MIGUEL MIES

The symbiotic relationship between *Symbiodinium* and coral reef larvae: gene expression, fatty acid biochemistry and responses to thermal stress

Thesis presented to the Instituto Oceanográfico da Universidade de São Paulo, as part of the requirements for obtaining the degree of PhD in Oceanography.

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Special dedication to:

Prof. Dr. Sergio Mies: father, friend, scientist, professor, role model and hero.

Your passionate dedication is what makes you simply the best at what you do; added to your incorruptible character and respect for the truth, you are the greatest source of inspiration I could ever have.

You have taught me not only how to stand up for the truth, but also how to be curious, which is the first requirement in order to become a scientist. Moreover, you taught me that science is science and not politics. While some still do not recognize that, I do and I will always put science, truth and respect ahead of personal gain, even if that adds significant hurdles to my life. Just like you did. I would never do it differently. I would be honored if I could trail a path like you did: morally, ethically and technically impeccable. Beyond science; it is about being fair.

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My champ.

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I hope to have remembered everyone.

---Miguel Mies---

"whether 'tis nobler in the mind to suffer
the slings and arrows of outrageous fortune,
or to take arms against a sea of troubles,
and by opposing end them?"

– *W. Shakespeare*

NIN

IN UTERO

GARBAGE
BLEED
IKE
ME

FILTER
amalgamut

HOOVERPHONIC BLUE WONDER POWER MILK

AIMEE MANN
Lost in Space

CHRIS CORNELL
EUPHORIA MORNING

MUSIC FOR THE MASSES
DEPECHE MODE

Tomahawk.

SWAMP

Harvest
Neil Young

WELLWATER CONSPIRACY

RESUMO

Muito pouco é conhecido sobre a associação entre dinoflagelados do gênero *Symbiodinium* e larvas de metazoários. Essa tese realizou três experimentos sobre a associação entre *Symbiodinium* e larvas de *Mussismilia hispida* (coral), *Berghia stephanieae* (nudibrânquio) e *Tridacna crocea* (vieira gigante). O primeiro experimento verificou a expressão de um gene específico para a relação simbiótica em *Symbiodinium* A associado com as larvas dos três hospedeiros. O segundo experimento quantificou a produção de ácidos graxos nos clados A-F de *Symbiodinium* também associados com as larvas dos três hospedeiros; o terceiro monitorou a perda de simbiontes nos três tipos larvais associados com os clados A-F, em temperaturas de 26, 29 e 32ºC. Os principais resultados mostram que: i) um gene específico para a simbiose é expresso por *Symbiodinium* A associado com *M. hispida* e *T. crocea*, mas não com *B. stephanieae*; ii) o ácido graxo DHA é produzido em quantidades significantemente maiores pelos clados A e C associados com *M. hispida* e *T. crocea*; e iii) *M. hispida* e *T. crocea* associadas com *Symbiodinium* A e C possuem taxas de perda de simbiontes significantemente menores do que os demais. Esses resultados mostram que os clados A e C estabelecem um mutualismo mais robusto com *M. hispida* e *T. crocea*, mas não há relação mutualística entre *Symbiodinium* e *B. stephanieae*.

Descritores: simbiose, ecologia larval, Scleractinia, Bivalvia, Gastropoda, zooxantelas

ABSTRACT

Very little is known about the association between *Symbiodinium* dinoflagellates, which perform the majority of primary production in coral reefs, and metazoan larvae. This thesis performed three experiments on the association between *Symbiodinium* and *Mussismilia hispida* (coral), *Berghia stephanieae* (nudibranch) and *Tridacna crocea* (giant clam) larvae. The first experiment monitored the expression of a symbiosis-specific gene in *Symbiodinium* clade A associated with the three larval forms during a 72-h window. The second experiment quantified the production of symbiosis-related ω3 fatty acids in *Symbiodinium* clades A-F also associated with the three larval hosts and the third experiment verified bleaching rates at 26, 29 and 32ºC in the larvae associated with clades A-F. The main results show that i) a symbiosis-specific gene is expressed by *Symbiodinium* A associated with *M. hispida* and *T. crocea* larvae, but not with *B. stephanieae*; ii) the DHA fatty acid is produced in significantly higher amounts by clades A and C associated with *M. hispida* and *T. crocea* larvae; and iii) that *M. hispida* and *T. crocea* larvae associated with *Symbiodinium* A and C have significantly lower bleaching rates. These findings suggest that clades A and C establish a more robust mutualism with *M. hispida* and *T. crocea* larvae, but there seems to be no mutualism between *Symbiodinium* and *B. stephanieae*.

Keywords: symbiosis, larval ecology, Scleractinia, Bivalvia, Gastropoda, zooxanthellae

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CHAPTER ONE: INTRODUCTION

A review on metazoan larvae associated with *Symbiodinium*: is there a mutualistic relationship?

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Abstract

The metazoan-*Symbiodinium* association has been extensively documented for adult hosts. However, very little is known about this association during host larval development. Four different metazoan phyla produce larvae associated with *Symbiodinium*, Porifera, Cnidaria, Acoelomorpha and Mollusca, all of which present significant diversity in larval forms, manner of symbiont acquisition and also different requirements on the symbiont presence for successful metamorphosis. Larvae have recognition mechanisms for selecting symbionts of different identities, which may have an impact on larval growth and fitness. Very little is known about metabolical exchange between coral reef larvae and *Symbiodinium*, but it seems to be active in some cases, despite being minimal. Molecular studies show that host larvae transcriptome is nearly unaltered after symbiont acquisition. However, a symbiosis-specific gene has been identified in *Symbiodinium* and similar genes are currently being described for host organisms. Climate change has a significant impact on the association, as coral reef larvae have a bleaching threshold of 28-30ºC, but symbiont identity may attenuate the negative effects. It is questionable whether a mutualistic symbiosis is usually active between *Symbiodinium* and coral reef larvae, as most studies have not been unable to confirm this. However, some reports of metabolite exchange and differential gene expression show that symbiosis is active in some cases.

Keywords: symbiosis, zooxanthellae, coral reef, larval ecology, planula, veliger

Introduction

Marine invertebrates typically present indirect development and their planktonic larvae act as the dispersive stage. This a particularly important adaptation, as it directly influences distribution and abundance, and also enables geographical range expansion and recovery of populations (Strathmann *et al*., 2002). Many factors influence larval development and its dispersal, such as oceanographic conditions, predation avoidance by planktivorous organisms and energy reserves and feeding abilities (Pechenik, 1990). As a response to such wide array of scenarios, there is a significant diversity of larval forms presented by metazoan organisms (Wray and Raff, 1991). Certain larval kinds are planktotrophic and required to feed, thus presenting specialized organs, while others are lecithotrophic and can survive through metamorphosis while relying solely on endogenous reserves (Thorson, 1950; Pechenik, 1999).

In coral reefs, however, certain larval forms must not only feed in the plankton, but also acquire symbionts (Fitt *et al*., 1986; Mieog *et al*., 2009; Ramos and Banaszak, 2014). Many metazoan phyla, especially scleractinian corals in Cnidaria, engage in a mutualistic symbiosis with the photosynthetic dinoflagellates of the genus *Symbiodinium* (Stat *et al*., 2006; Venn *et al*., 2008). The majority of these corals are broadcast spawners and newly-formed planula larvae, while lecithotrophic, must acquire their symbionts horizontally (Harrison and Wallace, 1990; Baird *et al*., 2009; Harrison, 2011). The planktonic larvae acquire different free-living *Symbiodinium* strains from the environmental pool before selecting a specific(s) strain(s) at a later stage (Belda-Baillie *et al*., 1999; Weis *et al*., 2001; Coffroth and Santos, 2005).

While studies on animal-dinoflagellate symbioses have advanced significantly, there is still an overall lack of knowledge about this relationship during host larval development, especially about metabolite exchange and symbiosis-related differential gene expression. In fact, this lack of information raises the question on whether the mutualistic relationship is already established during host larval development. Likewise, while there is much information about the impacts of climate change and anthropogenic influence on adult and symbiotic coral reef specimens (Hoegh-Guldberg, 1999; Hughes *et al*., 2003; Hoegh-Guldberg *et al*., 2007), very little is known about the impacts on *Symbiodinium*-associated larval forms. Therefore, this review has the purpose of compiling, describing and interpreting the available data on metazoan larva-

Symbiodinium associations, with emphasis on the ecological, molecular and biochemical relationship, evolution and influence of environmental change.

Metazoan larvae associated with *Symbiodinium*

Four different metazoan phyla produce larval forms that associate with *Symbiodinium*: Porifera, Cnidaria, Acoelomorpha and Mollusca (Table 1). Two protist taxa are also known to associate with *Symbiodinium*, Foraminifera (Pochon and Gates, 2010) and Ciliophora (Mordret *et al*., 2016). Metazoan larvae acquire symbionts in two different ways: vertically (transmitted to the offspring in parental oocytes) or horizontally by oral ingestion during larval development. Both strategies have their advantages. Vertical transmission ensures that a successful symbiont strain is supplied to the larvae (Wilkinson and Sherratt, 2001), while horizontal transmission allows for the larvae to acquire a diversity of symbionts, some of which may be more compatible to different environmental conditions encountered after dispersal (Baird *et al*., 2007).

Sponges and acoelomorph worms are the two groups in which significantly little is known about their association with *Symbiodinium*, especially during larval development. Within sponges, the association with *Symbiodinium* seems to be largely restricted to the family Clionaidae (Hill *et al*., 2011) and parenchymella larvae contain parentally-seeded symbionts (Mariani *et al*., 2000; 2001). In the case of acoelomorph worms, the genus *Waminoa* harbors two different symbiotic dinoflagellates, *Symbiodinium* and *Amphidinium*, which are also transmitted vertically (Barneah *et al*., 2007b). Other acoelomorph genera are known to associate with *Symbiodinium*, but no information regarding their larval development has been reported (Trench and Winsor, 1987).

In the case of cnidarians, while a massive amount of knowledge has been produced on their association with *Symbiodinium*, there are still relatively few studies on the larva-*Symbiodinium* association. Approximately 80-90% of reef-building coral species (Scleractinia) are broadcast spawners and acquire symbionts horizontally (Harrison and Wallace, 1990; Baird *et al*., 2009; Harrison, 2011). On the other hand, many of the less common brooding species transmit symbionts vertically, most notably in the family Pocilloporidae (Baird *et al*., 2009). In the case of soft coral species

Table 1 Host taxa and their larval stages associated with *Symbiodinium*, also describing mode of symbiont transmission and addressing whether the presence of *Symbiodinium* in larval tissues is critical for successfully attaining metamorphosis.

Host taxon	Mode of *Symbiodinium* transmission	Larval stages associated with *Symbiodinium*	*Symbiodinium* acquisition as a requirement for metamorphosis	References
Porifera	vertical	parenchymella	n/a	Mariani *et al*. (2000; 2001)
Cnidaria				
Scyphozoa	horizontal	planula	no	Hofmann *et al*. (1996); Sachs and Wilcox (2006); Mellas *et al*. (2014)
Actiniaria	vertical and horizontal	planula	no[a]	Bythell *et al*. (1997); Schwarz *et al*. (2002); Hambleton *et al*. (2014)
Alcyonacea	vertical and horizontal	planula	no	Benayahu *et al*. (1988; 1992); Achituv *et al*. (1992); Slattery *et al*. (1999); Barneah *et al*. (2004)
Scleractinia	vertical and horizontal	planula	no	Weis *et al*. (2001); Marlow and Martindale (2007); Harii *et al*. (2009); Mieog *et al*. (2009); Erwin and Szmant (2010)
Acoelomorpha	vertical	Müller's larva	n/a	Barneah *et al*. (2007a; b); Hikosaka-Katayama *et al*. (2012)
Mollusca				
Gastropoda	vertical and horizontal	veliger	yes[b]	Kempf (1984); Carroll and Kempf (1990) Banaszak *et al*. (2013); García Ramos and Banaszak (2014)
Bivalvia	horizontal	veliger and pediveliger	yes	Fitt and Trench (1981); Fitt *et al*. (1984; 1986); Heslinga *et al*. (1984; 1990); Mies *et al*. (2012)

[a] in the case of *Aiptasia* sp.; unknown for other anemones.

[b] in the case of the Queen Conch (*Strombus gigas*); unknown for nudibranchs.

n/a - not applicable.

(Alcyonacea), the incidence of vertical transmission seems to be higher (Benayahu *et al*., 1988; Achituv *et al*., 1992) than for scleractinians. Coral larvae are typically lecithotrophic (Morse *et al*., 1996) and while symbiont acquisition may improve host fitness, it does not seem to be a requirement for metamorphosis (Schwarz *et al*., 1999;

Mieog *et al*., 2009). In the case of other cnidarians such as anemones and jellyfish, there is still very little information on the role of *Symbiodinium* in their larval ecology.

Among mollusks, giant clams (subfamily Tridacninae) are possibly the most conspicuous species associated with *Symbiodinium*. In this group symbiont acquisition is horizontal (Fitt and Trench, 1981) and a requirement for successful metamorphosis (Mies *et al*., 2017a). Differently from other metazoan hosts, symbionts are only acquired during the second larval stage (veliger), as the first larval stage is a non-feeding trochophore (Fitt *et al*., 1984; Heslinga *et al*., 1984). To date, giant clams are also the only known *Symbiodinium* hosts that during larval development do not move symbionts from the digestive tract or equivalent to endodermal tissues (Schwarz *et al*., 2002; Marlow and Martindale, 2007; Huang *et al*., 2008), as they remain in the digestive tract throughout the larval development (Fitt *et al*., 1986; Hirose *et al*., 2006). The other mollusk group associated with *Symbiodinium* is Gastropoda. The Queen Conch *Strombus gigas* is the largest among them to do so and symbiont acquisition is also horizontal and apparently required for metamorphosis (Ramos and Banaszak, 2014). While a large diversity of nudibranchs (mainly aeolids) are known to associate with *Symbiodinium*, there is a scarcity of larval experiments, possibly due to their typically restricted diet (Dionísio *et al*., 2013). The only exception is *Berghia stephanieae*, a species that stores *Symbiodinium* in dorsal extensions of the digestive diverticulum. Its lecithotrophic veliger larvae go through metamorphosis in the absence of *Symbiodinium* (Carroll and Kempf, 1990; Kempf, 1991), but recent studies suggest that this species does not engage in a mutualistic relationship (Mies *et al*., this thesis chapter 2). While nudibranchs acquire symbionts horizontally (Loh *et al*., 2006; Burghardt and Wägele, 2014; Ahmadian *et al*., 2016), a case of vertical transmission has been recently reported (Wecker *et al*., 2015).

