings were performed at 10:00 am to avoid circadian rhythm variations (Levy et al., 2011).

Maintenance of cultured *Symbiodinium*

Three single-cell-derived cell lines of *Symbiodinium* spp. (clade temperate A) were originally isolated from a single specimen of *A. viridis* and established as clonal in vitro cultures at the University of Nice (MarEquipe Symbiose Marine, UMR 7138). The *Symbiodinium* cells were cultured in f/2 medium, supplemented with 1% antibiotic-antimycotic solution cocktail (Sigma). Cultures were routinely maintained at 24 °C and 90% humidity, under a standard 12 h/12-h light/dark cycle using an irradiance of $200 \mu\text{mol quanta/m}^2/\text{s}$ (JBL; Solar Ultra Marin Day) in a controlled cabinet (Witeg Labortecnik GmbH; WiseCube model WGC-450).

Immunofluorescence microscopy on tissue section

Tentacles of symbiotic *A.viridis* specimens were fixed overnight in 4% paraformaldehyde. Cryosections (12 µm) were incubated in PBS containing 0.1% Triton X-100 (PBT), then blocked for 2 hr in PBT containing 1% BSA. Primary antibody (rabbit anti-Av NPC2-d antibody) was incubated at 1:50 overnight at 4°C and secondary antibody (anti-rabbit Alexa 546 conjugated; Thermo Fisher Scientific Inc.) was used at 1:500 for 2 hr at room temperature (according to Dani et al. 2014). Fluorescence images were acquired using a Zeiss LSM 710 confocal laser-scanning microscope, on an inverted Axio Observer.Z1 stand (Carl Zeiss Microscopy GmbH, Jena, Germany), using objectives Plan Apo 63X oil 1.4 NA. The LASERs used were LASER diode 405 nm and/or Argon LASER (458, 488, 514 nm) and/or HeNe 594 nm.

Immunofluorescence microscopy on freshly isolated *Symbiodinium*

To obtain freshly isolated *Symbiodinium* (FIS) from *A.viridis*, gastrodermal (symbiont-containing tissue layer) and epidermal cell layers were separated as described previously (Richier et al., 2006). Gastrodermal cell layer were homogenized in filtered sea water (0.2 µm) and centrifuged at 500 x g for 3 min to pellet *Symbiodinium* cells. A 500 µl suspension of endodermal cell (>5.10⁵ cells/ml) was drawn gently back and forth through a 23 gauge metal hypodermic needle fitted to a 5 ml syringe. Three plunges of the syringe were required to remove gastrodermal tissue and to obtain intact symbiosome with the perisymbiont membrane. Additional plunges were required to obtain *Symbiodinium* free of perisymbiotic membrane without damaging the *Symbiodinium* cell integrity (Dani et al., en préparation). Three additional washing step were required to remove cell membrane fragments after syringe step.

To confirm the localization of AvNPC1 on the symbiosome, FIS samples were incubated in PBS containing 0.1% Triton X-100 (PBT), then blocked for 2 hr in PBT containing 1% BSA. Primary antibody (rabbit anti-Av NPC1 antibody, 1:100), was incubated overnight at 4°C and secondary antibody (anti-rabbit Alexa 546 conjugated (1:500; Thermo Fisher Scientific Inc.) for 2 hr at room temperature. Samples were wash 3 times in PBS and were trapped onto the

polycarbonate filter (Isopore, 0.4 μ m HTBP, HTBP01300, Millipore, Billerica, MA, USA) by gentle vacuuming. The filter was put onto a slide glass, seal with Fluoromount™ Aqueous Mounting Medium (F4680 Sigma) to prevent dehydration and oxidation, covered with a cover slip, and kept in the dark. Fluorescence images were acquired using a Zeiss LSM 710 confocal laser-scanning microscope.

Filter-aided sample preparation (FASP)

For shotgun proteomics analysis, FASP protocol was used as previously described (Zougman et al., 2009). We performed FASP using Microcon filter units YM-30 (Millipore). Trypsin (Promega) was used for protein digestion at 40 μ g/ml in 50 mM NH_4HCO_3 . The samples were incubated overnight at 37°C. The digests were collected by centrifugation, and the filter device was rinsed with 50 μ l of NaCl 0.5 M. Next, 5% TFA was added to the digests, and the peptides were desalted with a Millipore ZipTip device before LC-MS/MS analysis. Three biological replicates were analyzed: FIS were extracted from pools of 10 tentacles from 3 independent sea anemones.

LC MS/MS analysis

The samples were separated by an online reversed-phase chromatography using a Proxeon Easy-nLC1000 system (Thermo Scientific) equipped with a Proxeon trap column (100 μ m ID x 2 cm, Thermo Scientific) and a C18 packed-tip column (75 μ m ID x 50 cm, Thermo Scientific). The peptides were separated using an increasing amount of acetonitrile (5-35% over 100 min) at a flow rate of 300 nl/min. The LC eluent was electrosprayed directly from the analytical column, and a voltage of 1.7 kV was applied via the liquid junction of the nanospray source. The chromatography system was coupled to a Thermo Scientific Q Exactive mass spectrometer that was programmed to acquire in a data-dependent Top 10 MSMS method. The survey scans were acquired at a resolution of 70,000 at m/z 400 and MSMS scan with a resolution of 17,500 and an isolation window of 4 m/z.

Protein identification and bioinformatic analysis

MS/MS data were processed using Andromeda search engine (Cox et al., 2011) included in MaxQuant (Cox and Mann, 2008) (version 1.5.3.8). Proteins were identified by searching MS

and MS/MS data against Decoy version of the complete proteome for Homo sapiens of the UniProt database (Release June 2015, 147,854 entries) combined with a custom database of *Anemonia viridis* proteins and a 262 commonly detected contaminants. The *A. viridis* protein database was prepared from RNA sequencing of symbiotic and aposymbiotic specimens (SOLiD technology, clustering using TGICL, ORF prediction using transcoder and annotation using InterProScan). Peptides were also searched against the *Nematostella vectensis* predicted proteome. Digestion mode was set as Trypsin specificity, with methionine oxidation and N-terminal acetylation selected as variable, carbamidomethylation of cysteines set as a fixed modification and we allow up to two missed cleavages. For MS spectra an initial mass accuracy of 6 ppm was selected and the MS/MS tolerance was set to 20 ppm for HCD data. The FDR at the peptide spectrum matches (PSM) and protein level was set to 0.01. Relative label-free quantification (LFQ) of proteins was done using the MaxLFQ algorithm (Cox et al., 2014) with the default parameters.

Perseus software (<http://www.perseus-framework.org/>) (version 1.5.2.4) was used for analysis of the proteins identified. Results were filtered to remove proteins only identified with modified peptides, hits to the reverse database and potential contaminants. Then the LFQ intensity were logarithmized ($\log_2(x)$). Significantly differential expression were determined using a t-test and visualized by a volcano plot. ANOVA with a FDR of 5% was also performed. Visualization of the data was done by hierarchical clustering of significantly different proteins using Euclidean distance.

Chemicals and equipments for metabolomics analysis

All organic solvents used for material extraction were of analytical grade and purchased from Sigma-Aldrich. HPLC purifications were carried out on a Waters 600 system equipped with a Waters 717 plus autosampler, a Waters 996 photodiode array detector, and a Sedex 55 evaporative light-scattering detector (SEDERE, France). Detection wavelengths were set at 214, 254 and 280 nm. ^1H and ^{13}C NMR spectra were recorded with 500 MHz Bruker Avance NMR spectrometers. Chemical shifts (δ) are recorded in ppm with $(\text{CD}_3)_2\text{SO}$ (δ_{H} 2.5 ppm and δ_{C} 39.5 ppm) as internal standard with multiplicity (s singlet, d doublet, t triplet, m multiplet). HRESIMS was performed on a LTQ Orbitrap mass spectrometer (Thermo Finnigan). MS and MSn analysis were carried out on a Finnigan LCQ advantage ion trap mass spectrometer (Thermo, formerly Finnigan-MAT, San Jose, CA, USA) equipped with an ESI interface and an ion trap mass analyzer. The system is

managed by the Xcalibur software suite v2.0. The operating parameters were as follows: damping gas, helium (He); nebulizing gas, nitrogen (N₂); maximum injection time was set at 50 ms; the number of microscans was set at 3. For ESI in negative and positive mode, the source parameters were set as follow: Ionspray voltage 4.5kV, capillary temperature 250 °C; sheath gas (N₂), 50 (arbitrary units); auxiliary gas, 10 (arbitrary units). For MS/MS experiments, CID was carried out using Helium as collision gas. The collision energy is reported as a percentage of the maximum 5 V_{p-p} normalized for the parent ion *m/z* (NCE: Normalized Collision Energy). Collision energy for CID was optimized between 25% and 35% of maximum, and the isolation with of precursor ions was 2.0 amu.

Extraction and isolation of metabolites

A. viridis specimens were freeze-dried and ground to obtain a dry powder (8.5 g), which was exhaustively extracted with a mixture of MeOH/CH₂Cl₂ (1:1, v/v) to yield 1.7 g of the crude extract after concentration under reduced pressure. The crude extract was portioned between MeOH (F1 fraction) and cyclohexane (F2 fraction). The F1 fraction (600 mg) was portioned between dichloromethane (F3 fraction, 301 mg) and water (F4 fraction, 282 mg).

F4 fraction was then subjected to semi-preparative HPLC-DAD purification (Macherey Nagel HILIC, 250×10 mm id, 5 μm) with a gradient of H₂O/MeCN 10:90 to 67:33 over 70 min) (flow: 2.5 mL.min⁻¹, injection volume: 80 μL) to afford pure homarine (11.6 mg), trigonelline (5.1 mg), and zooanemonine (7.4 mg). All the compounds were identified by a combination of spectroscopic methods (1D and 2D NMR and/or MS and MS/MS) and comparison with the literature data.

F1 fractions of both *A. viridis* and cultured *Symbiodinium* were also subjected to LC-HRMS analyses (accucore C18 column, 100×2.1 mm id, 2.6 micrometers) to identify and compare less abundant metabolites.

MALDI Mass spectrometry imaging

DHB (20mg/ml dissolved in 7:3 methanol/0.1% TFA, v/v) was applied on the tissue section using a manual sprayer at 300μl/min flow rate. MALDI MSI experiments were performed on an Ultraflex II MALDI-TOF/TOF instrument (Bruker Daltonics, GmbH) equipped with a smartbean

II laser (Nd:YAG, 355nm). Mass spectra (mass range of m/z 100 to 1,000) were acquired in reflector positive ion mode. Distance between each points was set to 20µm and a total of 800 laser shots were accumulated at 200Hz repetition rate. Data visualization was performed using SCiLS lab (version 2015a). An alignment of the spectra between each image data set were performed.

Results

Targeted and shotgun proteomics

Based on the results obtained from transcriptome analyses used to decipher the molecular dialogue between *A. viridis* and its dinoflagellate symbionts, AvNPC proteins were further characterized by investigating their cellular localization. Specific antibodies were developed against AvNPC1 and AvNPC2-d, which corresponds to one of the duplicated AvNPC2. We used the anti-AvNPC2-d antibody in fluorescent immunolocalization experiments on tentacle tissue section. We demonstrated that this protein was mainly expressed in the gastrodermis of symbiotic sea anemones and was surrounding *Symbiodinium* cells (Fig.1a). As NPC2 is working with NPC1 within vertebrate endosomes, we hypothesized that AvNPC1 may be expressed at the host-derived symbiosome membrane in symbiotic cnidarians (Fig.1b). To confirm this hypothesis, freshly isolated *Symbiodinium* cells were therefore extracted from symbiotic sea anemones and hybridized with the specific anti-AvNPC1 antibody. Fig.1c showed that AvNPC1 was also surrounding *Symbiodinium* cells, a signal that supported the expected localization of AvNPC1 at the symbiosome membrane. In contrast, no labelling was recorded when the anti-AvNPC1 antibody was used on cultured *Symbiodinium* (Fig.1c).

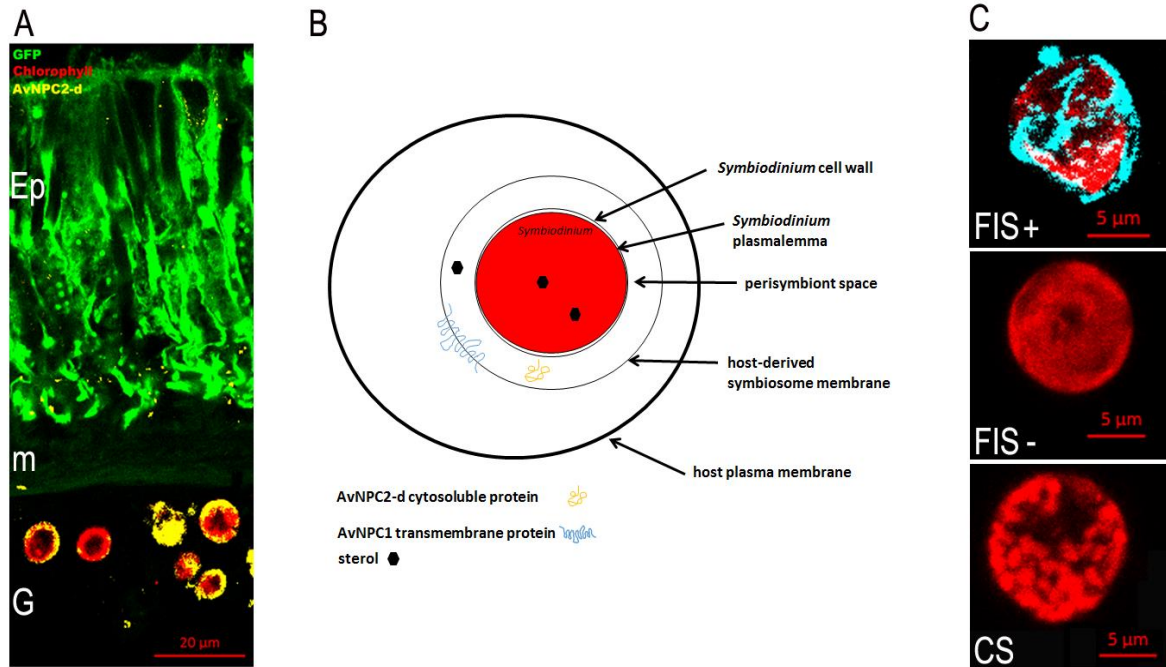


Figure 1: Immunolocalization of AvNPC1 and AvNPC2-d in the *A. viridis* holobiont. **A**, Immunolabelling showing the AvNPC2-d protein (yellow) surrounding dinoflagellate symbionts (red) within the gastrodermis of a tentacle cross section of *A. viridis*. Basal autofluorescence of the tissue (endogenous cnidarian GFPs in green and dinoflagellate chlorophylls in red). Ep, epidermis layer; m, mesoglea; G, gastrodermis layer. **B**, Model of the subcellular location of AvNPC proteins and their putative role in endosomal/lysosomal transport of symbiosis-derived sterols in a symbiotic gastrodermal cell. **C**, Immunolabelling of AvNPC1 protein (in blue) on *A. viridis* freshly extracted *Symbiodinium* (FIS) and cultured *Symbiodinium* (CS, lower panel). Half of the pool of FIS (FIS+, high panel) was treated by vigorous mechanical disruptions to remove the symbiosome membrane (FIS-, middle panel). The red corresponds to the natural autofluorescence of *Symbiodinium* chlorophyll pigments and the blue to the anti-AvNPC1 immunolabelling.

We therefore decided to use the anti-AvNPC1 as a specific marker of symbiosome membrane to follow symbiosomes purification in order to initiate a wider study dedicated to proteins expressed at the symbiotic interface using shotgun proteomics. First, a pool of FIS was prepared from symbiotic *A. viridis* tentacles and the presence of the symbiosome membrane was confirmed using the anti-AvNPC1 antibody (FIS+ sample, Fig.1c). Half of this pool, named FIS-, was then treated by vigorous mechanical disruptions using a 23-gauge syringe needle to remove the symbiosome membrane. Hybridization with the anti-AvNPC1 antibody showed that most of the treated FIS had lost their AvNPC1 labelling compared to the initial pool (Fig.1c), thus confirming the removal of the symbiosome membrane. Proteins were extracted from these two

samples, i.e. FIS with (FIS+) or without (FIS-) symbiosome membrane, digested by trypsin and analyzed by LC-MS/MS. Proteins were identified by searching against the human complete proteome as well as an *A. viridis* custom protein database. A total of 700 and 557 proteins were identified in the FIS+ and FIS- samples, respectively. Statistical analyses were performed to identify proteins that were significantly differentially expressed. A hierarchical clustering of differentially expressed proteins quantified in the three replicates is presented in Fig.2. ANOVA and t-test gave similar results, 303 proteins being identified as differentially expressed after the t-test and 299 proteins after the ANOVA test (all recorded within the 303 previously identified proteins).

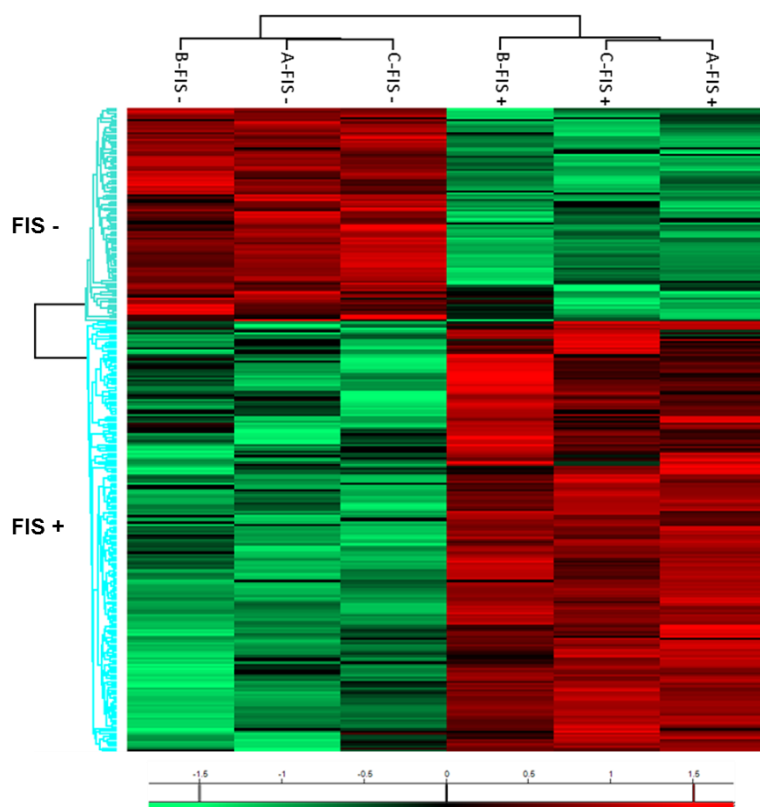


Figure 2: Comparison of symbiosome protein expression using label-free quantification (LFQ) protein analysis. Freshly isolated *Symbiodinium* cells (FIS) were extracted from symbiotic *A. viridis* sea anemones (n=3; A, B, C). Half of each pool of FIS (FIS+ sample) was treated by vigorous mechanical disruptions to remove the symbiosome membrane (FIS- sample). Protein were extracted from FIS with (FIS+) or without (FIS-) symbiosome membrane and analyzed by shotgun proteomics. The heatmap presents 303 differentially expressed proteins. Rows have been hierarchically clustered using the Euclidian distance between expression value vectors. Proteins grouped into two clusters on the basis of the expression similarity. Green and red indicate the lowest and the highest expression, respectively. The color scale bar is shown at the bottom of the figure.

Results from the t-test are presented as a volcano plot (Fig.3). Among the 303 differentially expressed proteins, 202 were specific of symbiosome samples (FIS+), and 101 were more expressed in the FIS samples that were depleted of symbiosome membrane (FIS-). Many of these protein sequences have not been functionally annotated because no known protein signature could be found using InterProScan. Nevertheless, Table 1 listed the proteins that were specifically expressed in symbiosome samples and previously reported as possible components of the symbiosome membrane in other symbiotic cnidarians. According to these previous studies, we found proteins involved in cell adhesion and recognition, such as lectins and Sym32, endocytosis and cytoskeleton remodeling, response to stress and solute/nutrients, including carbonic anhydrases, proton ATPases and AvNPC proteins. AvNPC2d was found specifically associated to symbiosome samples. However, AvNPC1, which is a transmembrane protein, was not identified in all replicates, probably because we failed to extract exhaustively and reproductively all the symbiosome transmembrane proteins. In contrast, most of the proteins overexpressed in the FIS- samples were involved in photosynthesis, such as chlorophyll binding proteins, response to stress and protein folding, such as chaperonins and heat-shock proteins, and recognition, including lectins.

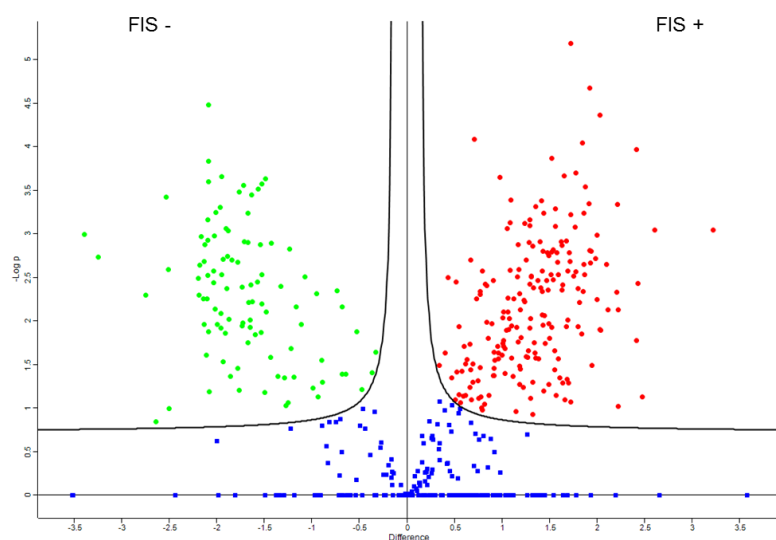


Figure 3: Volcano plot showing differentially expressed proteins in symbiosome using label-free quantification (LFQ) protein analysis. Freshly isolated *Symbiodinium* cells (FIS) were extracted from symbiotic *A. viridis* sea anemones (n=3). Half of each pool of FIS (FIS+ sample) was treated by vigorous mechanical disruptions to remove the symbiosome membrane (FIS- sample). Protein were extracted from FIS with (FIS+) or without (FIS-) symbiosome membrane and analyzed by shotgun proteomics. Volcano plot of protein intensity difference and significance ($-\log p$ -value) using a t-test. Protein intensity differences were identified in three biological replicates (A, B, C). Significantly overexpressed proteins in FIS+ are annotated as red circles (permutation-based false discovery rate 0.05).