Symbiodinium clades

Symbiodinium dinoflagellates may be found in two different life stages, a planktonic and free-living zoospore or a symbiotic and non-motile coccoid cyst (Freudenthal, 1962; Schoenberg and Trench, 1980; Stat *et al*., 2006). Thus, by presenting a free-living stage, the mutualistic association is not obligate for *Symbiodinium*. Based on ribosomal, plastid and mitochondrial phylogenies, they are divided in nine clades (A-I, Fig. 1) and

many subclades (Rowan and Powers, 1991; LaJeunesse, 2001; Pochon *et al*., 2014). Species-level diversity is still being assessed and many new strains have been described recently (LaJeunesse *et al*., 2012; Hume *et al*., 2015; Lee *et al*., 2015), with probably more than one hundred extant species (LaJeunesse, 2001). The higher diversity is found within clade C, that dominates Indo-Pacific coral reefs and is also one of the most abundant clades in the Atlantic (LaJeunesse, 2005). Host species tend to associate with a single *Symbiodinium* strain (Goulet, 2006), but multiple clades may be found within a single host specimen (Rowan and Powers, 1991; Carlos *et al*., 2000; Baker, 2003). *Symbiodinium* strain is a relevant factor for holobiont fitness, in both adult and larval stages (Schwarz *et al*., 1999; Mieog *et al*., 2009; Mies *et al*., this thesis chapter 4). While it has been reported that there is host life stage-specificity for symbiont strain, as juvenile and adult acroporid corals actively associate with different clades (Abrego *et al*., 2009), there is no life-stage specificity reported for larval stages yet.

Symbiont acquisition and larval response

Animal larvae acquire symbionts from different sources, but mainly from the water column and sediment (Adams *et al*., 2009; Cumbo *et al*., 2012). The amount of *Symbiodinium* cells is highly variable among species and may vary according to environmental conditions. For acroporid corals Cumbo *et al*. (2012) report less than 10 symbiont cells acquired by larvae, while Harii *et al*. (2009) registered an average of 50-60 symbiont cells, but with high variability. In another study, Mies *et al*. (this thesis chapter 3) report about 30 cells for giant clam and nudibranch larvae and more than 100 for coral larvae.

Metazoan hosts are able to select *Symbiodinium* strains through post-phagocytic recognition mechanisms involving lectin/glycan interactions (Koike *et al*., 2004; Wood-Charlson *et al*., 2006; Fransolet *et al*., 2012). These mechanisms are also active during larval development and desired strains are kept while undesired ones suffer apoptosis (Dunn and Weis, 2009). Other experiments also show that coral (Bay *et al*., 2011) and gastropod (Ramos and Banaszak, 2014) larvae actively select symbionts. However, there are reports that coral larvae may have no preference for different symbiont strains (Cumbo *et al*., 2012). In a study testing the offer of a homologous and two heterologous

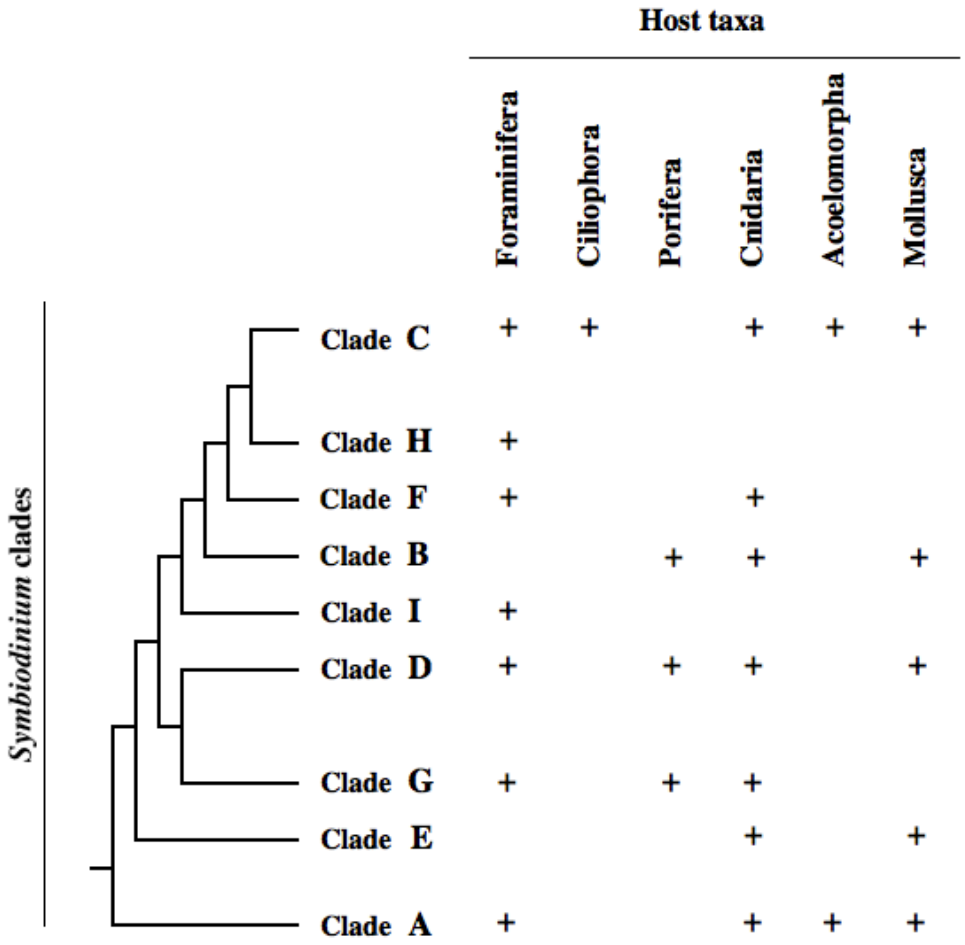

Fig. 1 Relationship between *Symbiodinium* clades (based on a multigene phylogeny – see Pochon *et al*., 2014) and their distribution in host taxa according to Stat *et al*. (2006); Barneah *et al*. (2007b) and Pochon *et al*. (2014).

strains to *Fungia scutaria* larvae, there was no statistical difference in acquisition among strains, except for one of the heterologous that had significantly lower acquisition (Rodriguez-Lanetty *et al*., 2004). Interestingly, homologous strains have been shown to distribute differently in larval tissues than heterologous strains (Rodriguez-Lanetty *et al*., 2006), and it has also been reported that homologous symbionts establish a more benefitting symbiosis (Trench *et al*., 1981; Schwarz *et al*., 1999; Dunn and Weis, 2009). However, Mies *et al*. (this thesis chapters 3 and 4) found no differences in survival in coral, nudibranch and giant clam larvae associated with both homologous and heterologous *Symbiodinium* clades.

The presence of symbionts in larval tissues may significantly contribute to its development. Giant clam larvae infected with *Symbiodinium* grow significantly larger than aposymbiotic larvae (Fitt *et al*., 1986; Mies *et al*., 2012) and *F. scutaria* larvae settle earlier in the presence of symbionts (Schwarz *et al*., 1999). However, higher

growth not always suggests the establishment of symbiosis as some coral larvae have been shown to digest the symbionts (Titlyanov *et al*., 1998).

Biochemical and molecular relationship

The metabolite exchange between *Symbiodinium* and its adult host has been extensively documented. The host supplies the symbiont with CO_2 and substrates for cellular synthesis such as nitrogen and phosphorus (Trench, 1979; Allemand *et al*., 1998; Leggat *et al*., 2003; Weis *et al*., 2008). In turn, the symbiont supplies the host with more than 90% of its metabolic requirement, in the form of organic compounds including glucose, glycerol, fatty acids and amino acids (Muscatine, 1990; Grant *et al*., 1997; Papina *et al*., 2003; Burriesci *et al*., 2012). But very little is known about the metabolite exchange between *Symbiodinium* and metazoan larvae. The only study confirming metabolite exchange is perhaps the one by Kopp *et al*., (2016), which shows that labeled ^{13}C and ^{15}N are translocated from symbiont to *Pocillopora damicornis* larvae. However, that same study found that these compounds contribute insignificantly to the larval nutrition. Other reports show that *Symbiodinium* clades A and C associated with coral and giant clam larvae produce a higher amount of the fatty acid DHA (docosapentaenoic acid, 22:6ω3 – see Mies *et al*., this thesis chapter 3) and that *Symbiodinium* associated with giant clam larvae express a glycerol-synthesizing gene (Mies *et al*., 2017b), but in neither of those cases translocation was effectively confirmed.

One of the main issues hindering the better understanding of this relationship is the lack of molecular studies and information for *Symbiodinium*, also addressed in Leggat *et al*. (2011). The genomes for clades A, B and F (Shoguchi *et al*., 2013; Lin *et al*., 2015; Aranda *et al*., 2016), transcriptomes for clades A-D (Bayer *et al*., 2012; Ladner *et al*., 2012) and the plastid genome for clade B (Mungpakdee *et al*., 2014) have been sequenced in recent years, but further information, especially on metabolic pathways, is still warranted. However, a few experiments performed differential gene expression on coral larvae with and without symbionts, all of which reported undetectable or inconclusive differences (deBoer *et al*., 2007; Voolstra *et al*., 2009; Schnitzler and Weis, 2010). The exception and explanation comes from Mohamed *et al*. (2016), that reports that less than 3% of the host transcriptome is altered after exposure

of *Acropora digitifera* planulae to *Symbiodinium*. While yet to be applied for larval studies, symbiosis-specific genes have been identified recently in the *Aiptasia* anemone (Bucher *et al*., 2016; Wolfowicz *et al*., 2016). From the symbiont perspective, apart from the identification of protein kinases that may be involved in the establishment of symbiosis (Rosic *et al*., 2014), a symbiosis-specific gene was identified in *Symbiodinium* clade A, an H^+-ATPase (Bertucci *et al*., 2010). This gene is the only symbiosis-specific gene that had its expression verified in *Symbiodinium* associated with metazoan larvae (Mies *et al*., 2017b; this thesis chapter 2). The expression of this gene was confirmed in the veliger larvae of the giant clams *Tridacna crocea* and *T. maxima* and in the planula larvae of the hermatypic coral *Mussismilia hispida*, demonstrating that symbiosis is active.

Environmental influence

Climate change is having a significant impact on coral reef ecosystems, particularly because of ocean acidification and the rise in seawater temperature (Hoegh-Guldberg, 1999; Hughes *et al*., 2003; Pandolfi *et al*., 2003; Hoegh-Guldberg *et al*., 2007). However, while the effects of ocean acidification have been investigated in metazoan larvae (Nakamura *et al*., 2011; Waldbusser *et al*., 2013; Rivest and Hofmann, 2014), they are yet to be assessed on metazoan larva-*Symbiodinium* associations. Defined by the loss of *Symbiodinium* cells from host tissue (Brown, 1997; Hoegh-Guldberg, 1999; Fitt *et al*., 2001), the bleaching phenomenon is the main effect of the rise in seawater surface temperature and has been widely documented.

The bleaching threshold for coral reef larvae is usually between 28-30ºC (Edmunds *et al*., 2005; Mieog *et al*., 2009), when the expression of heat-shock proteins and oxidative-stress genes becomes detectable (Rodriguez-Lanetty *et al*., 2009; Meyer *et al*., 2011). The findings in Mies *et al*. (this thesis chapter 4) show that coral and giant clam larvae bleach at 29ºC and hardly survive past 32ºC. However, the highly-tolerant larvae of *Pocillopora damicornis* have been shown to withstand a temperature of 32ºC, despite losing the majority of its symbiont cells (Haryanti *et al*., 2015). The symbiont clade associated with the larvae may also influence bleaching intensity, as coral and giant clam larvae associated with *Symbiodinium* clades A and C bleach at lower rates than clades B, D, E and F (Mies *et al*., this thesis chapter 4). However, further

investigation is required, especially for clade D, which increases the adult host threshold and becomes dominant during bleaching events (Berkelmans and van Oppen, 2006; Jones *et al*., 2008; Mieog *et al*., 2009; Ladner *et al*., 2012).

While symbiont presence is generally perceived as positive for coral reef larvae development and fitness, under thermal stress it may have the opposite effect. High temperatures cause the symbionts to become a source of reactive oxygen species (ROS), which leads to significantly higher larval mortality (Weis, 2008; Baird *et al*., 2009; Yakovleva *et al*., 2009; Schnitzler *et al*., 2012). Some of the effects of ROS include DNA damage and higher rates of antioxidant activity (Yakovleva *et al*., 2009; Nesa *et al*., 2012).

Is there a mutualism?

Symbiosis is usually defined as the long-term interaction between two different organisms and mutualism is defined as a beneficial relationship for both parties. Considering all of the information given in the previous sections, it is questionable whether a mutualistic symbiosis is in place for most metazoan larva-*Symbiodinium* associations. According to Davy *et al*. (2012), the establishment of symbiosis between *Symbiodinium* and its host is a complex process that involves several steps: symbiont uptake, phagocytosis by host cells (in the case of intracellular relationship), function integration, symbiont reproduction and long-term persistence. Therefore, for hosts in their larval development, the establishment of symbiosis would have to follow the steps in Fig. 2, which include symbiont acquisition, transfer of symbionts from gut to endodermal tissue, metabolite exchange and symbiont reproduction and persistence.

The vast majority of experiments performed on larva-*Symbiodinium* associations only visually observed the acquisition of symbionts and no metabolite exchange was detected, which fails to meet the criteria above. Especially considering reports that some larvae may digest the symbionts (Titlyanov *et al*., 1998; Mies *et al*., this thesis chapters 2, 3 and 4). There are several reports that come close to confirming the establishment of the mutualism, such as the transcriptional changes in coral larvae after symbiont exposure (Mohamed *et al*., 2016), although it is unclear if these changes are related to a mutualistic relationship or to the mere presence of a foreign algal body. The reports of higher production of fatty acids by *Symbiodinium* associated with coral and giant clam

larvae (Mies *et al*., this thesis chapter 3) and increases in larval caloric content and competence due to symbiont presence (Ben-David-Zaslow and Benayahu, 1998) also suggest but do not prove metabolite exchange. Perhaps the most compelling evidence of symbiosis between metazoan larvae and *Symbiodinium* comes from Mies *et al*. (2017b, this thesis chapter 2) and Kopp *et al*. (2016), which show the expression of a symbiosis-specific gene by *Symbiodinium* associated with coral (*Mussismilia hispida*) and giant clam (*Tridacna crocea* and *T. maxima*) larvae and the transfer of labeled carbon and nitrogen from symbiont to coral planulae (*Pocillopora damicornis*), respectively.