Table 1: Candidate proteins expressed at the host-symbiont interface

Protein name	Biological process	Reference
Sym32	Cell adhesion	Schwarz et al. 2003
Lectin C-type	Cell surface receptor	Vidal-Dupiol et al. 2009
Rab5	Endocytosis	Chen et al. 2004
HSP70	Response to stress	Peng et al. 2010
Actin	Cytoskeleton organization	Peng et al. 2010
V-type proton ATPase	Transmembrane transport	Barott et al. 2015
Carbonic anhydrase	Intracellular pH regulation	Bertucci et al. 2010
NPC2-d	Lipid transport	Dani et al. 2014
Enolase-1	Canonical glycolysis	Peng et al. 2010

Targeted metabolomics and mass spectrometry imaging

Lipid and other metabolites were investigated in order to identify compounds involved in the symbiotic interaction. Metabolites were extracted from *A. viridis* specimens using different combinations of solvents. Fractions were analyzed by HPLC and purified compounds were identified using NMR and/or MS. Comparison of samples, such as symbiotic vs aposymbiotic specimens and epidermis vs symbiont-containing gastrodermis tissue, first allowed the characterization of the main lipids found within the *A. viridis* holobiont (Revel et al.). Comparison of FIS and non-symbiotic cultured *Symbiodinium* cells was also investigated and several metabolites are still under identification. Bleaching of symbiotic *A. viridis* specimens was induced by exposition to menthol, using a protocol that was previously developed in the lab (Dani et al., soumise à publication). Differentially expressed metabolites were identified by comparison between tentacles that were sampled from healthy symbiotic sea anemones (day 0 of the experiment) and from menthol-induced bleached specimens (day 59).

Among the differentially expressed polar metabolites contained within the fraction 4, we first focused on UV-absorbing structures, a chemical property that was used to perform purification of these compounds. An HPLC-UV chromatogram of polar compounds extracted from symbiotic *A. viridis* specimens showed three main signals (Fig.4a and Fig.S1). These compounds were purified and characterized using a combination of ^1H and ^{13}C NMR spectroscopic methods. Isolation and purification of pure products have been performed using semi-preparative HPLC-DAD to obtain large quantities of compounds 1 to 3, 11.6 mg, 5.1 mg, and 7.4 mg, respectively. Peaks 1 and 2 are related to each other and have been identified as homarine and trigonelline, respectively. ^1H and ^{13}C NMR spectra are given as supplementary information for both homarine (Fig.S2 and S3) and trigonelline (Fig.S4 and S5). The third peak has been identified as zooanemonin (Fig.S6 and S7). These 3 compounds were also found in FIS extracts, while they were barely detectable in cultured *Symbiodinium*. An additional compound was detected, but at a low concentration that did not allow its purification. Comparison of HPLC-MS profiles (Fig.S8) showed that this compound was found both in the FIS fraction of symbiotic *A. viridis* and in cultured *Symbiodinium*, at a much higher concentration. MS/MS analyses were performed on these extracts and allowed the identification of this compound as the gonyol (Fig.S9 and Table S1). All these 4 structures corresponded to zwitterionic compounds that are derived from amino acids and belong to the chemical group of betaines.

Interestingly, HPLC profiles clearly showed that both homarine and trigonelline expression were much lower in menthol-induced bleached specimens compared to healthy symbiotic sea anemones (Fig.4b). In contrast, expression of zooanemonin remained unchanged during the bleaching stress experiment. As these compounds were detected in symbiotic *Symbiodinium* cells (FIS), but not in free-living cultured algae (CS), we hypothesized that these betaines might be related to the symbiosis.

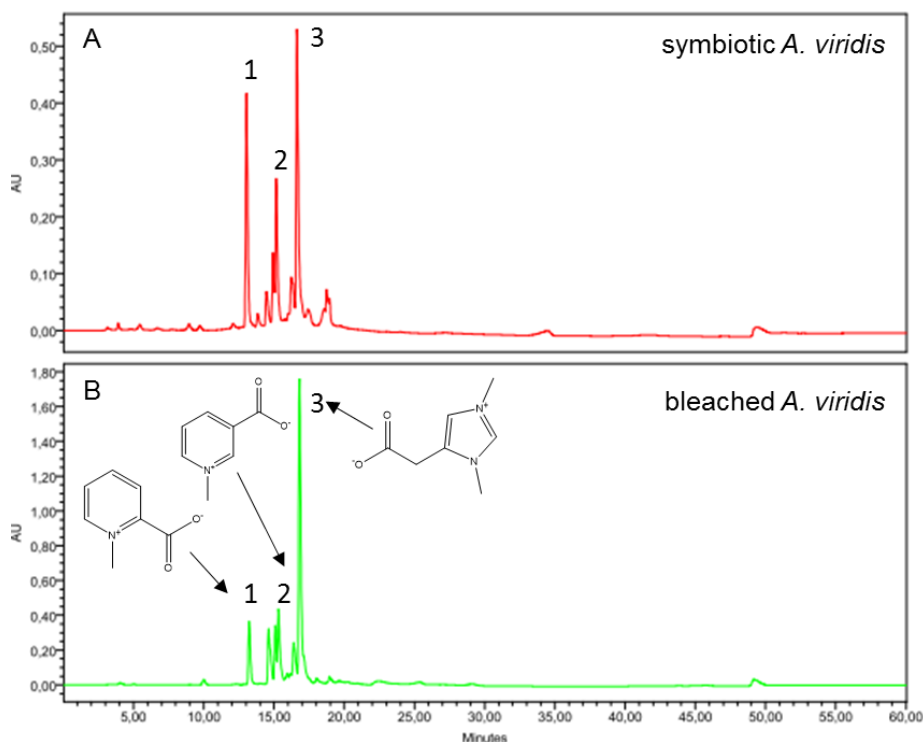


Figure 4: HPLC-UV chromatogram of *A. viridis* polar compounds (214 nm). **A**, symbiotic sea anemone; **B**, menthol-induced bleached sea anemone (after 59 days). Identified betaines: 1, homarine; 2, trigonelline; 3, zooanemonin.

In parallel to classical metabolomic analytical methods, that required large quantities of biological material, we adapted the MALDI-MS imaging technology to identify and localize metabolites directly in sea anemone tentacle sections (Kopp et al., 2015). Unfortunately, MALDI-MS imaging did not allow the detection of zwitterionic compounds that had molecular weight outside the mass range of the mass spectrometer. Symbiotic sea anemones were treated by menthol to induce symbiosis disruption, as described in Dani et al. (Dani et al., soumise à publication). Tentacle tissue sections were performed at each time point during 59 days and metabolites were recorded by MALDI-MS imaging (Fig.S10). Figure 5 illustrated changes in metabolite expression in menthol-treated *A. viridis* specimens. The remarkable ion m/z 872.761 colocalized with *Symbiodinium* cells within the gastrodermis tissue layer. The signal remained almost stable during the 5 first days of treatment but strongly decreased at day10, which corresponded to a massive symbiont loss (Dani et al., soumise à publication). Furthermore, this ion is almost undetectable in tentacles of bleached specimens at the end of the experiment (day59). A more accurate m/z value was obtained using MALDI-orbitrap analyses and comparison of this value with structures listed

in the LIPID MAPS database returned different possible isobaric structures, all referred as glycerophosphoserines. Identification of this compound is still under investigation.

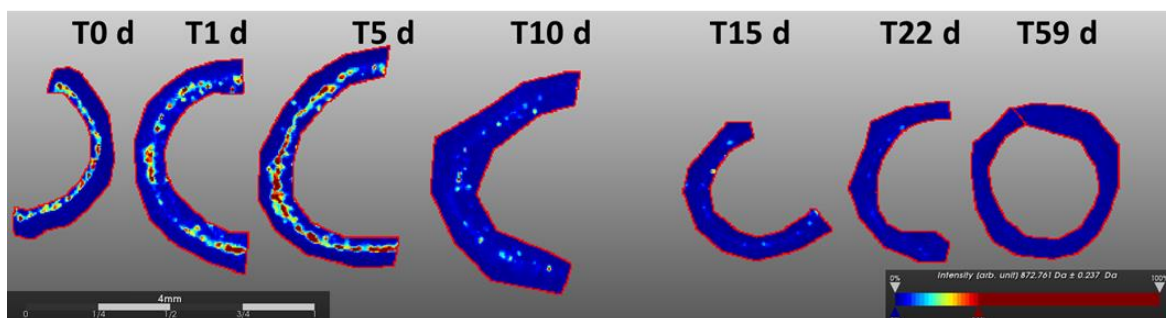


Figure 5: Molecular images of the metabolite detected at m/z 872.761 from a tentacle cross section using MALDI-MSI. *A. viridis* symbiotic specimens were treated with menthol to induce bleaching. Tentacle cryosections of 10 μm thickness were performed at the following days: d0, d1, d5, d10, d15, d22 and d59. Distribution of the m/z 872.761 ion in sections from day0 to day5 overlapped with *Symbiodinium* cells within the gastrodermal tissue (Fig. S10). The peak corresponding to this ion is absent from bleached specimens. Intensity color scale bar is given at the bottom right.

Discussion

Identification of symbiosis-related proteins or metabolites in both cnidarian host and dinoflagellate symbionts is crucial to unravel key processes involved in endosymbiosis (Davy et al., 2012a). In this study, we focused on the host-symbiont interface and we characterized healthy or stressed symbiosomes using proteomics and metabolomics. However, these investigations required preparation of samples without contamination from other tissues, such as host gastrodermal and epidermal cells. The first challenge was therefore to improve symbiosomes purification and to confirm their purity and intactness. Only two studies that described symbiosome preparation and characterization have been published so far. The first was reported for the zoanthid *Zoanthus robustus* (Kazandjian et al., 2008). In that study, the authors used mechanical disruption of host tissues then a discontinuous sucrose gradient centrifugation step to purify symbiosomes. However, cellular integrity of symbiosomes remained unclear because the authors used the fluorochrome FM 1–43, which is a non-specific membrane probe. An exciting study was published two years later by Peng and coworkers (Peng et al., 2010a). Intact symbiosomes were purified from the sea anemone *Aiptasia pulchella* and subjected to biotinylation in order to label membrane

surface proteins, which were separated by 2D-electrophoresis then identified by MS. However, if the identified proteins were likely candidates involved in symbiosis-related processes, only 17 known proteins were found using this protocol. To make a compromise between sample high purity and quantity we have chosen to use shotgun proteomics to perform a comparative study of intact symbiosomes *vs* FIS that were depleted from their symbiosome membrane (Dani et al., en préparation).

Previous transcriptomic studies proposed that the NPC genes are involved in the symbiotic interaction in various cnidarians (Bertucci et al., 2015b; Dani et al., 2014a; Ganot et al., 2011; Lehnert et al., 2014; Moya et al., 2012). As NPC are endosomal sterol binding proteins in vertebrates (Vance, 2010a), we hypothesized that they may be localized in the phagosome derived symbiosome of *A. viridis*. Using specific antibodies raised against AvNPC2d and AvNPC1, we demonstrated that these proteins are expressed around *Symbiodinium* cells (Fig.1), as expected previously (Dani et al., 2014a; Ganot et al., 2011). These antibodies were therefore used to optimize the protocol of symbiosome purification and to follow the purity of fractions. We finally chose to use the anti-AvNPC1 antibody to check the status of each fraction, because AvNPC1 was a transmembrane protein expressed at the symbiosome membrane (Fig.1c). The signal was more reproducible than with the anti-AvNPC2-d, which is a cytosoluble protein expected to be expressed within the perisymbiont space (Fig.1b; Dani et al. 2014). Following a protocol adapted from Trautman et al. that used mechanical disruption of membranes (Kazandjian et al., 2008), we managed to obtain fractions of FIS with intact symbiosome membrane (FIS+ sample) and FIS that were depleted of the symbiosome membrane (FIS- sample)(Dani et al., en préparation). Proteins were extracted from these samples and analyzed by shotgun proteomics.

Proteins were identified by searching against a custom *A. viridis* protein sequence database, but these sequences have not been fully functionally annotated. The FIS- dataset corresponded to proteins that were more expressed in freshly isolated *Symbiodinium* without perisymbiont membrane of animal origin, which was removed by mechanical treatment. This dataset mainly contained protein sequences of *Symbiodinium* origin, such as photosystem proteins, chlorophyll binding proteins and cytochrome c-550. Some peptides were identified as protein sequences of animal origin, such as actin, ATP synthase, lectin-C type and chaperones. The first hypothesis that may explain this result was a partial removing of the symbiosome membrane from some FIS. The

second hypothesis, which was more exciting, suggested that these proteins were tightly associated to the *Symbiodinium* cell wall, as it might be the case at least for the lectins.

Among the 202 proteins that were identified in *A. viridis* symbiosomes (FIS+), we found most of the proteins described by Peng et al., such as actin, HSP70, and enolase, but also many targeted proteins that were shown to be expressed at the symbiosome membrane in various species, using other techniques, including specific antibodies (Table 1). This included proteins involved in cytoskeleton remodeling (actin), endocytosis and vesicular trafficking (Rab proteins), cell adhesion and recognition (Sym32, enolase, lectins), solute transport and metabolism (V-type proton ATPase, carbonic anhydrase, enolase, NPC2-d) and response to stress (enolase, HSP70). NPC1, which was not systematically found in all FIS+ samples, was not statistically differentially expressed. This was probably because NPC1 is a multi-pass transmembrane protein and we did not optimize yet the purification protocol to exhaustively extract all membrane proteins. Nevertheless, the identification of both AvNPC2-d and AvNPC1 in our FIS+ dataset, in addition to proteins previously described to be expressed at the symbiosome membrane, clearly validated the sample preparation and analyses. All the proteins mentioned above were previously defined as symbiosis-related proteins because they displayed a transcriptional regulation and they were found overexpressed in symbiotic cnidarians compared to aposymbiotic or stressed/bleached specimens. Except for enolase, the possible role of these proteins in the symbiotic interaction has also been extensively discussed in previous studies (see references in Table 1), as well as in the review of Davy et al. (Davy et al., 2012a). Enolase is a multifunctional enzyme that was first described for its role in glycolysis (Pancholi, 2001). Besides its metabolic role, enolase plays a role in various processes such as growth control, hypoxia tolerance and immune responses in vertebrates (Pancholi, 2001). In cnidarians, enolase was not shown to be regulated at a transcriptional level, a result that might explain the relative lack of information. However, enolase was first reported to be expressed at the symbiosome membrane by Peng et al. (Peng et al., 2010a). Interestingly, the enzyme is functioning in acidic condition, which is also the property of the symbiosome microenvironment that is surrounding *Symbiodinium* cells (Barott et al., 2014). Enolase is usually cytoplasmic but can also translocate to the plasma membrane where it is expressed at the cell surface and acts as a plasminogen receptor (Pancholi, 2001) , or can be secreted in exosomes (Wubbolts et al., 2003). Once the plasminogen/plasmin system has been activated, it elicits cellular responses such as metalloprotease activation, as well as cell adhesion, migration and growth.

Interestingly, enolase of the protozoan parasite *Leishmania Mexicana* was found associated with the external face of the parasite plasma membrane and exerted the function of plasminogen receptor at the surface of the parasite. As such, enolase is thought to facilitate invasiveness and establishment of infection of parasite within the host. Given these details, we can hypothesize that enolase may promote the infection success of *Symbiodinium* within gastrodermal cells.

Betaine--homocysteine S-methyltransferase 1 (BHMT) was a protein found in the FIS+ dataset but not identified in all samples. This protein was somewhat related to the symbiotic interaction, because BHMT was shown to be transcriptionally upregulated during darkness-induced bleaching in two coral species, *Acropora palmata* and *Montastrea faveolata* (DeSalvo et al., 2012), and upregulated in aposymbiotic specimens of *Aiptasia* (Lehnert et al., 2014). BHMT is involved in the regulation of homocysteine metabolism, by converting betaine and homocysteine to dimethylglycine and methionine, respectively. The most interesting point is that BHMT has been found in human extracellular exosomes (Zhang et al., 2010), as already mentioned for enolase-1. Small portions of the plasma membrane are regularly internalized as endosomes, a phenomena that participates each hour to the plasma membrane recycling. Membranes of some endosomes may also be internalized as smaller vesicles, called multivesicular bodies. Exosomes are vesicles that derives from late endosomes. They are released from the cell when multivesicular bodies fuse with the plasma membrane. Exosomes can transfer molecules from one cell to another via membrane vesicle trafficking and therefore participates to cell-cell communication and intercellular signaling. Moreover, exosomes, may influence the immune responses to pathogens (Li et al., 2006). We might hypothesize that similar cellular processes are involved to promote the symbiotic interaction between competent gastrodermal cells and compatible *Symbiodinium*. We also have to keep in mind that we are looking at a very dynamic process, which implies that the composition of the symbiosome membrane may rapidly change depending of the physiological conditions of both host and symbiont. This is a very challenging question that requires accurate protocols and analyses. For example, all resident *Symbiodinium* cells did not exhibit the same physiological status within the same tentacle, some of them being healthy while others were degraded (Dani et al., soumise à publication; Hanes and Kempf, 2013). This should be reflected by the protein composition of the symbiosome membrane. This could also explain differences between FIS extractions from various tentacles or from different cnidarian hosts. Comparisons of protein datasets between FIS and cultured *Symbiodinium* on one hand, and gastrodermis vs epidermis on the other hand, are under

progress and should also help to decipher the molecular and cellular processes involved in the symbiotic interaction.

The same investigation approach was used to identify metabolites potentially involved in the symbiotic interaction. First, exhaustive metabolite characterization was performed on healthy symbiotic specimens using classical analytical techniques, in order to build a custom database of *A. viridis* main metabolites. Most of the molecules characterized so far were lipid compounds (Harland et al., 1991; Revel et al. submitted). Metabolite profiles were performed using Evaporating Light Scattering Detector and compared quantitatively between healthy and bleached specimens. Among the metabolite classes that were upregulated in symbiotic sea anemones, alkaloid-like structures were found in the aqueous fraction of the partitioned crude extract (fraction 4). Interestingly, all these structures were members of the chemical class of compounds named betaines. The main betaines identified within the *A. viridis* were homarine, trigonelline, zooanemonin and, in a smaller proportion, gonyol. Trigonelline has previously been isolated from many species, including plants (Minorsky, 2002), dinoflagellates (Gebser and Pohnert, 2013) and marine invertebrates (Welsh and Prock, 1958). Homarine has only been isolated so far from marine organisms. It was found in many invertebrates, including sea anemones (Mathias et al., 1960), but also in dinoflagellates (Gebser and Pohnert, 2013). Glycine has been shown to be the main precursor for the biosynthesis of both homarine and trigonelline. Regarding zooanemonin, this compound has been isolated from diverse sponges (Paul and Puglisi, 2004) and *Anemonia* (Ackermann and List, 1960) and seemed to be restricted to marine invertebrates. Gonyol was first isolated from the dinoflagellate *Gonyaulax polyedra* (Nakamura et al., 1993) and other microalgae (Gebser and Pohnert, 2013), and was shown to be biosynthesized from methionine (Nakamura et al., 1993). Our first investigations on the *A. viridis* holobiont demonstrated that gonyol was probably synthesized by symbionts, as it was found both in symbiotic *Symbiodinium* cells (FIS) and free-living *Symbiodinium*, in a higher concentration. Zooanemonin, which was not regulated during menthol-induced bleaching, might originate from the cnidarian host, according to previous reports. In contrast, biosynthesis of both homarine and trigonelline was reduced in bleached specimens. As both the cnidarian host and dinoflagellate symbionts may contribute to their synthesis, the decrease in homarine and trigonelline may be explained by either the loss of a high quantity of symbionts (definition of bleaching) and therefore of symbiont-derived compounds, or

by the consequent decline in host biosynthesis due to the absence of symbionts. The decrease in betaine abundance may also be explained by a decrease in their respective free fatty acid precursors, which have been shown to be translocated between the two partners (Davy et al., 2012a).

Many betaines have been described as osmolytes that protect host cells from various environmental stresses, such as osmotic stress, but also high salinity and high temperature. For example, betaines are crucial to maintain cellular volume by increasing water retention of the cells and replacing inorganic salts. In vertebrates, they have been shown to protect cell constituents from oxidative damages (Ganesan et al., 2011) and to protect cells against apoptosis via inhibition of the proapoptotic mitochondrial pathway (Graf et al., 2002). Betaines are also important methyl group donor and, as such, they are considered as a vital methylating agent (Barak and Tuma, 1982). This reaction is catalyzed by the BHMT enzyme, which was discussed above. Moreover, BHMT, which catalyzes the resynthesis of methionine using homocysteine and the methyl donor betaine, is a component of the methionine cycle. In plants and unicellular algae, betaines are well known to protect photosystem II of damages from various abiotic stresses, including high temperature and high UV irradiance (Chen and Murata, 2008; Gebser and Pohnert, 2013; Hema et al., 2007). Several betaines were recently recorded in various symbiotic reef-building corals, each species expressing its own set of betaines (Hill et al., 2010). Their relative concentrations were intraspecifically modulated in response to environmental conditions, especially irradiance conditions (Hill et al., 2010). It was therefore hypothesized that multiple betaines may protect symbiont photosystem from damages and thus contribute to the cnidarian host survival. Moreover, their role as mediators of acclimatization in defense systems in symbiotic cnidarians, including acclimatization of protection of photosystem II, has been suggested (Middlebrook et al., 2008). Nevertheless, even though betaines might exert their actions within the symbionts, the cnidarian host tissues could contribute to their biosynthesis. Indeed, we demonstrated that some betaines were still present in bleached specimens of *A. viridis*. Because such recorded concentrations of betaines could not be explained by biosynthesis from the few remaining symbionts, we hypothesized that these compounds were issued from the cnidarian host. Further experiments, such as betaine quantifications in separated tissues, are still under progress. Nevertheless, these results point to a role of the cnidarian host in betaine biosynthesis in order to reduce bleaching damages. Similarly, cnidarian hosts were shown to protect their algal symbionts by producing different substances, such as antioxidant systems (Richier et al., 2006), mycosporine-like amino acids and fluorescent

pigments (reviewed in Baird et al. 2009). The possible roles of betaines in marine invertebrates are still under debate, especially in cnidarians where no extensive investigations have been performed. In addition to their osmoprotectant and photoprotective roles (Hill et al., 2010), previous reports demonstrated that homarine and trigonelline may act as morphogens to inhibit pattern formation and metamorphosis in the hydroid *Hydractinia echinata* (Berking, 1987). In plants, trigonelline is known to regulate cell cycle through inducing G2 arrest (Minorsky, 2002). Furthermore, homarine and trigonelline have been reported to function as predator deterrents and antifouling agents (Paul and Puglisi, 2004; Targett et al., 1983). It was also described that homarine exerts antimicrobial activities and is thus an active constituent of the innate immune system in the gorgonian *Leptogorgia virgulata* (Shapo et al., 2007). The role of betaines in symbiotic cnidarian physiology remains to be investigated in closer detail. This includes localization of these compounds within the holobiont tissues and their modulation in both concentration and localization in different environmental conditions, including those known to induce bleaching.