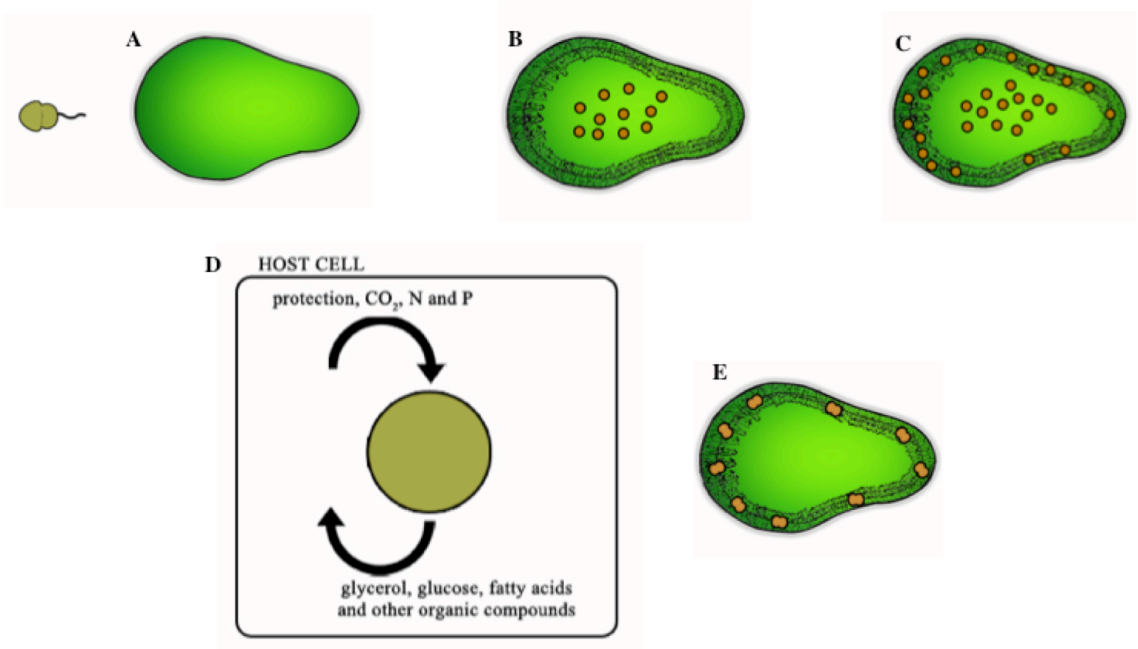

Fig. 2 Steps for the establishment of a mutualistic relationship between *Symbiodinium* and metazoan larvae, using a coral planula as example: **A** larval acquisition of free-living *Symbiodinium* zoospore, **B** symbiont inside gastrovascular vascular immediately after acquisition, **C** symbiont transfer to endodermal tissues, **D** metabolite/favor exchange, and **E** symbiont reproduction and long-term persistence.

This review provides an analysis on the relationship between *Symbiodinium* and metazoan larvae. The information compiled and discussed shows that symbiont presence may be imperative for the larval development and recruitment of certain host groups, while symbiont identity may improve larval fitness and better withstand thermal variations. However, for most cases investigated, the mutualistic relationship cannot be confirmed. The information presented may also be used for the improvement of aquaculture protocols, as well as provide insights for programs related to coral reef conservation and mitigation of climate change impacts.

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Author contributions

M.M. and P.Y.G.S. wrote the manuscript.

CHAPTER TWO

Expression of a symbiosis-specific gene in *Symbiodinium* clade A1 associated with coral, nudibranch and giant clam larvae

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Abstract

Coral reefs are highly diverse marine environments. Such biodiversity is supported by the primary production performed by dinoflagellates of the genus *Symbiodinium*. These dinoflagellates are found in a mutualistic symbiosis with multiple animal phyla. However, very little is known about the establishment of this symbiosis and whether it initiates during host larval development. To address this question, we monitored the expression of a putative symbiosis-specific gene (H^+-ATPase) in *Symbiodinium* A1 *ex hospite* and in association with larvae of a scleractinian coral (*Mussismilia hispida*), a nudibranch (*Berghia stephanieae*) and a giant clam (*Tridacna crocea*). We acquired broodstock for each invertebrate host, induced spawning and collected the larvae, which were placed in plankton kreisels. *Symbiodinium* A1 cells were offered and larvae samples taken for each host at 0, 12, 24, 48 and 72 h after symbiont addition. Control samples such as free-living *Symbiodinium* and broodstock tissue containing symbionts for each host were collected. Total RNA extraction and RT-PCR were performed and amplified products cloned and sequenced. The H^+-ATPase was found expressed in coral and giant clam larvae, but not for nudibranch larvae, that were digesting the symbionts. Broodstock tissue for coral and giant clam also expressed H^+-ATPase, but not the

nudibranch tissue sample. Our results of the H^+-ATPase expression suggest that symbiosis between *Symbiodinium* and *M. hispida* and *T. crocea* is established during host larval development. Conversely, case of *B. stephanieae*, the absence of the H^+-ATPase expression show that *Symbiodinium* is ingested, but no mutualistic relationships are established. In conclusion, our study supports the utilization of H^+-ATPase gene expression as a marker for assessing *Symbiodinium*-invertebrate relationships with applications for the differentiation of symbiotic and non-symbiotic associations.

Keywords: zooxanthellae, *Tridacna*, Scleractinia, sea slug, larval ecology, ATPase

Introduction

Coral reefs are marine coastal environments found in tropical areas, noted for their remarkable biodiversity (Sheppard *et al*., 2009). This diversity is supported by the complexity of habitats created by the $CaCO_3$ structure produced by reef-building corals (Kleypas and Yates, 2009) and by the high primary production performed by symbiotic dinoflagellates, also called zooxanthellae (Cesar, 2000; Hughes *et al*., 2003; Pandolfi *et al*., 2003). These dinoflagellates (genus *Symbiodinium*) are found in an endosymbiotic association with multiple metazoan and protist phyla (Stat *et al*., 2006), being harbored inside the host tissues at high densities, typically 10^{10} cells per m^2 of coral reef (Baker, 2003).

Before the widespread availability of mainstream molecular biology techniques, a single species of zooxanthella had been described (*Symbiodinium microadriaticum*) and considered pandemic (Freudenthal, 1962; Taylor, 1974). However, through phylogenetic analyses combining ribosomal (nuclear), chloroplast and mitochondrial genes (Rowan and Powers, 1991; LaJeunesse, 2001; Pochon *et al*., 2014), *Symbiodinium* dinoflagellates have been proposed to be categorized in nine clades, A-I (Pochon and Gates, 2010). Current efforts are concentrating on the formal description of species within the clades (LaJeunesse *et al*., 2012; Hume *et al*., 2015; Lee *et al*., 2015). Each clade tends to associate with a particular selection of hosts (Baker, 2003; Coffroth and Santos, 2005; Goulet, 2006), and in cases of acquisition of heterologous clades, the host typically displays reduced fitness and growth (Weis *et al*., 2001; Rodriguez-Lanetty, 2003).

The symbiosis between *Symbiodinium* and their hosts is mutualistic. In exchange for protection, CO_2, nitrogen and phosphorus (Trench, 1979; Allemand *et al*., 1998; Leggat *et al*., 2003), *Symbiodinium* supplies the host with several organic

compounds, including glycerol, glucose, fatty acids and amino acids (Grant *et al*., 1997), which may contribute to more than 90% of metabolic requirements of the host (Muscatine, 1990). While this metabolite exchange is very well researched for adult hosts, there is scarcity of information for the relationship between *Symbiodinium* and hosts still in their larval stages. The majority of zooxanthellate organisms acquire their symbionts horizontally (Fitt *et al*., 1986; Harrison and Wallace, 1990; Baird *et al*., 2009), but it is still unknown when and if metabolite exchange initiates during larval development, which may have a crucial impact on the recruitment of coral reef organisms. In addition to these considerations, metabolite exchange and molecular signals are important to ascertain whether a mutualism is in place. Recent studies have sequenced genes in cnidarians that may be symbiosis-specific markers (Baumgarten *et al*., 2015; Mohamed *et al*., 2016; Wolfowicz *et al*., 2016), but are yet to be tested. While *Symbiodinium* genomes for clades A, B and F have been sequenced recently (Shoguchi *et al*., 2013; Lin *et al*., 2015; Aranda *et al*., 2016), only a single symbiosis-specific marker has been suggested to date. This marker is the H^+-ATPase, a proton pump that transports cations across the cell membrane (Møller *et al*., 1996; Kühlbrandt, 2004) and that is only expressed by *Symbiodinium* engaged in the mutualistic symbiosis (Bertucci *et al*., 2010). At present, this gene has only been characterized for *Symbiodinium* A1 (Bertucci *et al*., 2010). Also, it seems to be little conserved among clades, with a variation in the amount and size of introns, making it more difficult to detect and amplify for *Symbiodinium* species belonging to other clades (M. Mies, unpublished data).

In order to better understand the symbiotic relationship between *Symbiodinium* and coral reef larvae and to assess the general suitability of H^+-ATPase as a symbiosis marker, we investigated H^+-ATPase expression by *Symbiodinium* when associated with larvae of three different hosts: i) *Mussismilia hispida*, a scleractinian coral; ii) *Berghia stephanieae*, a nudibranch and iii) *Tridacna crocea*, a giant clam. By investigating these relationships we hope to not only determine if and when the mutualistic relationship between these organisms is established during larval development, but also to increase our current understanding of coral reef larval ecology, with potential implications for recruitment and dispersal.

Materials and methods

The experiment was designed with the purpose of amplifying the H^+-ATPase in *Symbiodinium* associated with coral, slug and clam larvae. Therefore, we cultured *Symbiodinium*, spawned and cultured the offspring of the three hosts, offered the cultured *Symbiodinium*, took samples periodically, performed RNA extraction, RT-PCR, cloning and sequencing.

Symbiodinium *culture*

Symbiodinium cells (ITS2 clade A1) were cultured using the f/2 medium (Guillard and Ryther, 1962), at a temperature of 23ºC and a photon flux of 100 μE m^{-2} s^{-1} with a photoperiod of 12L:12D. The antibiotics penicillin and streptomycin were added together with the culture medium, at a final concentration of 1.0 and 0.5 g L^{-1}, respectively.

Broodstock maintenance and spawning

The *Symbiodinium* hosts selected for this experiment (Table 1) were *Mussismilia hispida*, a reef-building coral endemic to Brazil with a latitudinal distribution of 2,500 km (Castro and Pires, 2001); the stenophagous nudibranch *Berghia stephanieae* (formerly known as *Aeolidiella stephanieae* and often mistaken for *Berghia verrucicornis*) that feeds exclusively on zooxanthellate anemones of the genus *Aiptasia* (Carroll and Kempf, 1990); and the smallest species of giant clams, *Tridacna crocea*. All of these hosts naturally house *Symbiodinium* strains belonging to clade A and all of them acquire symbionts horizontally (Carroll and Kempf, 1990; Neves and Pires, 2002; Mies *et al*., 2012). All organisms were kept under conditions that simulated tropical reef waters, *i.e*., temperature at 27ºC, specific gravity at 1,024 kg m^{-3} and nutrient concentrations near zero. Thirty *M. hispida* colonies (17.5 ± 3.5 cm in approximate diameter) were collected at the Recife de Fora (16º25'S, 38º59'W), near the Abrolhos Reefs in northeastern Brazil. Colonies were kept in semi-closed nursery tanks and naturally spawned gamete bundles containing both spermatozoa and oocytes were

Table 1 Ecological aspects of the three *Symbiodinium* hosts used in this experiment, including their range distribution, spawning mode, larval size, and mode, stages and total duration of larval development (according to Carroll and Kempf, 1990; Mies and Sumida, 2012; Pires *et al*., 2016). Larval stages in bold denote the stages used in this experiment, at 4, 10 and 3 days post-fertilization, respectively.

Host organism	Distri-bution	Spawning mode	Larvae size at hatching (μm)	Larval development mode	Larval development stages	Larval development duration
Mussismilia hispida (Scleractinia)	Tropical Brazil	broadcast spawner	≈ 300	lecithotrophic	**planula**	≈ 12 days
Berghia stephanieae (Gastropoda)	Gulf of Mexico	benthic spawner	≈ 200	facultative planktotrophic	**veliger**	1-2 days
Tridacna crocea (Bivalvia)	Tropical Indo-Pacific	broadcast spawner	≈ 95	planktotrophic	trochophore, **veliger** and pediveliger	≈ 17 days

collected immediately after release. Bundles were dispersed and oocytes fertilized in 60 L tubs and kept for four days until planulae had open digestive tracts. Water changes of 90% were performed daily and strong aeration was provided in order to keep the extremely buoyant eggs from becoming trapped in the surface tension. One hundred thirty broodstock individuals of *B. stephanieae* (1.7 ± 0.3 cm in length) were kept in two 60 L black round tubs in a recirculating aquaria system of 250 L. They were fed 250 individuals of the glass anemone, *Aiptasia* sp. (harboring *Symbiodinium* clade A1) and egg masses spawned overnight were collected in the next morning. Embryos were then kept for ten days under strong aeration in order to stimulate the release of veliger larvae (Carroll and Kempf, 1990). Finally, 10 *T. crocea* broodstock individuals (7.6 ± 0.9 cm in shell length) were maintained in a 350-L recirculating system for three months in order to stimulate gamete production (Beckvar, 1981; Mies and Sumida, 2012). They were then induced to spawn with an intragonadal injection of 1.0 mL of a serotonin (5-hydroxytryptamine, 1.0 g L^{-1}) solution (Braley, 1985; Alcazar *et al*., 1987; Mies *et al*., 2013). Fertilization was performed according to Heslinga *et al*. (1990) and eggs and, subsequently, trochophore larvae were kept in 60 L black round tubs for three days until all larvae attained the veliger stage. Water changes of 50% were performed daily.

Larval cultures, Symbiodinium *offering and sampling*

For each host, larvae were placed in three (replicates) 1.2-L plankton kreisels kept in water baths at 27ºC. *Mussismilia hispida* planulae were stocked at 0.8 larva mL^{-1}, *B. stephanieae* veligers at 1.0 mL^{-1} and *T. crocea* veligers at 2.0 mL^{-1}. *Symbiodinium* A1 was then offered at a final concentration of 10^3 cells mL^{-1} to all kreisels. At 11 h post-symbiont offering (PSO), a water change of 100% was performed in all kreisels in order to remove *Symbiodinium* cells that had not been acquired. Symbiont acquisition was recorded at this point. Samples of 50, 50 and 250 larvae were taken for *M. hispida*, *B. stephanieae* and *T. crocea*, respectively, at 0, 12, 24, 48 and 72 h PSO. As a positive control for the expression of H^+-ATPase, tissue (containing symbionts) was retrieved from adult individuals of each host. To confirm that cultured (free-living) *Symbiodinium* do not express H^+-ATPase, a sample containing 1.0 x 10^6 cells was obtained.