In addition to classical metabolomic analytical methods, we adapted the MALDI-MS imaging technology to identify and localize metabolites directly in sea anemone tentacle sections (Kopp et al., 2015). In a first attempt to identify compounds that exhibited differences in quantity and/or localization in response to stress, we performed a follow-up of metabolites in *A. viridis* specimens exposed to menthol-induced bleaching. Sampling were performed at day 0, 1, 5, 10, 22 and 59 and tentacle cross sections were imaged to record metabolite quantity and distribution. Unfortunately, MALDI-MS imaging did not allow the detection of zwitterionic compounds, such as betaines, which are not ionisable molecules. We particularly focused on the 700-950 m/z range, which corresponds to phospholipids. Phospholipids play multiple roles in cells, from membrane structure and function, including phagocytosis, to cell signaling in response to both internal and external stimuli (Bohdanowicz and Grinstein, 2013). The signal at m/z 872.761 was exclusively observed in the internal part of tentacles, which corresponded to gastrodermal-containing symbiont cells. The signal was intense until day5 and was dramatically reduced at day10, which corresponded to a massive loss of symbionts in response to menthol exposition (Dani et al., soumise à publication). These results supported the hypothesis that this compound may be of *Symbiodinium* origin. Another possibility is that this compound may be synthesized by host gastrodermal cells in the presence of resident *Symbiodinium*. Interrogation of lipid databases returned possible structures identified as glycerophosphoserines. Confirmation of the lipid assignment will be achieved by

tandem mass spectrometry (MS/MS) and its identification might give additional clues about its origin and putative role in the symbiotic interaction.

Overall, these results demonstrated that both shotgun proteomics and metabolomics are very promising techniques to study the cnidarian-dinoflagellate interaction. Nevertheless, the preparation of suitable samples for comparative studies remains the major bottleneck. The same is true for the interpretation of results, which requires both specific and accurate databases. However, these techniques will be very useful to complement transcriptomic studies in order to unravel the cellular mechanisms involved in the symbiotic interaction. Proteomic studies are necessary because post-translational mechanisms might be important in the symbiosis. For example, the recruitment of Sym32 from plasma membranes to the symbiosome during early stages of symbiosome formation, as proposed in Ganot et al. (Ganot et al., 2011) was independent of transcriptional changes, but was confirmed by our proteomic analysis of symbiosomes. Similarly, the recruitment of enolase-1 at the symbiosome membrane in order to promote *Symbiodinium* infection success was also an interesting hypothesis, which would require further attention. These results provide the first insight into membrane trafficking and recycling that are required to achieve *Symbiodinium* phagocytosis and symbiosome formation. However, we might expect these processes to be highly dynamic, depending of the physiological state of either the cnidarian host or the symbiont. Changes in environmental conditions, such as temperature or irradiance, may impact the symbiosome microenvironment and therefore the protein composition of the host-symbiont interface. Complementary imaging techniques such as fluorescence recovery after photobleaching (FRAP) will be useful to study protein dynamics and activity and thus unravel the dynamics of cellular events involved in the symbiosis establishment (Davy et al., 2012a). Metabolomics will also contribute to understand the symbiotic interaction. There are still some limitations to study host-symbiont interface, such as required quantity of biological material, availability of accurate databases for metabolite identification and spatial resolution for metabolite imaging. But these problems should be solved in the next few years.

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1.1. Discussion et Perspectives

La caractérisation des acteurs moléculaires situés à l'interface de communication entre les deux partenaires de la symbiose chez les Cnidaires est cruciale pour une meilleure compréhension des mécanismes impliqués dans la reconnaissance, l'internalisation, le maintien de l'association et le dialogue moléculaire entre les deux partenaires (Davy et al., 2012a). Dans une étude précédente, nous avons défini la protéine AvNPC1 comme un marqueur de la membrane pérисymbiotique dans notre modèle d'étude *A. viridis*, mais également dans deux espèces de corail (Dani et al., en préparation). Nous avons également établi un protocole d'enrichissement de symbiotes possédant ou non la membrane pérисymbiotique. Une partie de cette étude constitue alors les premiers résultats de la caractérisation du protéome de la membrane pérисymbiotique chez *A. viridis* et nous a permis de valider, de par les résultats obtenus, le protocole d'enrichissement préalablement établi.

Premièrement, nous avons validé la protéine AvNPC1 comme un marqueur de membrane. En effet, même si la protéine AvNPC1 n'as pas été retrouvée dans l'ensemble des réplicats biologiques utilisés dans cette étude, ce qui peut s'expliquer par la taille importante et le caractère transmembranaire de la protéine NPC1. Néanmoins, l'amélioration du protocole d'extraction des protéines actuellement en cours nous permet de retrouver la protéine AvNPC1 de façon plus reproductible. De plus, la présence dans les données obtenues de la protéine AvNPC2d, probablement localisée dans l'espace pérисymbiotique, nous permet de valider notre approche permettant d'identifier d'une part les protéines associées à la membrane, mais également celles exprimées dans l'espace pérисymbiotique.

Les premiers résultats sont très encourageants et parmi les protéines identifiées dans la condition enrichie en membrane pérисymbiotique, nous avons retrouvé la majorité des cibles rapportées par Peng, par purification de la membrane (Peng et al., 2010a), ainsi que d'autres protéines identifiées individuellement, comme Sym32 par exemple. Parmi ces protéines, la présence de l'actine et de protéines liant ou modifiant l'actine confirme la forte implication du cytosquelette de l'hôte au niveau du symbiosome. On retrouve également l'énolase-1, une enzyme impliquée dans de nombreux processus tels que la glycolyse, le contrôle du cycle cellulaire, la tolérance à l'hypoxie et la réponse immunitaire chez les vertébrés (Pancholi, 2001). Par ailleurs, l'énolase est également impliquée dans les mécanismes de reconnaissance et d'établissement de l'infection chez *Leishmania mexicana* (Vanegas et al., 2007). Des études complémentaires

pourront être engagées pour déterminer son rôle dans les processus de mise en place de la symbiose chez les Cnidaires symbiotiques. En effet, dans d'autres modèles symbiotiques, des molécules responsables de la reconnaissance et/ou de l'attraction des symbiotes ont été mises en évidence. C'est le cas de la galaxin dans la symbiose Seiche-Vibrio (Heath-Heckman et al., 2014), de la protéine Mermaid chez le Némotode *Laxus oneistus* (Bulgheresi et al., 2011) et de flavones chez *Rhizobium* (Redmond et al., 1986).

Cette première étude nous a permis d'identifier 202 protéines surexprimées chez les symbiotes isolés avec leur membrane pérисymbiotique par rapport à ceux qui en sont dépourvus. Ceci constitue à l'heure actuelle la plus grande caractérisation du protéome de la membrane pérисymbiotique chez les Cnidaires symbiotiques. Suite à cette étude préliminaire, nous avons engagé la caractérisation du protéome des cellules de *Symbiodinium* en culture. Par comparaison avec les symbiotes isolés des cellules gastrodermiques, nous pourrions ainsi déterminer la modification du protéome de *Symbiodinium* en réponse à la condition symbiotique des cellules. Nous avons également engagé la caractérisation du protéome du compartiment épidermique. Ceci nous permettra d'écarter les contaminants épidermiques de notre analyse finale. Nous avons également complété ce protocole par une étape de tri préalable par cytométrie de flux (FACS) basée sur la granulométrie et l'autofluorescence des *Symbiodinium*. Cette étape permettra d'éliminer de façon systématique les contaminants cellulaires tels que des débris de cellules gastrodermiques et les cnidocytes représentés par la population P4 (Figure 18B), afin de travailler sur une population uniquement constituée de cellules de *Symbiodinium* représentée par la population P6 (Figure 18B).

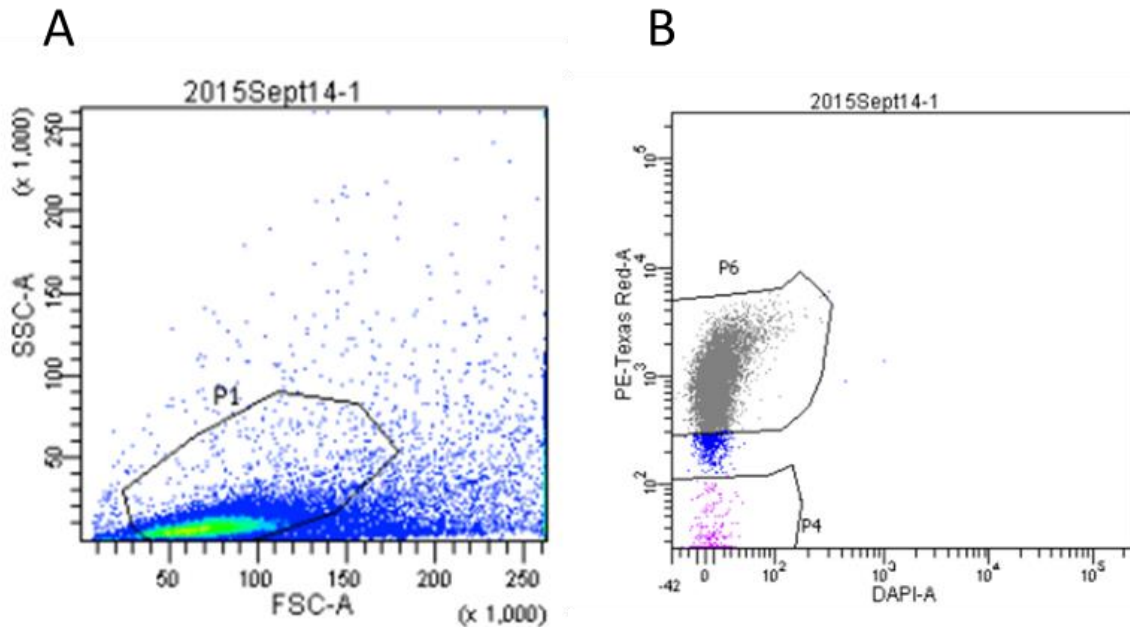


Figure 18: Résultats du tri par FACS de l'échantillon de *Symbiodinium* extraits d'*Anemonia viridis*, triés en fonction de la granulométrie et de l'aire (A) et de l'autofluorescence (B). SSC-A : granulométrie ; FSC-A : aire, PET-Texas Red : autofluorescence de la chlorophylle

Nous avons démontré auparavant la présence d'une population hétérogène de symbiotes *in hospite*. En effet, grâce au marquage au CalcoFluor White, nous avons observé la présence de symbiotes ayant une forte concentration en contenus cellulosesiques ou dépourvu de contenus cellulosesiques (Dani et al., en préparation). Dans une précédente étude (Kwok and Wong, 2003), la concentration en contenus cellulosesiques a été partiellement corrélé avec les stades du cycle cellulaire des symbiotes. En combinant ces différentes techniques de marquage et purification, nous envisageons alors de déterminer s'il existe une modification du protéome exprimé au niveau du symbiosome, par le recrutement différentiel de protéines, en fonction des phases du cycle cellulaire du symbiote.

De façon globale, les premiers résultats montrent que certaines des protéines identifiées chez l'anémone sont des orthologues de protéines impliquées dans les processus de migration/prolifération chez l'homme. Par exemple, nous retrouvons associées à la membrane pérисymbiotique, de nombreuses Rab GTPases (dont Rab11A), protéines impliquées dans la régulation du trafic vésiculaire. Les protéines Rab semblent avoir un rôle dans la régulation de la

mise en place et le maintien de la symbiose chez les Cnidaires symbiotiques (Chen et al., 2005). Par ailleurs, la protéine Rab11, impliquée dans la migration cellulaire, est fortement surexprimée dans les cancers ovariens et du poumon (Welz et al., 2014). Nous retrouvons également la périostine, impliquée chez l'homme dans la migration et l'adhésion des cellules souches cancéreuses (Malanchi et al., 2012). Il est à noter que les domaines protéiques retrouvés dans la protéine Sym32 sont homologues aux domaines Fascicline qui composent la périostine. Egalement, l'énolase que l'on retrouve associée à l'extrait enrichi en membrane pérисymbiotique, est impliquée chez l'homme dans la croissance cellulaire, la migration et l'invasion des cellules tumorales (Song et al., 2014)

Il est très intéressant de noter que la plupart des protéines que nous avons identifiées se répartissent en trois groupes : i) des protéines impliquées dans le transport du calcium (Ca^{2+} ATPases, TRPC4), dans la signalisation calcique (calmoduline) ou liant le calcium (protéines pourvues d'un domaine EF-hand, dont la calumenine), ii) des protéines pourvues de domaines ANKIRIN, connus comme étant impliquées dans les interactions protéines-protéines, et iii) des protéines du cytosquelette ou liant le cytosquelette. La signalisation calcique est importante pour l'établissement de la symbiose Cnidaire-Dinoflagellés (Davy et al., 2012a; Ganot et al., 2011) et sa perturbation a été observée lors de la rupture de symbiose (Ganot et al., 2011; Moya et al., 2012). De la même façon, une altération de la signalisation calcique a été observée dans de nombreuses pathologies cancéreuses, et est liée en particulier aux processus de prolifération et de migration (Stewart et al., 2014). En ce qui concerne les répétitions ANK, elles sont retrouvées dans de très nombreuses protéines et facilitent les interactions protéines-protéines nécessaires à la transduction du signal, le trafic vésiculaire, la réponse inflammatoire et l'intégrité du cytosquelette. On les retrouve aussi associées à des pathologies cancéreuses. Enfin, le remodelage du cytosquelette est également un point commun observé entre l'établissement de la symbiose et la transition épithélio-mésenchymateuse. Certaines protéines de Cnidaire associées à la membrane pérисymbiotique possèdent des assemblages de domaines protéiques particuliers par rapport à leurs orthologues humains. Leur étude permettra sans doute d'apporter un éclairage sur l'évolution comparée de ses protéines, entre les Cnidaires et les Vertébrés.

Dès lors, nous avons déjà entrepris la validation d'un certain nombre de protéines identifiées par protéomique par des expériences d'immunolocalisation sur des *Symbiodinium* isolés du gastroderme (Figure 19). Par exemple, la coronine est une protéine se liant à l'actine et se

retrouve impliquée dans les processus cellulaires de remodelage du cytosquelette, l'endocytose, la phagocytose et la motilité cellulaire chez les vertébrés (Gandhi and Goode, 2000). Chez *A. viridis*, nous avons retrouvé la coronine surexprimée dans les *Symbiodinium* fraîchement isolés possédant la membrane pérисymbiotique et nous avons montré que cette protéine était colocalisée avec la membrane pérисymbiotique par immunolocalisation (Figure 19).

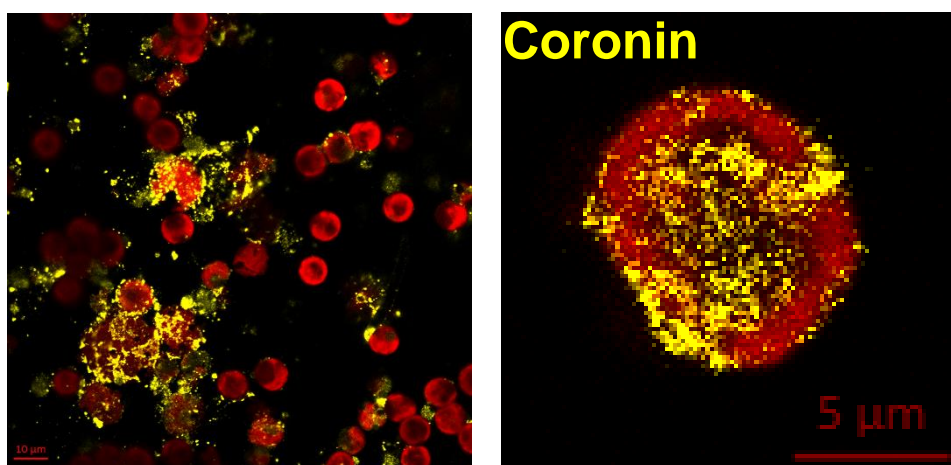


Figure 19: Localisation de la coronine (jaune) sur des FIS possédant la membrane pérисymbiotique. En rouge l'autofluorescence de la chlorophylle de *Symbiodinium*

Il s'agira donc de déterminer le rôle de la coronine dans les processus de migration des symbiotes lors de l'internalisation et du maintien dans les cellules du gastroderme de l'hôte. Dans un deuxième temps, il s'agira de comparer les résultats obtenus avec ceux de laboratoires effectuant des recherches sur des pathologies cancéreuses et avec lesquels nous collaborons étroitement : le laboratoire PRISM de l'Université de Lille 1 (essentiellement cancers de l'ovaire et glioblastomes), et l'Institut de Biologie de Valrose (nombreuses équipes travaillant sur diverses pathologies cancéreuses, et diversité des modèles biologiques tels que l'oursin et la drosophile). Cette comparaison devrait nous permettre d'identifier des protéines à la fois impliquées dans l'établissement de la symbiose et retrouvées dans certains cribles spécifiques à des pathologies cancéreuses. Nous pourrions ainsi préciser le rôle potentiel de nouveaux candidats et définir les bases scientifiques d'un projet à plus long terme destiné à réaliser une étude fonctionnelle de ces protéines.

Chapitre 5 : Conclusions et perspectives générales

Ce travail doctoral, mené dans la continuité de mon stage de Master 2 a eu pour principal objectif une meilleure compréhension de l'interface symbiotique dans la symbiose Cnidaire-Dinoflagellés. Avec pour outil de départ les gènes *npc1* et *npc2*, surexprimés en condition symbiotique par rapport à la condition aposymbiotique (Ganot et al., 2011), nous avons développé de nouvelles approches expérimentales. Les résultats obtenus nous ont permis d'acquérir de nouvelles connaissances sur le fonctionnement de la symbiose dans notre modèle d'étude, et de façon globale chez les Cnidaires symbiotiques. Si la symbiose chez les Cnidaires est de plus en plus considérée comme un continuum entre mutualisme et parasitisme (Lesser et al., 2013), la conception de la symbiose qui était la mienne au départ de mon projet doctoral a également évolué au cours de nos différentes études.

Au cours de ma première année de doctorat, j'avais pour vision des Cnidaires des organismes ayant une organisation cellulaire simple, définis comme tels dans les ouvrages pédagogiques et de vulgarisation, de par leur position à la base de l'arbre phylogénétique des Métazoaires. La première étude de microscopie que nous avons conduite afin de décrire l'organisation au niveau cellulaire et subcellulaire des tissus de l'anémone de mer *A. viridis*, a permis de confirmer un niveau de complexité bien supérieur. Nous avons ainsi décrit une organisation particulière des fibres de collagène contenues dans la mésoglée encore jamais décrite chez les Cnidaires, mais également la présence de cellules de type améobocytes circulants impliquées notamment dans les processus de l'immunité. Au final, cette étude permet une meilleure connaissance au niveau subcellulaire de notre modèle d'étude, mais également de dégager de nouvelles problématiques. A ce titre, nous avons eu l'occasion d'engager des discussions avec l'équipe « Phylogénie, Anatomie, Evolution » de l'Université Pierre et Marie Curie (UMR 7138) sur des questions d'organisation cellulaire chez les Cnidaires.

Nous avons également adapté pour la première fois chez un Actiniaire, la méthode de blanchissement au menthol (Wang et al., 2012). Cette méthode permet l'obtention d'individus aposymbiotiques d'*A. viridis* de façon plus graduelle, plus efficace et en absence de mortalité. A ce jour, le laboratoire possède donc un protocole permettant de disposer rapidement d'un stock d'individus dépourvus de symbiotes. Notre suivi au niveau cellulaire et subcellulaire (Dani et al.,

soumise à publication), montre que cette technique de blanchissement induit principalement un mécanisme d'autophagie, en comparaison avec le blanchissement induit par un traitement hyperthermal dont les mécanismes majoritaires observés sont l'apoptose, l'exocytose et la nécrose. Ces mécanismes de rupture de symbiose montrent conceptuellement qu'une relation de type mutualiste à l'état stable, peut rapidement évoluer vers une relation de type parasitique lors d'un stress au niveau de l'organisme, et entraîner le déclenchement d'une réponse de type immunitaire chez les Cnidaires symbiotiques (Miller et al., 2007). L'observation de mécanismes de type mort cellulaire programmée dans notre modèle, déjà largement décrit dans des modèles insectes (Schmitz et al., 2012), nous ont permis d'établir des parallèles avec les mécanismes de l'immunité innée présents chez des insectes symbiotiques (Schulenburg et al., 2007). Le séquençage des transcrits exprimés lors du stress menthol permettra de connaître les acteurs moléculaires impliqués dans ce type de rupture de symbiose. Par comparaison avec les mécanismes induits lors de l'augmentation de la température, nous disposerons alors de nouvelles cibles d'études. L'ensemble de ces projets permettrait d'avoir une compréhension globale des acteurs moléculaires impliqués dans les mécanismes précédemment mis en évidence au niveau subcellulaire.

Durant ce projet doctoral, nous avons également montré une hétérogénéité des symbiotes *in hospite* en condition symbiotique et une dynamique de l'état des symbiotes lors de divers stress. En effet, nous avons utilisé le marqueur (Calcofluor White) comme indicateur du stade du cycle cellulaire des symbiotes *in hospite* dans des conditions stables et de rupture de symbiose (Dani et al., en préparation). Cette étude suggère qu'il existe un mécanisme de contrôle de l'hôte en condition symbiotique permettant de maintenir une population stable de symbiotes. L'hôte aurait donc la capacité de maintenir une part de symbiotes en phase de production active, hypothèse formulée il y a quelques années par la théorie de « l'élevage » (Wooldridge, 2010). En revanche, lors de la diminution des apports hétérotrophiques et/ou en condition de stress pouvant entraîner la rupture de la symbiose, la diminution des transferts entre les deux partenaires entraîne un blocage du passage des symbiotes de la phase G1/G0 vers la phase de réplication, incapables d'atteindre la taille seuil nécessaire au passage dans la phase de division. Nous pouvons donc poser l'hypothèse que l'hôte maintient alors une partie plus importante de symbiotes en phase de production photosynthétique pour augmenter la part de production autotrophique. Lors de la rupture de symbiose, l'arrêt des transferts hôte-symbiotes semble conduire à un blocage des symbiotes en phase G1/G0.