Primer design

In order to confirm the identity of the *Symbiodinium* culture we amplified the internal transcribed spacer 2 (ITS2), using primers designed by LaJeunesse and Trench (2000). Two *Symbiodinium* genes were targeted for this experiment, H^+-ATPase (Enzyme Commission number 3.6.3.6) and RuBisCO (Ribulose-1,5-bisphosphate carboxylase oxygenase, EC number 4.1.1.39), with the latter chosen as a positive control. Primers for H^+-ATPase (5'-GCACTTCTTGGGCTTGCTGC-3' and 5'-ATCTTCCGGGACTCCACCAC-3') were designed in adjacent regions of two conserved amino acids motifs that are diagnostic for this protein (Meade *et al*., 2000), the ATP phosphorylation site (DKTGTLT) and the ATP binding site (TGDGVND). The design was based on conserved regions from the alignment of several sequences obtained from transcriptomes and expressed sequence tags of multiple *Symbiodinium* clades and other dinoflagellates (Hackett *et al*., 2005; Patron *et al*., 2005; Karako-Lampert *et al*., 2006; Leggat *et al*., 2007; Toulza *et al*., 2010; Bayer *et al*., 2012; Ladner *et al*., 2012; Baumgarten *et al*., 2013; Rosic *et al*., 2014). The RuBisCO primers (5'-ACCGGCGTGGGCAAGCTGTTCTCT-3' and 5'-TGGGAGTGGTCTGCTTCATG-3') were taken from Bertucci *et al*. (2010).

RNA extraction and RT-PCR reaction

Total RNA was extracted from all samples, including the cultured *Symbiodinium* and the tissues from coral, nudibranch and giant clam broodstock. Samples were macerated with a mortar and a pestle, and TriReagent (Ambion) was used for the extraction with modifications suggested in Rosic and Hoegh-Guldberg (2010). Extracted RNA was then treated with the Turbo-DNA-Free kit (Ambion) and the cDNA was generated using the SuperScript First Strand Synthesis III kit (Invitrogen). Approximately 50 ng of cDNA was used in the RT-PCR in a reaction volume of 25 μL, with final concentrations of 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.15 mM for both forward and reverse primers and 0.04 units μL^{-1} of GoTaq DNA polymerase (Promega). Cycling conditions for H^+-ATPase and RuBisCO were the following: 3 min at 94ºC, 35 cycles of 1 min at 94ºC, 1 min at 54ºC and 1.5 min at 72ºC and termination at 72ºC for 5 min. Cycling conditions for the ITS2 were according to LaJeunesse and Trench (2000).

Cloning and sequencing

The amplicons produced were separated on 0.8% agarose, band-purified using the Nucleospin Extract II kit (Macherey-Nagel) and ligated into pGEM T-Easy vector (Promega). Vectors were transformed into electrocompetent cells (DH10B) according to standard practices described in Sambrook *et al*. (1989). Plasmid minipreparations, also according to Sambrook *et al*. (1989), were performed for each RT-PCR reaction and sequenced on a 3130XI sequencer using T7 vector primer.

Phylogenetic analysis

Nucleotide sequences related to both H^+-ATPase and RuBisCO sequences produced in this experiment were retrieved from the National Center for Biotechnology Information (NCBI) using the BLAST algorithm (Sayers *et al*., 2012). Maximum-likelihood phylogenies for both genes were generated in MEGA5 (Tamura *et al*., 2011) using the optimal model of nucleotide substitution (default settings) and a bootstrap of 1,000 replicates.

Results

More than 99% of all host larvae acquired symbionts. The number of symbionts acquired varied greatly among hosts. Symbiont acquisition per planula larva of *M. hispida* was 194.5 ± 31.6 cells, while *B. stephanieae* and *T. crocea* veligers acquired 19.2 ± 5.0 and 36.6 ± 6.5 cells, respectively. *Tridacna crocea* veligers kept the symbionts in the digestive tract throughout the duration of the experiment, while *M. hispida* planulae seemed to move them from the gastrovascular cavity to different areas in the endoderm (Fig. 1). Surprisingly, stereomicroscope observations showed that *B. stephanieae* larvae digested the symbionts and did not move them to specialized tissues or cells. Many *B. stephanieae* individuals underwent metamorphosis after 48 h PSO and all of them had become juveniles at 72 h PSO.

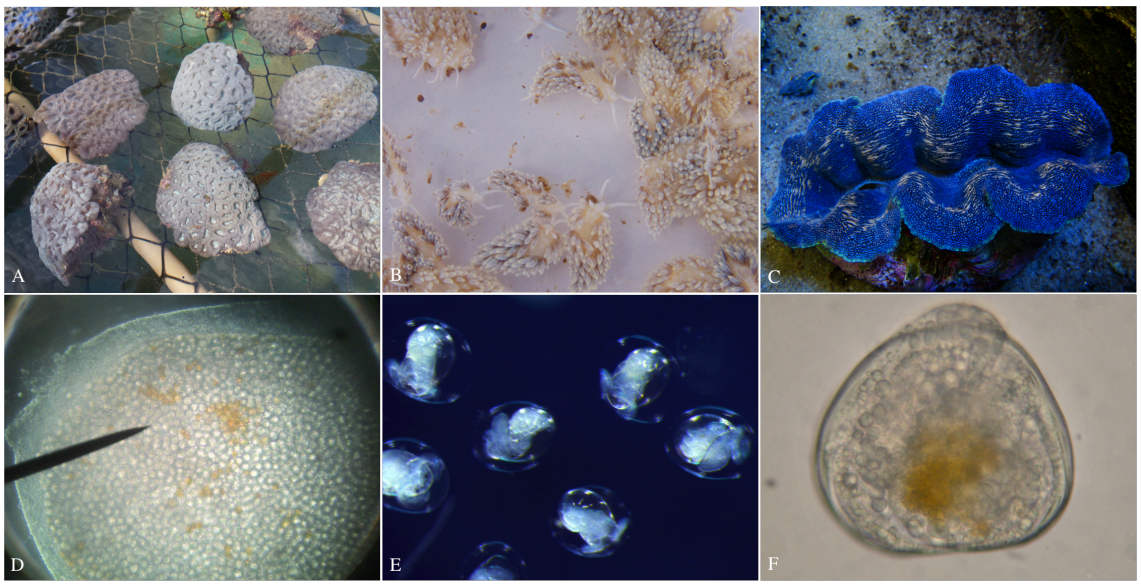

Fig. 1 Host broodstock and larvae used in the experiment. **A** *Mussismilia hispida* colonies collected at Recife de Fora, **B** *Berghia stephanieae* spawning individuals (note brownish area in the cerata, harboring *Symbiodinium* cells captured from the anemone *Aiptasia* sp.), **C** *Tridacna crocea* broodstock clam, **D** *M. hispida* planula after acquiring multiple *Symbiodinium* A1 cells, **E** *B. stephanieae* veliger larvae immediately before hatching and **F** *T. crocea* veliger larva with *Symbiodinium* A1 cells inside the digestive tract.

The ITS2 amplification confirmed that the *Symbidiodinium* cells belonged to clade A1. The amplicons produced for H^+-ATPase and RuBisCO genes had a size of 460 and 430 bp, respectively (GenBank accession numbers KY483989-997). The BLAST searches and phylogenetic trees (Fig. 2) confirmed that the sequences obtained belong to *Symbiodinium* A1. All H^+-ATPase and RuBisCO sequences obtained were 99

and 100% identical in pairwise comparisons, respectively. Phylogenetic analyses (Fig. 2) and BLAST results confirm that the targeted gene were amplified and belonged to *Symbiodinium* A1. The H^+-ATPase sequences were 99% identical to *Symbiodinium* A1 (GenBank accession number FJ807389) and RuBisCO sequences were 95% identical to *Symbiodinium* (GenBank accession number JX465541).

Free-living *Symbiodinium*, as expected, did not express the H^+-ATPase. Broodstock tissue containing symbionts from both *M. hispida* and *T. crocea* did express the H^+-ATPase, while tissue from *B. stephanieae* did not. Out of the three replicated larval cultures for *M. hispida*, the H^+-ATPase gene was expressed in only one replicate, at 72 h PSO (Table 2). This gene was not expressed by any *B. stephaniae* larval replicates, at any time. For *T. crocea*, one of the replicates expressed the H^+-ATPase at 24, 48 and 72 h PSO, the second replicate only at 24 and 72 h PSO and the third replicate did not express the gene. The RuBisCO gene was expressed for all broodstock tissue and larval samples, as well as for the free-living *Symbiodinium* in culture.

Discussion

The establishment of a mutualistic symbiosis is a process that requires the successful completion of many steps, such as symbiont acquisition, transfer to specialized cells/tissues, metabolite and/or favor exchange, and long-term persistence (Davy *et al*., 2012). However, very little is known about the biochemical and molecular mechanisms involved in the establishment of this relationship. The only difference reported is the expression of H^+-ATPase, a symbiosis-specific gene (Bertucci *et al*., 2010). Although a number of studies have investigated differential expression in marine invertebrate hosts with and without *Symbiodinium* (deBoer *et al*., 2007; Voolstra *et al*., 2009a; Voolstra *et al*., 2009b; Polato *et al*., 2010; Schnitzler and Weis, 2010; Wolfowicz *et al*., 2016), few have attempted to suggest symbiosis marker genes that can be reliably used to assess symbiotic states. The protein coded for by the H^+-ATPase is responsible for several reactions, particularly in generating protons gradients across the plasma membrane and dehydrating HCO_3^- (Goiran *et al*., 1996; Allemand *et al*., 1998; Kühlbrandt, 2004). This gene is also present in other photosynthetic eukaryotes such as

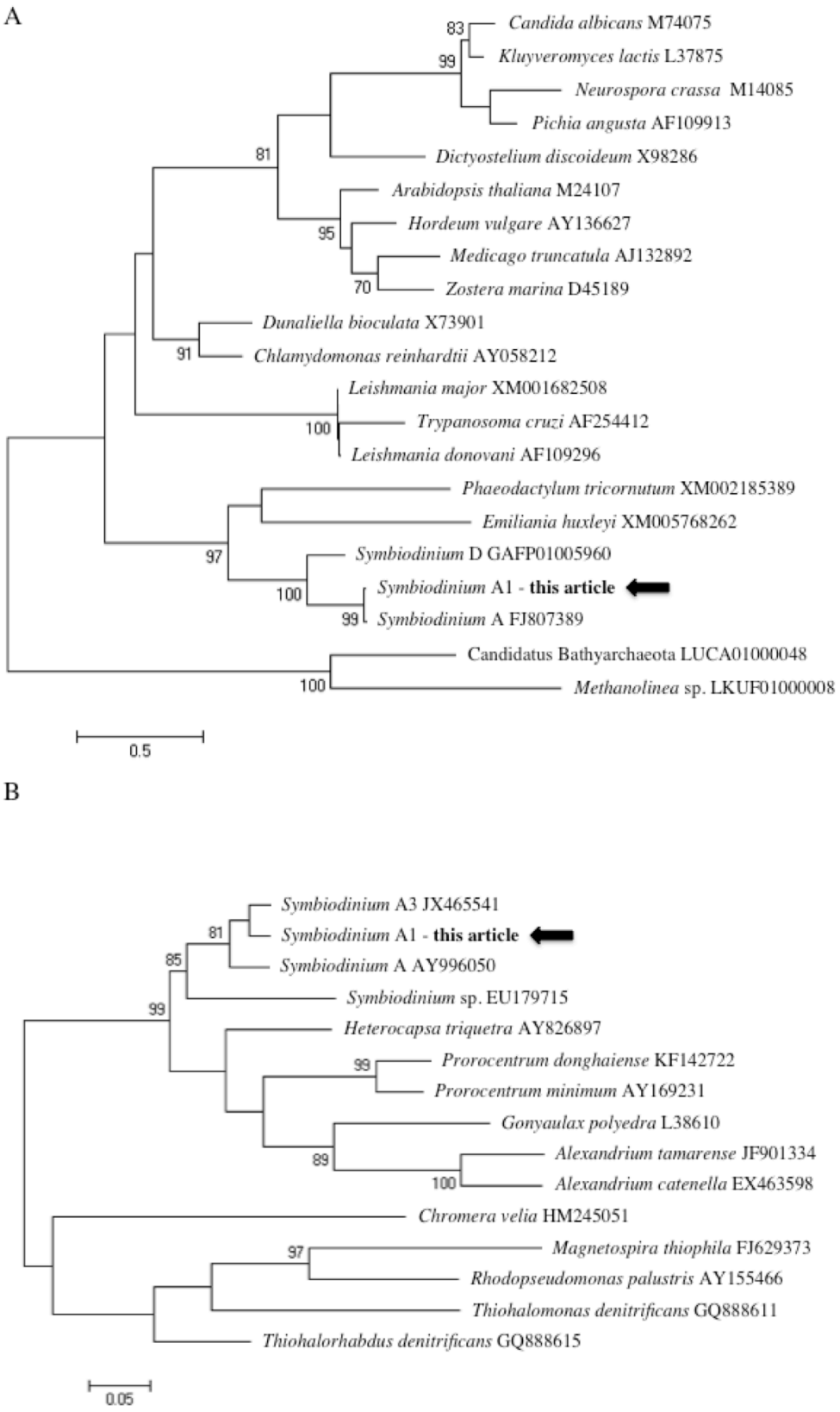

Fig. 2 Phylogeny of (**A**) H^+-ATPase and (**B**) RuBisCO genes of *Symbiodinium* A1 in this experiment. Trees were constructed using maximum likelihood analysis and 1,000 bootstrap replicates; only values above 70 are shown. Accession numbers are from the National Center of Biotechnology Information (NCBI) database.

Table 2 Expression of H^+-ATPase by *Symbiodinium* A1 acquired by *Mussismilia hispida* (scleractinian coral), *Berghia stephanieae* (nudibranch) and *Tridacna crocea* (giant clam) larvae over 72 h after acquisition. Expression for cultured *Symbiodinium* A1 (free-living) and tissue of host broodstock was also monitored. All samples exhibited expression of RuBisCO (positive control).

Sample	Control samples	0 h	12 h	24 h	48 h	72 h
Symbiodinium A1 culture	-	n/a	n/a	n/a	n/a	n/a
Mussismilia hispida						
Broodstock tissue	**+**	n/a	n/a	n/a	n/a	n/a
Larvae replicate 1	n/a	-	-	-	-	-
Larvae replicate 2	n/a	-	-	-	-	**+**
Larvae replicate 3	n/a	-	-	-	-	-
Berghia stephanieae						
Broodstock tissue	-	n/a	n/a	n/a	n/a	n/a
Larvae replicate 1	n/a	-	-	-	-	-
Larvae replicate 2	n/a	-	-	-	-	-
Larvae replicate 3	n/a	-	-	-	-	-
Tridacna crocea						
Broodstock tissue	**+**	n/a	n/a	n/a	n/a	n/a
Larvae replicate 1	n/a	-	-	**+**	**+**	**+**
Larvae replicate 2	n/a	-	-	**+**	-	**+**
Larvae replicate 3	n/a	-	-	-	-	-

+ : positive expression
- : no expression
n/a : not applicable

the angiosperm *Arabidopsis thaliana* (see Pardo and Serrano, 1989) and the planktonic green alga *Platymonas viridis* (see Popova and Balnokin, 1992). While many studies have observed symbiont acquisition by metazoan larvae (Fitt *et al*., 1986; Schwarz *et al*., 1999; Weis *et al*., 2001; Rodriguez-Lanetty *et al*., 2006; Marlow and Martindale, 2007; Harii *et al*., 2009; Cumbo *et al*., 2012; Ramos and Banaszak, 2014; Wolfowicz *et al*., 2016), very few tested metabolite exchange or symbiosis-specific molecular signals (Kopp *et al*., 2016; Mies *et al*., 2017b). For that purpose, we decided to investigate whether larvae of several marine invertebrate coral reef taxa express H^+-ATPase as a result of *Symbiodinium* acquisition.

While only few and nearly undetectable differences were found in comparative analyses of the transcriptomes of symbiotic and aposymbiotic coral larvae (deBoer *et*

al., 2007; Voolstra *et al*., 2009b; Schnitzler and Weis, 2010), we did find expression of H^+-ATPase in *Symbiodinium* acquired by the larvae of the coral *Mussismilia hispida*. This expression was found in only one of the triplicates, at 72 h PSO, however. While monitoring the expression of this gene for a longer period of time would probably give a broader view on the establishment of this mutualistic symbiosis, our results do show that *Symbiodinium* A1 and coral larvae are able to engage in symbiosis. These findings relate to reports that *Symbiodinium* A1 acquired by *M. hispida* larvae produce a higher amount of fatty acids and present lower bleaching rates than most of the other *Symbiodinium* clades (Mies *et al*., this thesis chapters 3 and 4).

For the experiments with the nudibranch *Berghia stephanieae*, a valuable product in the marine ornamental trade (Olivotto *et al*., 2011), the expression of H^+-ATPase was not detected in any of the larval samples, and, more importantly, neither in the broodstock tissue sample. In fact, host larvae were digesting the symbionts. Despite evidence that *Symbiodinium* mutualistically translocates photosynthetically-fixed carbon to the nudibranch *Pteraeolidia ianthina* (Hoegh-Guldberg and Hinde, 1986), this does not seem to be the case for *B. stephanieae*. There is much evidence that goes against the idea of mutualism in this species, especially from the *Symbiodinium* perspective: *B. stephanieae* are nocturnal organisms and remove the symbionts from the *Aiptasia* anemone, which is found in sunlit areas, and later defecates the non-motile *Symbiodinium* cysts after 3-6 days from acquisition (Kempf, 1991). This not only deprives *Symbiodinium* from light, but also renders it an easy prey in the benthos. Furthermore, it has been reported that some nudibranchs sequester *Symbiodinium* from their prey, but may not engage in symbiosis (Rudman, 1981; Burghardt *et al*., 2005). Therefore, the association between *B. stephanieae* and *Symbiodinium* does not seem to fit the requirements for a mutualistic symbiosis. Regardless, this example supports that the expression of H^+-ATPase is not an endocytosis signal.

In the case of *Tridacna crocea*, an important commodity for both the food and aquarium trade (Mies *et al*., 2017a), many studies based on morphological examinations suggested that symbiosis was not established until metamorphosis (Fitt *et al*., 1986; Hirose *et al*., 2006), when symbionts migrated to post-metamorphic diverticulae called zooxanthellal tubular system (Norton *et al*., 1992). However, studies show that veliger larvae grow faster and have increased survival if symbionts are available (Fitt *et al*., 1986; Mies *et al*., 2012). Our results show that *Symbiodinium* cells in two of the three

replicates of *T. crocea* larvae expressed H^+-ATPase, in agreement with the findings of Mies *et al*. (2017b) for *T. maxima*. Similarly to that reported for coral larvae, *Symbiodinium* acquired by *T. crocea* veliger also seem to produce more fatty acids and to be more resistant to bleaching (Mies *et al*., this thesis chapters 3 and 4). However, the expression of H^+-ATPase was intermittent for one of the replicates (Table 2). While there is very little information available in the literature to explain why this would happen, we argue it may be related to the intense changes in circadian rhythm in *Symbiodinium* and other dinoflagellates (Rees *et al*., 1993; Yacobovitch *et al*., 2004; Pizay *et al*., 2009). Nonetheless, this event reinforces that the modulation of the expression H^+-ATPase requires further investigation.

It is important to note that the host organisms selected for this experiment, and particularly their larval ecology, are very different (Table 1). Scleractinian coral larvae are known lecithotrophs and go through metamorphosis without any exogenous feeding (Morse *et al*., 1996), while giant clam veligers are planktotrophs and must feed before attaining the juvenile stage (Fitt *et al*., 1984; 1986; Mies *et al*., 2012). Based on H^+-ATPase expression, our findings argue that giant clam larvae establish symbiosis with *Symbiodinium* earlier than coral larvae, which could point to their higher need of exogenous nutrition. While the nudibranchs also produce lecithotrophic larvae, they are facultative planktotrophs and the *Symbiodinium* seems to be more of a prey item than a symbiont, as H^+-ATPase was never expressed and *Symbiodinium* cells were digested.

While our experiments may contribute to the knowledge on the state of the symbiotic relationship between *Symbiodinium* and coral reef larvae by mean of H^+-ATPase expression, there is still an overwhelming lack of marker genes for *Symbiodinium*. This hinders functional genomics studies (Leggat *et al*., 2011). Investigating comparative differential gene expression in the free-living and coccoid stages (symbiotic) is crucial for further understanding the relationships between coral reef organisms and their symbionts. As an example, the expression of H^+-ATPase may be tied to the non-motile coccoid life stage of *Symbiodinium*. Interestingly, the shift from free-living to coccoid stage has been shown to be chemically stimulated by lectin produced by the coral host (Kita *et al*., 2015). Regardless, our results support the application of H^+-ATPase gene expression as a molecular symbiosis-specific marker for *Symbodinium*-invertebrate associations. This gene may be used for distinguishing between symbiotic and non-symbiotic associations (*e.g*., the case of the nudibranch *B.*

stephanieae). Our findings may also provide insights for coral reef restoration and aquaculture protocols (Pomeroy *et al*., 2006; Guest *et al*., 2010), as early symbiont acquisition and mutualism establishment may improve survival and metamorphic competence.

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Author contributions

M.M. designed the experiment, M.M. performed the experiment, C.R.V., C.B.C., D.O.P. and E.N.C. contributed with infrastructure/material/technical support, M.M. analyzed the data and M.M., P.Y.G.S., C.R.V. and M.A.V.S. wrote the manuscript.

CHAPTER THREE

Production of three symbiosis-related fatty acids by *Symbiodinium* clades A-F associated with coral reef larvae

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Abstract

Symbiodinium are phototrophic dinoflagellates engaged in a mutualistic symbiosis with multiple coral reef taxa. They are divided in nine different clades (A-I), which typically associate with different hosts. However, it is still unknown if *Symbiodinium* clades present differences in metabolism, especially when associated with metazoan larvae. We tested if three ω3 fatty acids (SDA, DPA and DHA) that are typically translocated from *Symbiodinium* to its host are produced in *Symbiodinium* clades A-F associated with *Mussismilia hispida* (scleractinian coral), *Berghia stephanieae* (nudibranch) and *Tridacna crocea* (giant clam) larvae. For that purpose, we acquired and spawned broodstock for each host, cultured their larvae and offered *Symbiodinium* clades A-F. Samples were taken during a 72-h window after the offer of *Symbiodinium* and fatty acids were extracted and analyzed by gas chromatography. The concentrations for SDA and DPA for all host larvae-dinoflagellate associations were low and variable, without any trends. However, *M. hispida* planula larvae associated with *Symbiodinium* A and C had a statistically significant higher amount of DHA. The veliger larvae of *B.*

stephanieae digested the *Symbiodinium* and the amount of DHA remained constant throughout the experiment. The veliger larvae of *T. crocea* associated with *Symbiodinium* A and C also presented a higher amount of DHA, although not statistically different from the other clades. These results show that *Symbiodinium* clades A and C, homologous to *M. hispida* and *T. crocea*, may contribute with a small amount of DHA to the symbiotic associations within these host larvae and form a stronger mutualism than the other clades.

Keywords: zooxanthellae, Scleractinia, *Tridacna*, gas chromatography, lipids, larval ecology

Introduction

Symbiodinium are photosynthetic dinoflagellates responsible for the majority of the primary production in coral reefs (Muscatine and Porter, 1977; Muscatine *et al*., 1981). These organisms are found in either a free-living or a non-motile coccoid stage (Freudenthal, 1962; Schoenberg and Trench, 1980), with the latter found in higher abundances and engaged in a mutualistic symbiosis with several metazoan and protist phyla (Stat *et al*., 2006; Venn *et al*., 2008). Based on molecular phylogenies inferred on ribosomal genes (Rowan and Powers, 1991; LaJeunesse, 2001), *Symbiodinium* dinoflagellates are divided in nine clades, A-I (Pochon and Gates, 2010). The ancestral clade A originated in the Eocene about 50 million years ago, with most extant strains diversifying about 15 million years go in the Miocene (Tchernov *et al*., 2004; Pochon *et al*., 2006).

Each *Symbiodinium* clade tends to associate with specific hosts (Baker, 2003; Coffroth and Santos, 2005) and usually the host taxon associates with a single symbiont strain (LaJeunesse, 2002; Goulet, 2006). For instance, clades A-D are usually found in cnidarians and mollusks, while clades F-I are typically found in foraminiferans (Stat *et al*., 2006; Pochon and Gates, 2010). However, *Symbiodinium* can be diverse within the host, as multiple clades have been found in a single host individual on numerous occasions (Rowan and Powers, 1991; Rowan and Knowlton, 1995; Carlos *et al*., 2000; Baker, 2003; Loh *et al*., 2006; Picciani *et al*., 2016). Host-symbiont recognition mechanisms take place during initial infection and the host organism may exert some control on which *Symbiodinium* strains it will harbor (Belda-Baillie *et al*., 1999; Weis *et al*., 2001; Little *et al*., 2004; Coffroth and Santos, 2005), and also on its density inside the tissues (Gordon and Leggat, 2010). *Symbiodinium* clades can also be specialized in

certain niches, as some strains are more adapted to lower light intensity found in higher depths (Cooper *et al*., 2011) and others more tolerant to high temperatures (van Oppen *et al*., 2005; Ladner *et al*., 2012).

The symbiosis between *Symbiodinium* and its host is mutualistic. In exchange for protection, CO_2 and substrate for cellular synthesis, the symbiont supplies the host with metabolites such as glycerol, glucose and fatty acids, among other organic compounds (Trench, 1979; Muscatine, 1990; Grant *et al*., 1997; Allemand *et al*., 1998). Specifically, three ω3 fatty acids (18:4ω3 - SDA, 22:5ω3 - DPA and 22:6ω3 - DHA) are considered dinoflagellate markers and are translocated from *Symbiodinium* to host (Graeve *et al*., 1994; Zhukova and Aizdaicher, 1995; Papina *et al*., 2003). However, it is unknown if the *Symbiodinium* clades may present differences in their metabolic contributions to the host. Furthermore, while translocation of photosynthates to the adult host has been well described, it remains largely untested for hosts undergoing larval development.

In light of such issues, this experiment intends to answer the question whether different *Symbiodinium* clades present different production rates of symbiosis-related ω3 fatty acids when associated with coral reef larvae. For that purpose, we cultured the larvae of a scleractinian coral, a nudibranch and a giant clam and offered *Symbiodionium* clades A-F. Then we monitored the increase and production of the three symbiosis-related ω3 fatty acids SDA, DPA and DHA. Studying the metabolic relationship between coral reef larvae and *Symbiodinium* is of paramount importance, as it may provide insights for the recruitment in coral reefs and also perhaps aid in programs related to coral reef restoration and the mitigation of climate change impacts.

Materials and methods

The experimental design had the purpose of detecting increments in the concentration of three ω3 fatty acids, 18:4ω3 (stearidonic acid, SDA), 22:5ω3 (docosapentaenoic acid, DPA) and 22:6ω3 (docosahexaenoic acid, DHA), in the association between *Symbiodinium* clades A-F and the larvae of *Mussismilia hispida* (hermatypic coral), *Berghia stephanieae* (nudibranch) and *Tridacna crocea* (giant clam). For that, we cultured the larvae, offered cultured *Symbiodinium*, collected samples and performed fatty acid extraction and gas chromatography analysis.

Symbiodinium *culture*

The *Symbiodinium* (ITS2 types A1, B1, C1, D1, E1 and F1) were cultured in f/2 medium (Guillard and Ryther, 1962), at 23ºC and with a photon flux of 100 μE m^{-2} s^{-1} in a photoperiod of 12L:12D. The antibiotics streptomycin (0.5 g L^{-1}) and penicillin were used (1.0 g L^{-1}).