Ces résultats suggèrent néanmoins une dynamique de la composition de la paroi cellulaire des symbiotes en fonction des conditions environnementales. En parallèle avec les données obtenues lors des stress entraînant la rupture de la symbiose, nous pourrions envisager de déterminer les acteurs moléculaires présents à l'interface pérисymbiotique qui réguleraient la dynamique de l'interaction *in hospite*.

En parallèle, nous avons obtenu le répertoire complet des gènes *npc1* et *npc2* chez *A. viridis* et caractérisé leur régulation et localisation *in situ* au niveau des transcrits et des protéines (Dani et al., en préparation; Dani et al., 2014). A notre connaissance, il s'agit de la caractérisation la plus complète de gènes potentiellement impliqués dans la relation symbiotique chez les Cnidaires. En effet, nous avons décrit l'évolution phylogénétique des gènes *npc1* et *npc2* chez les Métazoaires mettant en évidence des duplications spécifiques aux Cnidaires des gènes *npc2*. Nous avons ensuite décrit leur régulation et localisation tissulaire spécifique. Ceci nous a permis de mettre en évidence une sub-fonctionnalisation de la protéine AvNPC2-d. De plus, la protéine AvNPC2-d pourrait constituer un marqueur de l'état de santé de l'association symbiotique. Le rôle majeur que jouerait cette protéine dans les échanges métaboliques entre les deux partenaires, nous a conduits à développer la caractérisation et la purification de stérols potentiellement impliqués dans la communication métabolique entre les deux partenaires. Ainsi, d'après leur profil d'expression compartiment spécifique, douze stérols potentiellement impliqués dans la symbiose ont pu être caractérisés. Dans le but de connaître l'interaction entre les stérols identifiés et la protéine AvNPC2-d, nous avons engagé une étude préliminaire de modélisation *in silico*. Un modèle moléculaire de la protéine AvNPC2-d a pu être proposé à partir de sa séquence protéique chez *A. viridis* et par comparaison avec la protéine NPC2 de *Bos taurus* et *Apis mellifera*. Ce modèle nous permettra de tester *in silico* la spécificité des ligands stérols potentiels, en amont des études d'interaction envisagées par la technique de résonance plasmonique de surface (Biacore). Ces analyses nous permettront de clarifier le rôle fonctionnel de la protéine AvNPC2-d dans l'interaction symbiotique.

Nous avons également proposé la protéine AvNPC1 comme marqueur de la membrane pérисymbiotique dans notre modèle d'étude mais également chez plusieurs coraux symbiotiques. Ce marqueur constituerait une avancée majeure dans les projets impliquant la détermination du protéome de l'interface symbiotique, étant donné qu'aucun marqueur universel de la membrane

périsymbiotique n'est disponible à l'heure actuelle. De plus, ce type de marqueur fait l'objet de nombreuses investigations de la part de la communauté scientifique (Davy et al., 2012a) et les protéines NPC avaient déjà été mentionnées comme des cibles prioritaires (Ganot et al., 2011; Lehnert et al., 2014). Ainsi, même si ces trois différentes études n'ont pas de dénominateur commun hormis une meilleure connaissance des relations symbiotiques chez les Cnidaires, chacune apporte un élément nous permettant de réaliser l'objectif initial de caractérisation de l'interface symbiotique. En effet, au cours de la mise au point du protocole de purification de symbiotes isolés du gastroderme et pourvus de leur membrane périsymbiotique, la meilleure connaissance de la structure ultra-cellulaire de notre modèle nous a permis d'affiner ou d'écartier certains protocoles par observation en microscopie électronique des échantillons obtenus. De manière décisive, la mise au point de AvNPC1 comme un marqueur de la membrane périsymbiotique nous a permis de disposer d'un test spécifique pour établir la proportion de symbiotes possédant ou non la membrane périsymbiotique, par visualisation directe par immunofluorescence en microscopie confocale. D'ores et déjà, nous disposons du protéome le plus complet des protéines présentes au niveau du symbiosome et/ou exprimées en conditions symbiotiques par rapport à des *Symbiodinium* en culture. Les résultats préliminaires (Revel et al., soumise à publication) valident notre protocole en accord avec les résultats précédemment obtenus dans la littérature, et proposent également de nouvelles pistes d'études dans la régulation de l'association symbiotique.

L'ensemble des résultats obtenus découle de la mise un point et/ou de l'adaptation aux spécificités d'*A. viridis* d'un grand nombre de protocoles expérimentaux. Ainsi, la volonté de pouvoir étudier les mécanismes symbiotiques *in hospite* nous a incité à développer des protocoles pour des analyses en microscopie confocale et électronique. Par ailleurs, une partie des protocoles mis en place ont été conditionnés par l'utilisation d'un milieu d'enrobage permettant l'analyse par imagerie MALDI-MS des composés peptidiques et métabolites directement sur coupe (Kopp et al., 2015), compatible avec les autres expériences d'immuno-localisation. Le laboratoire possède ainsi une banque d'échantillons de plusieurs individus pour chacune des conditions utilisées dans cette étude (contrôle, stress nutritif, stress hyperthermique et stress menthol) qui pourra servir dans les projets futurs. Les résultats obtenus permettront également au laboratoire de participer à un projet collaboratif de recherche (ANR) afin de comparer les interactions biotiques chez plusieurs modèles

de symbioses photosynthétiques, benthique (*A. viridis*) et pélagique (radiolaire en symbiose avec des Dinoflagellés).

Finalement, la symbiose chez *A. viridis* correspond en partie à la définition de Van Beneden qui définit la symbiose mutualiste comme un bénéfice réciproque entre les deux partenaires (Van Beneden, 1878) et par l'apparition de nouvelles voies de biosynthèse partagées (Redman et al., 2001). C'est le cas par exemple de la biosynthèse de certains stérols, dont les premières étapes seraient réalisées par le symbiote. Nos études montrent que la duplication des gènes *npc2* pourrait avoir favorisé l'émergence (sub-fonctionnalisation) d'une protéine spécifiquement impliquée dans le transport de stérols (ou de ses précurseurs) à l'interface symbiotique.

A l'inverse, dans certaines conditions de stress, nous avons montré un réengagement de la maturation du phagosome contenant le symbiote, conduisant à terme à la dégradation du symbiosome (Dani et al., soumis à publication). En effet, les parasites intracellulaires du groupe des Apicomplexes, groupe frère des *Symbiodinium*, tels que *Plasmodium* et *Toxoplasma*, manipulent le système immunitaire de l'hôte pour se maintenir dans une vacuole parasitaire (Bradley and Sibley, 2007; Martin et al., 2007). Dans des conditions de stress, la symbiose Cnidaire-Dinoflagellés correspond plus alors à une définition de type parasitaire.

La relation symbiotique est alors régit par une balance entre bénéfice-conflit entre les deux partenaires qui évolue de façon dynamique, en fonction des conditions environnementales (Lesser et al., 2013). Une étude menée chez la méduse *Cassiopea*, en symbiose avec *Symbiodinium* et capable de passer d'un modèle mutualiste à parasitique, illustre parfaitement la nature dynamique de la symbiose et le continuum entre ces deux modes d'interaction (Sachs and Wilcox, 2006b).

Les résultats obtenus lors de la caractérisation du symbiosome avec ou sans membrane pérисymbiotique apportent déjà des éléments de réponse par l'identification de protéines impliquées dans la reconnaissance cellulaire, le remodelage du cytosquelette, la réponse au stress et le transport de métabolites (Revel et al., soumise à publication). Finalement, la caractérisation du protéome du symbiosome des différents types de symbiotes à l'état stable, lors de la rupture de symbiose et par comparaison avec des symbiotes en culture, apportera davantage de réponses sur la transition parasitisme/mutualisme.

La symbiose repose alors sur une relation instable. La rupture de la symbiose a pour conséquence une diminution de la croissance et une augmentation de la mortalité chez les Cnidaires symbiotiques (Douglas, 2003), incapable de retrouver un partenaire symbiotique adapté aux nouvelles conditions environnementales (Berkelmans and van Oppen, 2006). En revanche, les spécimens de *Symbiodinium* peuvent vivre à l'état libre et les preuves expérimentales démontrent que les cellules de *Symbiodinium* se multiplient de façon plus active en dehors de l'hôte (Jones and Yellowlees, 1997; Wilkerson et al., 1983; Wilkerson et al., 1988). Des études montrent également que le clade A, par exemple, serait plus adapté au mode de vie libre et entrerait en symbiose avec certains coraux, de façon opportuniste (Mieog et al., 2009; Stat et al., 2008). Dès lors, de nombreuses études complémentaires seront nécessaires pour définir la logique de l'interaction.

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MALDI-MS and NanoSIMS imaging techniques to study cnidarian–dinoflagellate symbioses[☆]



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ABSTRACT

Cnidarian–dinoflagellate photosynthetic symbioses are fundamental to biologically diverse and productive coral reef ecosystems. The hallmark of this symbiotic relationship is the ability of dinoflagellate symbionts to supply their cnidarian host with a wide range of nutrients. Many aspects of this association nevertheless remain poorly characterized, including the exact identity of the transferred metabolic compounds, the mechanisms that control their exchange across the host–symbiont interface, and the precise subcellular fate of the translocated materials in cnidarian tissues. This lack of knowledge is mainly attributed to difficulties in investigating such metabolic interactions both in situ, i.e. on intact symbiotic associations, and at high spatial resolution. To address these issues, we illustrate the application of two in situ and high spatial resolution molecular and ion imaging techniques—matrix-assisted laser desorption ionization mass spectrometry imaging (MALDI-MSI) and the nano-scale secondary-ion mass spectrometry (NanoSIMS) ion microprobe. These imaging techniques provide important new opportunities for the detailed investigation of many aspects of cnidarian–dinoflagellate associations, including the dynamics of cellular interactions.

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1. Introduction: cnidarian–dinoflagellate symbioses

Symbioses between members of the phylum Cnidaria, such as scleractinian corals and sea anemones, and photosynthetic dinoflagellates of the *Symbiodinium* genus are commonly found in both temperate and tropical ecosystems (Muller-Parker and Davy, 2001). This cnidarian–dinoflagellate mutualistic association has special ecological significance in often nutrient-poor

environments, such as (sub-)tropical shallow seawater, where it plays an important role in maintaining the health and development of coral reef ecosystems (Hoegh-Guldberg, 1999). One of the main recognized benefits of dinoflagellate symbionts is their ability to supply substantial amounts of photosynthesis-derived nutrients (photosynthates) to their cnidarian host partner, which help support its respiration, growth, reproduction, as well as biocalcification for reef-building corals (Venn et al., 2008; Yellowlees et al., 2008; Stambler, 2011; Davy et al., 2012). The nature of translocated photosynthates (“mobile compounds”) ranges from soluble low molecular weight compounds, such as glycerol, glucose, amino acids and organic acids (Muscatine and Cernichiaro, 1969; Trench, 1971; Burriesci et al., 2012), to more complex molecules such as free fatty acids (Papina et al., 2003; Mortillaro et al., 2009; Teece et al., 2011), glycoconjugates (Markell and Trench, 1993) and even intact lipid droplets (Patton et al., 1977; Kellogg and Patton, 1983; Patton and Burris, 1983; Peng et al., 2011).