Broodstock maintenance and spawning

All hosts selected for this experiment are simultaneous hermaphrodites and acquire symbionts horizontally (Carroll and Kempf, 1990; Neves and Pires, 2002; Mies *et al*., 2012). Broodstock for all hosts was maintained in conditions that simulated tropical reef waters, with temperature at 27ºC, specific gravity at 1,024 kg m^{-3} and nutrients at undetectable levels. *Mussismilia hispida* colonies (n = 30, 17.5 ± 3.5 cm in approximate diameter) were collected at the Recife de Fora (16º25'S, 38º59'W) in northeastern Brazil and kept in semi-closed tanks. Natural spawns occurred in the evenings of September 2015 and gamete bundles were collected immediately after release and later fertilized in 60-L round tanks. Strong aeration was provided in order to prevent larvae from becoming trapped in the surface tension. Water changes of 90% were performed daily for four days until planulae were capable of acquiring symbionts. Broodstock of the nudibranch *B. stephanieae* was produced in the lab from originally 12 individuals according to the recommendations in Carroll and Kempf (1990) and Mies *et al*. (in preparation). A total of 95 broodstock individuals (1.5 ± 0.2 cm in length) were produced and kept in a recirculating aquaria system of 250 L. A mesh of 250 μm installed on the drainpipe prevented the nudibranchs from escaping the tank. The broodstock was heavily fed with 100 individuals of the glass anemone *Aiptasia* sp. (*B. stephanieae* are stenophagous nudibranchs that acquire both nutrition and symbionts exclusively from *Aiptasia* spp. and present a short larval development of about 48 h – see Carroll and Kempf, 1990 and Leal *et al*., 2012). Spawns were collected in the next morning and kept for 10 days under strong aeration in order to stimulate the release of veliger larvae (Carroll and Kempf, 1990). Ten broodstock *T. crocea* clams (7.6 ± 0.9 cm in shell length) were kept in a recirculated system of 350 L and induced to spawn by an intragonadal injection of 1.0 mL of serotonin (5-hydroxytryptamine, 1.0 g L^{-1})

according to the procedures in Braley (1985) and Mies *et al*. (2013). Spawned gametes were fertilized in 60 L round tubs according to the procedures in Heslinga *et al*. (1990). Daily water changes of 50-60% were performed during the embryonic and larval development, until all larvae had reached the veliger larval stage, at 72 h post-fertilization.

Larval cultures, Symbiodinium *offering and sampling*

The larvae produced for each host were stocked in 1.2-L plankton kreisels with filtered seawater. Four days post-fertilization (PF) *Mussismilia hispida* planulae were stocked at 0.8 larva mL^{-1}, ten days PF *B. stephanieae* veligers at 1.0 mL^{-1} and three days PF *T. crocea* veligers at 2.0 mL^{-1}. The kreisels were placed in a water bath at 27ºC. Twenty-one plankton kreisels were used for each host larvae; three replicated kreisels for each one of the six *Symbiodinium* clades (A-F) and one control group to which no symbionts were offered. The *Symbiodinium* were then offered at a final concentration of 10^3 cells mL^{-1}. At 11 h post-symbiont offering (PSO), *Symbiodinium* acquisition was recorded and a water change of 100% was performed in all kreisels in order to prevent symbiont reacquisition. Samples of 25, 25 and 200 larvae were taken for *M. hispida*, *B. stephanieae* and *T. crocea*, respectively, at 0, 12, 24, 48 and 72 h PSO. Together with each sample, three larvae were collected, measured (length) and squashed under the microscope and the *Symbiodinium* mitotic index (Wilkerson, 1983) was recorded. Survival was also monitored during sampling.

Fatty acids extraction and analysis

Collected samples were macerated with glass beads, a mortar and a pestle in 120 μL of water for 2 minutes, before being vortexed for 3 minutes and centrifuged for collection. Extraction was performed in similar fashion to Masood *et al*. (2005). Briefly, 90 μL of the sample was incubated with 50 μL of margaric acid (C17:0, 1.0 mg mL^{-1}), 1.85 mL of methanol and 100 μL of acetyl chloride for one hour at 100ºC. Hexane was added and the organic layer transferred to a fresh tube. The solution was evaporated under N_2 until dry and the residue dissolved in 100 μL of hexane. The individual fatty acid methyl esters (FAMEs) were analyzed by a Trace 1310 gas chromatograph (Thermo

Scientific) equipped with a flame ionization detector. FAMEs were separated with a DB-FFAP column of 15 m x 0.1 mm ID x 0.1 μm film thickness (J & W Scientific, Agilent Technologies). The temperature program started at 150ºC with a 15-second hold, before increasing 35ºC per minute to 200ºC, 8ºC per minute to 225ºC with a 3.2-minute hold, 80ºC per minute to 245ºC and finishing with a 4.75-minute isothermal period. A mixture of hydrogen and nitrogen was used as carrier gas. The FAMEs were identified by direct comparison of their retention times with the PUFA Nº1 Marine Source (Sigma-Aldrich) standard mix. Each individual peak was integrated and normalized by the internal standard (C17:0). The amount of FAMEs detected was normalized by the number of larvae in the samples.

Statistical analysis

Statistical analyses were performed independently for each host larvae. In order to verify if time and *Symbiodinium* clade influence the amount of SDA, DPA and DHA, a two-way analysis of variance (ANOVA) was performed. To verify which treatments (clades) were statistically different at the end of the experiment, a one-way ANOVA was performed on the 72 h samples, with a *post-hoc* Tukey's HSD test. Finally, a one-way ANOVA was performed on the 72 h samples for survival and growth to test if there were any significant differences between larvae associated with *Symbiodinium* clades A-F.

Results

Symbiont acquisition was higher than 99% for all *Symbiodinium* clades and all host larvae. While the amount acquired for *Berghia stephanieae* and *Tridacna crocea* larvae was similar for all clades, *Mussismilia hispida* acquired *Symbiodinium* A1 cells nearly twice as much as the other clades (Table 1). While symbionts remained intact in *M. hispida* and *T. crocea* larvae throughout the experiment, *B. stephanieae* larvae appeared to digest most of the symbiont cells acquired for all clades. The mitotic index (Table 1) shows that approximately 10-15% of the *Symbiodinium* cells (for all clades) acquired by *M. hispida* and *T. crocea* were undergoing division while only 6% for *B. stephanieae* larvae.

Table 1 Acquisition of *Symbiodinium* clades A-F by *Mussismilia hispida* (scleractinian coral), *Berghia stephanieae* (nudibranch) and *Tridacna crocea* (giant clam) larvae after 11 hours of exposure. The average mitotic index of acquired *Symbiodinium* during the 72-h experiment is also presented.

	Mussismilia hispida		*Berghia stephanieae*		*Tridacna crocea*	
Clade	*Symbiodinium* cells acquired	*Symbiodinium* mitotic index	*Symbiodinium* cells acquired	*Symbiodinium* mitotic index	*Symbiodinium* cells acquired	*Symbiodinium* mitotic index
Control	0.0 ± 0.0	n/a	0.0 ± 0.0	n/a	0.0 ± 0.0	n/a
A	194.5 ± 31.6	0.14 ± 0.04	19.2 ± 5.0	0.05 ± 0.03	36.6 ± 6.5	0.09 ± 0.03
B	123.2 ± 22.9	0.12 ± 0.02	18.5 ± 5.4	0.04 ± 0.04	33.5 ± 10.7	0.14 ± 0.07
C	125.2 ± 18.6	0.14 ± 0.07	19.5 ± 3.1	0.10 ± 0.03	38.1 ± 3.2	0.14 ± 0.06
D	119.0 ± 30.2	0.19 ± 0.05	16.5 ± 1.0	0.08 ± 0.03	31.7 ± 9.0	0.11 ± 0.04
E	123.5 ± 21.9	0.12 ± 0.05	18.2 ± 2.9	0.06 ± 0.03	32.0 ± 8.4	0.06 ± 0.05
F	102.2 ± 27.9	0.13 ± 0.05	13.5 ± 2.3	0.05 ± 0.02	31.1 ± 7.7	0.14 ± 0.01

n/a : not applicable

For all host larvae, no significant differences were found in survival at the end of the experiment between the control group (larvae without *Symbiodinium*) and the treatments for the six clades. The same was verified for growth. While *M. hispida* and *T. crocea* larvae grew steadily during the experiment, *B. stephanieae* larvae showed a remarkable increase in size at 48 h post-hatch and PSO (Fig. 1). This is related to the fact that veliger larvae underwent metamorphosis and juvenile nudibranchs discarded their shells and fully extended their body.

The results for SDA and DPA showed no patterns and their content was highly variable throughout the experiment, for all host larvae (Fig. 2). In the case of DPA, it was nearly undetectable for *B. stephanieae* and *T. crocea* larvae and in fact undetectable for some *T. crocea* samples. In order to detect if time and treatment could influence the SDA and DPA content, a two-way ANOVA was performed, but no significant differences were found for all host larvae. In the case of DHA, it is possible to observe a slight increase in its content for all host larvae associated with most clades at 12 h PSO (Fig. 3). Another increase takes place at 48 h PSO for *M. hispida* and *T. crocea* larvae associated with *Symbiodinium* A and C. Time does influence the amount of DHA for *M. hispida* larvae (two-way ANOVA, $F = 7.0$, $df = 4$, $p < 0.001$), but there is no influence of *Symbiodinium* clade. However, the interaction between time and clade does influence ($F = 3.0$, $df = 24$, $p < 0.001$). Furthermore, there are significant differences

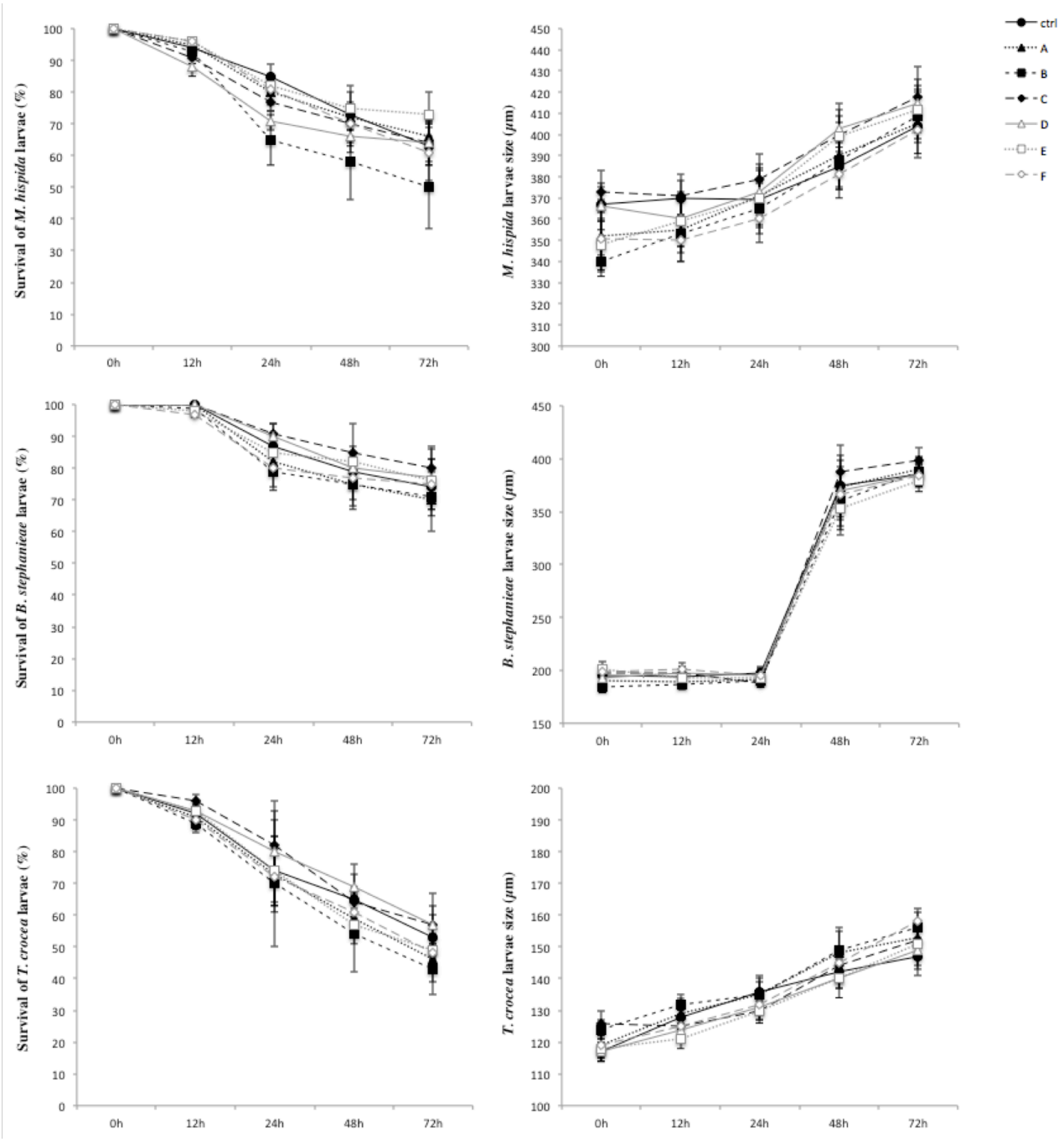

Fig. 1 Survival and growth (at 27ºC) of *Mussismilia hispida* (scleractinian coral), *Berghia stephanieae* (nudibranch) and *Tridacna crocea* (giant clam) larvae during a 72-h window after acquiring *Symbiodinium* clades A-F.

in the amount of DHA in the 72 h samples for *M. hispida* larvae associated with *Symbiodinium* clades A and C when compared to the control and other clades (one-way ANOVA, $F = 10.4$, $df = 6$, $p < 0.001$). The amount of DHA for *M. hispida* larvae associated with *Symbiodinium* clades A and C at 72 h PSO was 20% and 22%,

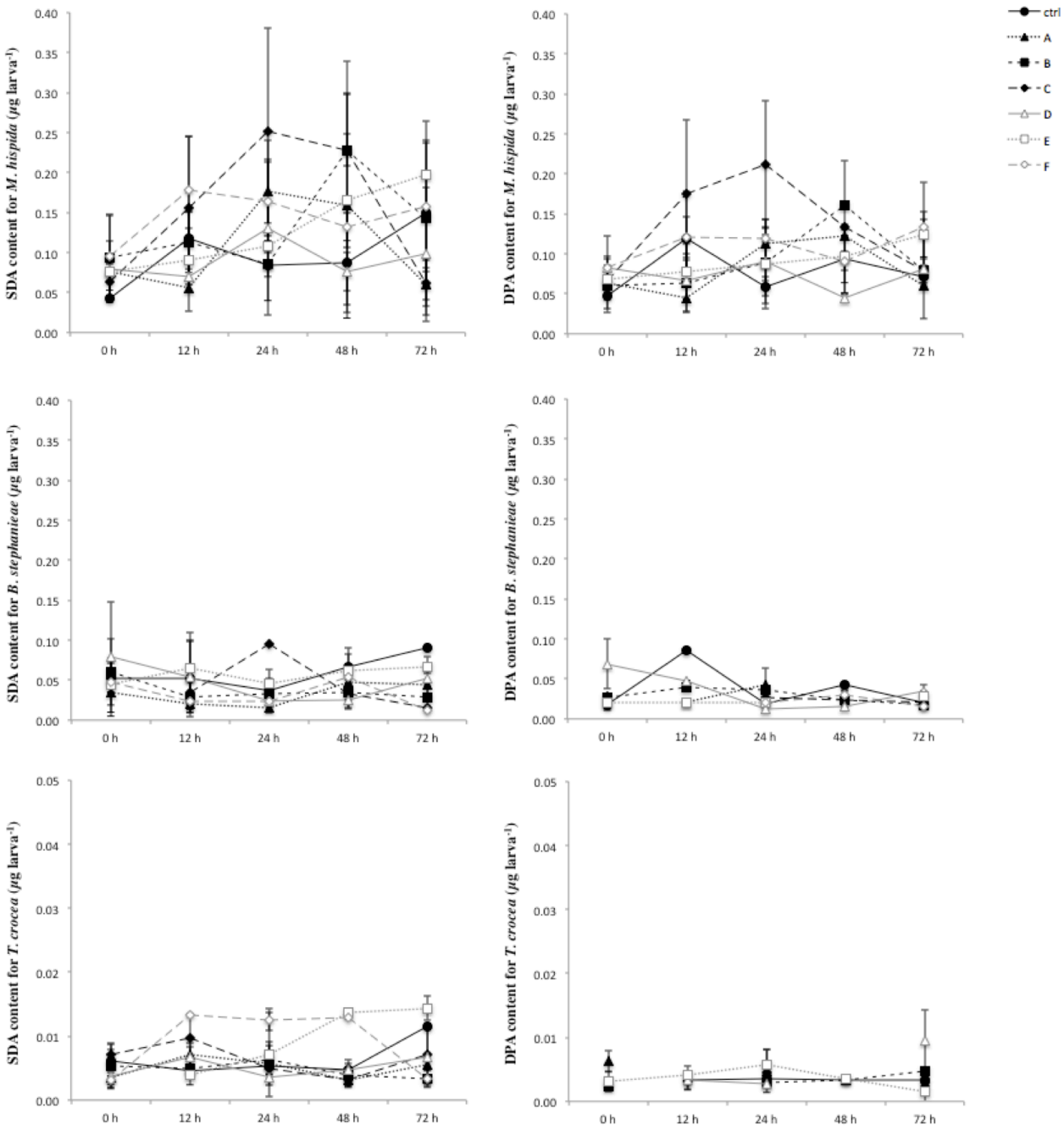

Fig. 2 Content of two fatty acids, SDA (18:4ω3) and DPA (22:5ω3) in *Mussismilia hispida* (scleractinian coral), *Berghia stephanieae* (nudibranch) and *Tridacna crocea* (giant clam) larvae during a 72-h window after acquisition of *Symbiodinium* clades A-F. Data missing for DPA in *T. crocea* larvae is due to undetectably low concentrations.

respectively, higher than at 12 h PSO. In the case of *B. stephanieae*, DHA amount was approximately constant throughout the experiment and neither time, *Symbiodinium* clade nor their interaction presented any significant differences. For *T. crocea* larvae, time did influence the amount of DHA (two-way ANOVA, $F = 6.3$, $df = 4$, $p < 0.001$), but no differences were found for *Symbiodinium* clade or its interaction with time.

However, *T. crocea* larvae associated with *Symbiodinium* clades A and C showed higher amounts of DHA than larvae associated with other clades and the control group despite not showing statistically significant differences (Fig. 3). The amount of DHA for *T. crocea* larvae associated with *Symbiodinium* clades A and C at 72 h PSO was 28% and 27%, respectively, higher than at 12 h PSO.

Discussion

Fatty acids are important tools for investigations in trophic ecology. When a given organism consumes certain fatty acids, these are often routed to energy reserves or tissues with little or no modification, becoming a record of dietary habit (Ruess *et al*., 2005; Budge *et al*., 2006). Highly unsaturated fatty acids are particularly useful as they are produced by phytoplankton species and cannot be synthesized *de novo* by animals (Volkman *et al*., 1989; Dalsgaard *et al*., 2003). While, on rare occasions, some animals are able to elongate and desaturate fatty acids and convert them into highly unsaturated compounds, this is performed at low and insignificant amounts (Nevejan *et al*., 2003). This is mostly related to the fact that, contrary to photoautotrophs, animals lack enzymes required to produce double bonds at certain positions in the carbon chain (Bachok *et al*., 2006). Highly unsaturated fatty acids must therefore be acquired from dietary items and thus are important trophic markers in marine ecology, as extensively reviewed in Dalsgaard *et al*. (2003), Bergé and Barnathan (2005) and Budge *et al*. (2006).

In light of such context, we tested if the ω3 fatty acids SDA, DPA and DHA are produced in different rates by *Symbiodinium* clades A-F associated with coral, nudibranch and giant clam larvae. These fatty acids are symbiosis-related as they are translocated from *Symbiodinium* to its host (Papina *et al*., 2003). Considering that these fatty acids are found in both *Symbiodinium* (Mansour *et al*., 1999; Zhukova and Titlyanov, 2003; Mortillaro *et al*., 2009) and coral reef larvae (Arai *et al*., 1993; Figueiredo *et al*., 2012; Leal *et al*., 2012), although the latter are incapable of producing them, our intention was to find increments in the amounts of these fatty acids in host larvae. As they are only produced by the symbiont and no other organisms other than the larvae were present in the filtered seawater used for the larval cultures, any increments would have to result from the production by *Symbiodinium*.

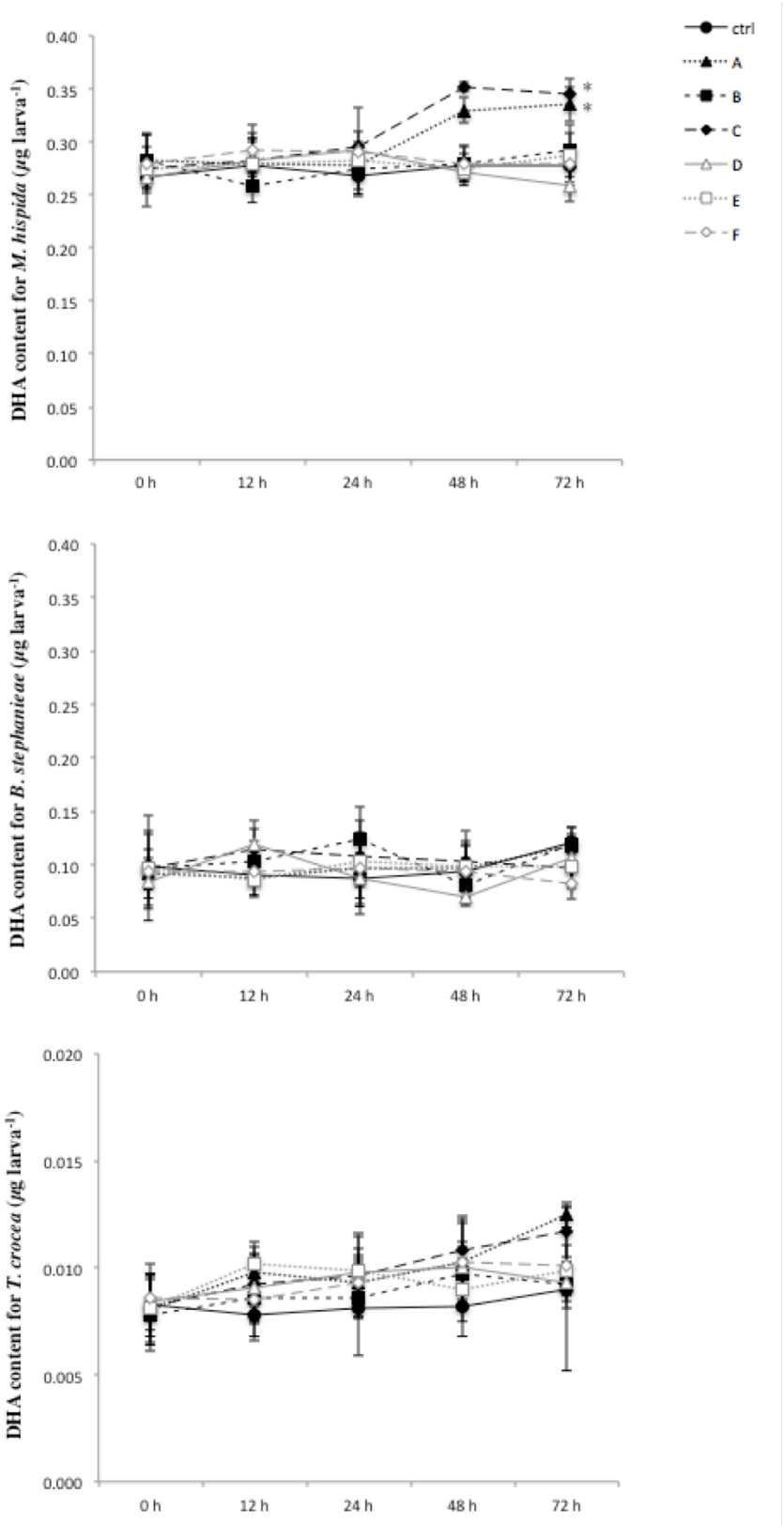

Fig. 3 DHA (22:6ω3) content in *Mussismilia hispida* (scleractinian coral), *Berghia stephanieae* (nudibranch) and *Tridacna crocea* (giant clam) larvae during a 72-h window after acquisition of *Symbiodinium* clades A-F. Asterisks denote significantly different (Tukey's HSD, $p < 0.05$) DHA content at the end of the experiment.

SDA and DPA are considered dinoflagellate-specific markers, despite occurring in other marine phytoplankton species at lower concentrations (Graeve *et al*., 1994; Zhukova and Aizdaicher, 1995). The amount of both fatty acids found in the larvae of *Mussismilia hispida*, *Berghia stephanieae* and *Tridacna crocea* associated with *Symbiodinium* clades A-F was very low (Fig. 1). In the case of DPA for *T. crocea* it was below the detection limit of the gas chromatograph for some samples. Higher amounts were found in the *M. hispida* samples, possibly because the larvae are larger and also acquired a higher number of symbionts (Table 1). Nonetheless, the concentrations for both SDA and DPA were highly variable for all host larvae and all *Symbiodinium* clades and no practical conclusions can be reached for this experiment. However, this does not exclude the possibility that these compounds may have a role in metazoan larvae-*Symbiodinium* associations.

In the case of DHA, however, the data produced show interesting trends. For all host larvae, there was an increase in DHA content in the 12 h sample (Fig. 3). This is most likely related to the acquisition of symbionts that took place in between the 0 and 12 h sampling periods. However, a higher increase was found at 48 and 72 h PSO for *M. hispida* and *T. crocea* larvae associated with *Symbiodinium* clades A and C, statistically significant in the case of *M. hispida*. While our experimental design cannot account for translocation of photosynthates from these *Symbiodinium* strains to host larvae, it is highly possible as *Symbiodinium* are known to translocate more than 90% of its carbon production to the host (Muscatine and Porter, 1977; Muscatine, 1990). Furthermore, the increase in DHA amount for *M. hispida* and *T. crocea* larvae associated with clades A and C is higher than 20%, which is more than the 10-15% reproductive stage cells recorded during their larval development (Table 1). This suggests that more DHA was being produced than the necessary for reproduction. Our findings are in accordance with reports that *M. hispida* and *T. crocea* larvae associated with *Symbiodinium* clade A1 engage in symbiosis-specific gene expression and have lower bleaching rates than larvae associated with other clades (Mies *et al*., this thesis chapters 2 and 4). While DHA seems to be a relevant trophic marker and may be translocated from symbiont to host larvae, we found no significant differences in survival and growth for larvae associated with *Symbiodinium* clades A and C. This is in agreement with reports that *Symbiodinium* does not contribute significantly to the nutrition of larvae of the scleractinian coral *Pocillopora damicornis* (Kopp *et al*., 2016).

Therefore, it is possible that this contribution becomes significant only at later stages in the development. In the case of *B. stephanieae,* however, there is little indication of symbiotic exchange during the larval development as both veliger larvae and juvenile individuals sampled in this experiment were digesting the *Symbiodinium* cells.

The reason why *Symbiodinium* clades A and C presented conspicuous data when associated with *M. hispida* and *T. crocea* larvae in this experiment may be related to symbiont homology. Apart from *Symbiodinium* A and C being the most abundant and well-adapted clades (Pochon *et al*., 2006; Stat *et al*., 2006), both host species naturally harbor these two strains (DeBoer *et al*., 2012; Picciani *et al*., 2016). It has been widely reported that hosts have recognition mechanisms for selecting homologous symbiont clades (Weis *et al*., 2001; Fransolet *et al*., 2012), and these would establish a more robust symbiosis, with positive impacts on host fitness (Trench *et al*., 1981; Weis *et al*., 2001(Belda-Baillie *et al*., 2002; Rodriguez-Lanetty *et al*., 2003).

The findings in this study suggest that early acquisition of homologous symbionts may contribute to a higher content of ω3 fatty acids that are important for coral reef larvae nutrition. Apart from being relevant to the current understanding on animal-dinoflagellate symbioses, our results are particularly important for coral reef conservation and restocking programs that rely on aquaculture. Furthermore, our findings may be useful for recruitment prediction models, which have become increasingly important in the current scenario of climate change.

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Author contributions

M.M. designed the experiment, M.M., A.B.C.F. and A.A.T. performed the experiment, A.B.C.F., S.M., C.B.C., D.O.P. and E.N.C. contributed with infrastructure/material/technical support, M.M., A.B.C.F., S.M. and A.Z.G. analyzed the data and M.M. and P.Y.G.S. wrote the manuscript.

CHAPTER FOUR

Bleaching in coral reef larvae associated with *Symbiodinium* clades A-F

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Abstract

Coral reefs are diverse ecosystems because of the presence of dinoflagellates (genus *Symbiodinium*) that are found in symbiotic association with multiple phyla and perform the majority of primary production. However, coral reefs are currently threatened by climate change and, especially, the bleaching phenomenon, which is caused by increase in seawater temperature. While bleaching has been well documented for host organisms, it is still poorly understood in larval stages. We offered *Symbiodinium* clades A-F to the larvae of *Mussimilia hispida* (scleractinian coral), *Berghia stephanieae* (nudibranch) and *Tridacna crocea* (giant clam) and gradually manipulated temperature to 26, 29 and 32ºC. Samples were taken at 0, 12, 24, 48 and 72 h post-temperature increase, chlorophyll-*a* (chl-a) was extracted and its content measured in a fluorometer. Results show that *Symbiodinium* clade, temperature and time all influence in the chl-a content. *Mussismilia hispida* larvae displayed a bleaching threshold at 29ºC, and larvae containing clades A-F all bleached at 32ºC, but with larvae associated with clade A showing a significantly lower bleaching. *Berghia stephanieae* digested the symbionts

and while chl-a content decreased over time in equal fashion for all clades, it is not possible to determine if it is related to bleaching. *Tridacna crocea* larvae at 29ºC bleached for all clades, except for A. At 32ºC, all clades bleached but clade A bleached significantly less. These findings show that clade A seems to be more thermo-tolerant in coral reef larvae, but this may be related to the fact that it is homologous to both *M. hispida* and *T. crocea*. Therefore, symbiont type may have an important role in coral reef larval development and present relevant implications for recruitment.