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2006-2009	Licence en Biologie des Organismes et des Ecosystèmes, Université de Nice

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Etude du maintien et de la rupture de l'association symbiotique cnidaire-dinoflagellés : Approches cellulaires et moléculaires chez l'anémone de mer *Anemonia viridis*

- Monitorat/Enseignement, service d'enseignement (203h sur 3 ans) comme suit :

Titre UE	Détails	Niveau	Nombre d'heures
Diversité du Vivant Animal	Mise en place du nouveau TP en collaboration avec J. De Vaugelas, F. D'Autreaux et P. Guidetti	L1	108
Organisation du Vivant Animal	TP Cnidaires	L2	57
Projet Biodiversité	Encadrement d'étudiants lors du projet campus Gypse-Cimiez en 2013	L2	12
ID-Bio	TD et mise en place de supports de cours	L3	20
Origine de la Vie	TP Théorie des jeux	L1	6

- Biochimie : Extraction de protéines, SDS-PAGE, Western-Blot, protéomique Shotgun
- Biologie moléculaire : Extractions ADN/ARN, PCR, clonage, qPCR, Hybridation *in situ*
- Biologie cellulaire : Cryocoupes de tissus, colorations, Immunomarquage par fluorescence, microscopie confocale et électronique (TEM)
- Bioinformatique : Outils d'analyses de séquences (Blast, Primer, analyses de domaines protéiques, phylogénie)
- Encadrement : 1 Master 1 et 1 Master 2

Chef de projet bénévole Cybelle-Planète 2012 - en cours



- Développement de partenariats inter-associatifs
- Eco-guide : encadrement de groupes lors de sorties en mer

Chargé de Mission Terre de Vie et Nature 83 – FNE Octobre 2011- Février 2012



- Education à l'environnement et campagnes de sensibilisation
- Proposition et gestion de projets ; Recherche de financements ; Webmaster

RESPONSABILITES ANNEXES

Membre du comité scientifique Les Aquanauts 2013- en cours



- Avis scientifique et conseils stratégiques sur les projets élaborés
- Vérification des articles et des publications édités

Rédacteur et Correcteur scientifique DORIS FFESSM 2012- en cours



Rédaction et correction de fiches présentant les espèces (groupe Cnidaire)

Consultant Aquascience et ECOMERS Université de Nice S-A Octobre 2011- 2014



- Conception d'iGuide multimédia de découverte du patrimoine culturel et environnemental
- Conduite de projet et promotion auprès des services territoriaux ; Force de proposition
- Réalisation d'un topoguide en collaboration avec la Mairie de Nice et le CME-CPIE

STAGES

Stage Master 2 Recherche Equipe "Symbiose Marine" Université de Nice S-A Janvier-Juin 2011

Sujet : Caractérisation des transporteurs du cholestérol chez l'anémone de mer *Anemonia viridis*



- Biologie moléculaire : qPCR, RT-PCR, RACE-PCR, Clonage, Séquençage
- Analyses Bioinformatiques et Biostatistiques

Assistant chercheur UMR 7138 Equipe "Symbiose Marine" Université de Nice S-A Juin-Juillet 2010

Sujet : Biologie évolutive de la gorgone symbiotique de Méditerranée *Eunicella singularis*



- Mesure de croissance et de fitness en condition contrôlé
- Dosage de protéines et de chlorophylle ; Utilisation et optimisation du logiciel ImageJ

Observateur des pêches Campagne Medits IFREMER Corse Est et Golfe du Lion7 Mai 2010



- Inventaire et identification ; mesure de taille et sexage d'espèces Méditerranéennes
- Notion de pêche scientifique, connaissance du protocole d'échantillonnage

INFORMATIONS COMPLEMENTAIRES

Langues : Anglais, niveau professionnel

Loisirs : Apnée, randonnée, voyage, nouvelles technologies

Niveau 3 Plongée bouteille FFESSM
Plongeur professionnel Classe IB INPP

Niveau 2 Biologie subaquatique FFESSM

Informatique : Microsoft Office (Word, Excel, Power Point) ; Photoshop ; Pinnacle ; ImageJ ; BioStat ; PhyML, ZEN

PRODUCTION SCIENTIFIQUE

Publications de rang A:

1. Johana Revel*, **Vincent Dani***, Fabrice Priouzeau, Mohamed Mehiri, Lionel Massi, Laure Capron, Maxence Wisztorski, Dounia Mouajjah, Kévin Lebrigand, Virginie Magnone, Sophie Pagnotta, Sandra Lacas-Gervais, Marjolijn Mertz, Pascal Barbry, Michel Salzet, Cécile Sabourault The symbiotic interface in the *Symbiodinium-Anemonia* interaction: Insight from proteomics and metabolomics studies (soumise à Symbiosis) IF=1.438
2. **Dani, V**, Priouzeau, F, Pagnotta, S, Carette, D, Laugier, J-P, Sabourault, C. Thermal and menthol stress induce different cellular events during sea anemone bleaching. *Symbiosis* (soumise) IF=1.438
3. **Dani, V**, Priouzeau, F, Pagnotta, S, Mertz, M, Lacas-Gervais, S, Sabourault, C. NPC1 and NPC2d are expressed at the symbiosome and play a role in the symbiotic interaction between *Anemonia viridis* and *Symbiodinium* spp (en préparation).
4. **Dani, V**, Priouzeau, F, Pagnotta, S; Loubat, A, Sabourault, C. Cell-cycle state of *in hospite Symbiodinium* is dependent of symbiotic condition in the cnidarian-dinoflagellate association (en préparation)
5. Kopp C, Wisztorski M, Revel J, Mehiri M, **Dani V**, Capron L, Carette D, Fournier I, Massi L, Mouajjah D, Pagnotta S, Priouzeau F, Salzet M, Meibom A & Sabourault C. (2015) MALDI-MS and NanoSIMS imaging techniques to study cnidarian–dinoflagellate symbioses. *Zoology*, 118(2):125-131, IF=1.65
6. **Dani, V**, Ganot, P, Priouzeau F, Furla P. and Sabourault, C. (2014) The NPC team mediates cholesterol transfer within symbiotic cells in *A. viridis*. *Molecular Ecology*, 23(18):4527-4540, IF=6.23

Autres publications non indexés:

1. **Dani, V.**, Descombes, J-M., Ferrand, Y., Gerbier, E., Gerriet, A., Ruffin, F., De Vaugelas, J. (2013) “Mieux comprendre le gypse : colline de Cimiez. Topoguide Centre Méditerranéen de l'Environnement, série : Pour en savoir plus sur les risques majeurs

Présentation orales :

1. **V. Dani**, F. Priouzeau, C. Sabourault. "Role of NPC protein in host/symbiont communication" Journée des doctorants de l'UMR 7138. Paris, Février 2015
2. **Sabourault C, Dani V**, Revel J, Wisztorski M, Mehiri M, Priouzeau F, Mouajjah D, Massi L, Pagnotta S, Capron L, Fournier I, Salzet M. L'interaction Cnidaire-Dinoflagellés photosynthétiques : de la transcriptomique à l'imagerie par spectrométrie de masse. 1ères journées GDR MediatEC, 28-30 Octobre 2014, Paris, France.
3. **V. Dani**, F. Priouzeau, S. Pagnotta, D. Carette, M. Mertz, C. Sabourault. "NPC proteins are key players in molecular interactions between cnidarians and their dinoflagellate endosymbionts" Journée des doctorants de l'UMR 7138. Paris, Mai 2014
4. **V. Dani**, F. Priouzeau, S. Pagnotta, D. Carette, M. Mertz, C. Sabourault. "NPC proteins are key players in molecular interactions between cnidarians and their dinoflagellate endosymbionts" 42th ImmunInv at Dijon, 12-14 May, 2014
5. **V. Dani** ; J. Revel ; L. Capron; I. Fournier ; S. Lacas-Gervais ; L. Massi ; M. Mehiri ; S. Pagnotta ; F. Priouzeau; M. Salzet ; M. Wisztorski ; **C. Sabourault** "Metabolite exchange in the cnidarian-dinoflagellate interplay" 8th International Conference on Coelenterates Biology at Eilat, Israel, 2-5 December, 2013
6. **P-L. Merle**, T. Zamoum, **V. Dani**, C. Sabourault and P. Furla "Post-genomic study of carbonic anhydrase enzymes in the cnidarian-dinoflagellate symbiosis" 1er Colloque d'EcoPhysiologie Animale, Lyon, Novembre 2013
7. **V. Dani**, P. Ganot, F. Priouzeau, S. Pagnotta, P. Furla and C. Sabourault "Gene duplication and adaptation to endosymbiosis in Cnidarians : Focus on the NPC team" 17th Evolutionary Biology Meeting at Marseille, France, September, 2013
8. **V. Dani**, J. Revel, C. Sabourault "Etude de la symbiose Cnidaire/Dinoflagellé" Journée des doctorants de l'UMR 7138. Paris, Juin 2013

Posters:

1. **V Dani**, F Priouzeau, S Pagnotta, S Lacas-Gervais, J-P Laugier, M Mertz, and C Sabourault. "NPC proteins are key players in molecular interactions between cnidarians and their dinoflagellate endosymbionts" 8th Congress of the International Symbiosis Society, Lisbon, Portugal, 12-18 July 2015.
2. **C Sabourault, V Dani**, F Priouzeau, J Revel, M Mehiri, L Massi, L Capron, S Pagnotta, S Lacas-Gervais, J-P Laugier, K Lebrigand, P Barbry, M Wisztorski, Mouajjah, M Salzet. "Symbiosome membranes characterization, or how to decipher molecular interactions between cnidarians and their dinoflagellate symbionts" 8th Congress of the International Symbiosis Society, Lisbon, Portugal, 12-18 July 2015.
3. **V. Dani**, J. Revel, F. Priouzeau, S. Pagnotta, D. Carette, M. Mertz, L. Massi, M. Mehiri and C. Sabourault "Are NPC proteins key players in the molecular interactions between cnidarians and their dinoflagellate endosymbionts?" 1st Labex SIGNALIFE Meeting Cell Signaling, Nice, France, 2014

4. **V. Dani**, P. Ganot, F. Priouzeau, S. Pagnotta, P. Furla and C. Sabourault “Gene duplication and adaptation to endosymbiosis in Cnidarians : Focus on the NPC team” 17th Evolutionary Biology Meeting at Marseilles, France, 2013
5. S. Barnay-Verdier, R. Christen, **V. Dani**, D. Forcioli, P. Furla, C. Mallien, P-L. Merle, B. Ponderini, F. Priouzeau, J. Revel, C. Sabourault, P. Ventura and T. Zamoum “*Anemonia viridis* : A top model to study the Cnidarian-Dinoflagellate symbiosis under ecophysiological stressors” 1^{er} Colloque d’Eco-Physiology Animal, Lyon, France, 2013

Contribution DORIS:

1. BRUCY Claire, BERTONCELLO Danièle, **DANI Vincent**, LAMARE Véronique, *in* DORIS, 30/9/2013. *Anemonia viridis* (Forskal, 1775)
2. CONTET Julian, **DANI Vincent**, SITTLER Alain Pierre, *in* DORIS, 6/9/2013. *Parazoanthus anguicomus* (Norman, 1868)
3. PROUZET Anne, **DANI Vincent**, SCAPS Patrick, *in* : **DORIS**, 3/11/2013 : *Isaurus tuberculatus* (Gray, 1828)
4. CONTET Julian, PROUZET Anne, **DANI Vincent**, *in* DORIS, 12/1/2014 : *Telmatactis cricoides* (Duchassaing, 1850)
5. CONTET Julian, SITTLER Alain Pierre, **DANI Vincent**, *in* DORIS, 7/1/2014 : *Phymanthus pulcher* (Andrès, 1883)