Keywords: zooxanthellae, *Tridacna*, Scleractinia, larval ecology, chlorophyll, temperature

Introduction

Coral reefs are the most diverse marine ecosystems, and despite occupying only 0.2% of the ocean area, they harbor millions of eukaryotic and prokaryotic species (Connell, 1978; Sheppard *et al*., 2009; Knowlton *et al*., 2010 and references therein). Besides presenting such high biodiversity, coral reefs are economically valuable and worth hundreds of billions (USD) yearly (Moberg and Folke, 1999). Apart from supplying animal protein to local communities, coral reefs are an important resource for commercial fisheries, tourism and recreation, pharmaceuticals and the aquarium trade (Brander *et al*., 2007; Newton *et al*., 2007; Olivotto *et al*., 2011; Lima *et al*., 2013). The existence of coral reefs is much dependent on the symbiosis between reef-building corals and photosynthetic dinoflagellates of the genus *Symbiodinium* (Cesar, 2000; Hughes *et al*., 2003; Pandolfi *et al*., 2003). *Symbiodinium* dinoflagellates are found in an endosymbiotic association with several metazoan and protist phyla, including Cnidaria, Mollusca, Porifera, Acoelomorpha, Foraminifera and Ciliophora (Stat *et al*., 2006; Barneah *et al*., 2007; Venn *et al*., 2008). This symbiosis is important because, apart from both parties exchanging metabolites (Muscatine, 1990; Grant *et al*., 1997; Allemand *et al*., 1998; Leggat *et al*., 2003), *Symbiodinium* facilitates calcium deposition in their reef-building hosts (Muscatine, 1990; Furla *et al*., 2005; Colombo-Pallotta *et al*., 2010).

However, this symbiotic relationship and coral reefs in their entirety are currently threatened by climate change and anthropogenic influence. Among the main impacts are ocean acidification (Hoegh-Guldberg *et al*., 2007), pollution (Hughes *et al*., 2003) and, possibly the most damaging to date, the bleaching phenomenon (Hughes *et al*., 2003; 2007; Baker *et al*., 2008). The bleaching event is defined by the loss of

Symbiodinium from host tissue which, associated with the loss of non-photosynthetic pigments and tissue breakdown turns the coral pale white, as a consequence of the exposure of its calcareous skeleton (Brown, 1997; Hoegh-Guldberg, 1999; Fitt *et al*., 2001). The main cause of bleaching is the increase in seawater temperature, which damages the symbiont photosystem II (Iglesias-Prieto *et al*., 1992; Fitt *et al*., 2001). This results in the production of reactive oxygen species, which causes cellular damage in both host and symbiont and culminates in the expulsion of the symbiont and disruption of the symbiotic relationship (Lesser, 2006; Weis, 2008; Yakovleva *et al*., 2009). Considering that, in most cases, the symbiotic relationship is obligatory for the host, the bleaching phenomenon often leads to host death (Glynn, 1996). This highlights the crucial importance of *Symbiodinium* dinoflagellates in the survival of coral reef ecosystems.

The response of *Symbiodinium* to fluctuations in abiotic parameters is variable among strains. *Symbiodinium* are divided in nine clades, A-I (Rowan and Powers, 1991; Pochon and Gates, 2010), with clade D being notoriously more thermo-tolerant than others (Chen *et al*., 2003; Mieog *et al*., 2009; Ladner *et al*., 2012), while clade C is known to be adapted to low irradiance (Cooper *et al*., 2011). *Symbiodinium* clades also occupy different host niches, as cnidarians and mollusks usually associate with clades A-D, sponges with clades B, D and G and foraminiferans with clades F-I (Stat *et al*., 2006; Pochon and Gates, 2010). In addition, more than a single clade can be found within the tissues of an individual host (Rowan and Powers, 1991; Carlos *et al*., 2000; Baker, 2003).

Most of the research performed on bleaching impacts has been carried out on adult hosts, with several reports produced on the bleaching intensity and rates (Ralph *et al*., 2005; Strychar *et al*., 2005; Venn *et al*., 2006). However, only a few bleaching experiments were performed for hosts undergoing larval development (in the brooding corals *Porites astreoides* and *Pocillopora damicornis*) and the symbiont clade in the larval tissue was not identified in any of those cases (Edmunds *et al*., 2005; Haryanti *et al*., 2015). Therefore, our study aims to answer two different questions: i) if bleaching occurs in different types of coral reef larvae and ii) if there is any *Symbiodinium* clade that is more resistant to bleaching when associated with larvae. For those purposes, we investigated the bleaching rates in *Symbiodinium* clades A-F associated with the larvae of *Mussismilia hispida* (scleractinian coral), *Berghia stephanieae* (nudibranch) and

Tridacna crocea (giant clam). Considering that bleaching events should intensify in the coming times (Hoegh-Guldberg, 1999; Baker *et al*., 2008), by investigating such symbiotic relationships we aim to contribute to the understanding of animal-dinoflagellate symbioses during larval stages, which may be important for recruitment prediction models and contribute to conservation programs and assessments of climate change scenarios.

Materials and methods

The experiment was designed with the purpose of verifying bleaching in the larvae of three different host organisms associated with *Symbiodinium* A-F. Therefore we acquired broodstock for each host, spawned them and cultured the larvae, offered the *Symbiodinium* clades, manipulated three different temperature treatments, took samples and performed fluorimetric analysis.

Symbiodinium *culture*

Symbiodinium cultures (ITS2 types A1, B1, C1, D1, E1 and F1) were maintained in f/2 medium (Guillard and Ryther, 1962) with the addition of streptomycin (0.5 g L^{-1}) and penicillin (1.0 g L^{-1}). Cultures were kept at 23ºC, with a photon flux of 100 μE m^{-2} s^{-1} in a photoperiod of 12L:12D.

Broodstock maintenance and spawning

The three hosts selected for this experiment were i) *Mussismilia hispida*, a colonial scleractinian coral endemic from Brazil, ii) *Berghia stephanieae*, a stenophagous nudibranch that feeds exclusively on *Aiptasia* anemones, from where they acquire *Symbiodinium* (Leal *et al*., 2012) and iii) *Tridacna crocea*, the smallest species of giant clam. All hosts are simultaneous hermaphrodites and acquire symbionts horizontally (Carroll and Kempf, 1990; Neves and Pires, 2002; Mies *et al*., 2012). Broodstock for all hosts were kept in water conditions that simulated tropical reefs, with temperature at 26ºC, specific gravity at 1,024 kg m^{-3} and nutrients at undetectable levels. Five *M. hispida* colonies (18.5 ± 4.4 cm in approximate diameter) were collected at the Recife

de Fora (16º25'S, 38º59'W) in northeastern Brazil, near the Abrolhos Reefs, and kept in semi-closed tanks. Natural spawns were collected from tanks in September 2015 and gamete bundles were collected immediately after release. They were subsequently fertilized in 60-L round tubs and cultured until planulae reached four days post-fertilization (PF). Daily water changes of 90% were performed. Eighty-five *B. stephanieae* individuals (1.6 ± 0.1 cm in length) were kept in two 60-L round tubs in a recirculating aquaria system of 250 L. Broodstock was heavily fed with 100 *Aiptasia* sp. individuals and egg masses produced were collected with a plastic pipette in the next morning. Egg masses were kept under strong aeration for 10 days to stimulate the release of veliger larvae (Carroll and Kempf, 1990). *Tridacna crocea* broodstock (n=10, 7.6 ± 0.9 cm in shell length) was imported from Vietnam and kept in a 350-L recirculating system. Induced spawning was performed by the intragonadal injection of 1.0 mL of a serotonin solution (5-hydroxytryptamine, 1.0 g L^{-1}). Spawned gametes were fertilized in a volumetric ratio of 1:200 (sperm:oocyte) to avoid polyspermy (Heslinga *et al*., 1990) and placed in 60-L round tubs with daily water changes of 50%. Cultures were kept until all larvae had attained the veliger stage, at 72 h PF.

Larval cultures, Symbiodinium *offering, temperature manipulation and sampling*

Host larvae were stocked in 1.2-L plankton kreisels containing filtered seawater. Planulae of *M. hispida* (4 days PF) were stocked at 1.2 larva mL^{-1}, *B. stephanieae* veligers (10 days PF) at 1.2 mL^{-1} and *T. crocea* veligers (3 days PF) at 2.0 mL^{-1}. Three groups of seven kreisels were used for the larvae of each host. To each group, *Symbiodinium* cultures (A-F) were offered separately at a final concentration of 10^3 cells mL^{-1}, with symbionts being withheld in the remainder kreisel (control group). The three groups were maintained in a water bath at 26ºC at the time of symbiont offering. At 12 h after the offering of symbionts, a water change of 100% was performed in all kreisels in order to remove *Symbiodinium* cells that had not been acquired and symbiont acquisition was recorded. The temperature of the water baths for the three groups was then increased to 26, 29 and 32ºC, over a 6-h period (Fig. 1). Samples of 150 larvae were then taken at 0, 12, 24, 48 and 72 h post-temperature increase (PTI) for size measurements and fluorimetric analysis.

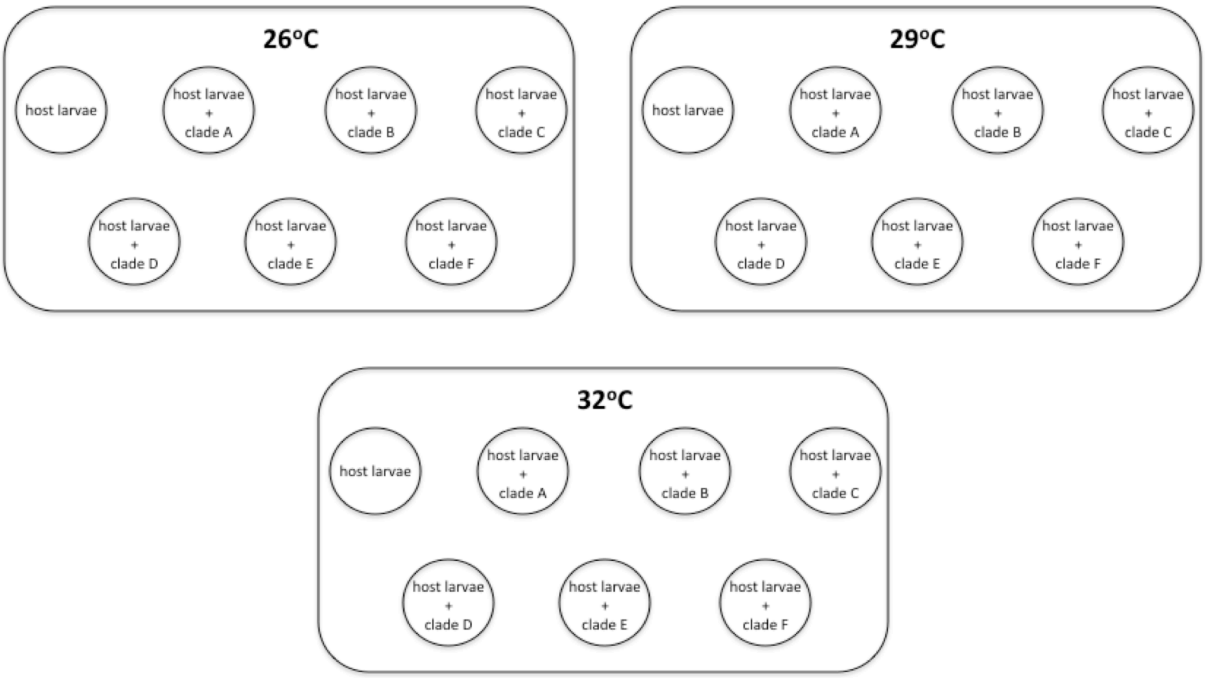

Fig. 1 Experimental design for bleaching in *Mussimilia hispida* (scleractinian coral), *Berghia stephanieae* (nudibranch) and *Tridacna crocea* (giant clam) larvae associated with *Symbiodinium* clades A-F: for each host, larvae were stocked in seven plankton kreisels, one for host larvae associated each *Symbiodinium* clade and a control group (aposymbiotic larvae), in water baths at 26, 29 and 32ºC.

Fluorimetric analysis

Collected samples were pelleted in a centrifuge at 6,000 g for 2 minutes. The supernatant was discarded and sample mas macerated by the use of glass beads, a mortar and a pestle. Chlorophyll-*a* (chl-a) extraction was performed in the dark, with acetone (90%) and refrigeration at 4ºC for 24 hours, as according to Wasmund *et al*. (2006). Samples were read in a 10-AU Turner Fluorometer and chlorophyll-*a* concentration was calculated according to the procedures in Welschmeyer (1994).

Statistical analysis

Statistical analyses on the larval experiments were performed independently for each host larvae. A three-way ANOVA was performed to check if *Symbiodinium* clade, time and temperature influence on the relative change in chl-a content (taking initial and final concentrations into consideration and excluding control larvae data). To verify which clades presented significantly different relative changes in chl-a content, a one-way

ANOVA was performed on the 72 h samples for the three temperatures tested (except for *T. crocea*, for which the 48 h sample was used for the 32ºC temperature as the 72 h sample larvae had died), independently. Significant differences between pairs were tested with Tukey's HSD test.

Results

Symbiodinium acquisition was higher than 99% for all host larvae used in this study. While *B. stephanieae* and *T. crocea* larvae acquired a similar amount of symbionts for all clades (Table 1), *M. hispida* larvae acquired *Symbiodinium* A more than twice than most other clades. While *Symbiodinium* clade did not influence larval survival for all three hosts, the temperature treatments did, as mortality increased with temperature for all host larvae (Fig. 2). In fact, all *T. crocea* veligers at 32ºC died before the end of the 72-h window. Similarly to survival, larval growth was not influenced by *Symbiodinium* clade, but by temperature as all larvae at 32ºC presented a smaller size than larvae at 26ºC and 29ºC (Fig. 3). *Berghia stephanieae* veliger larvae did not grow during their development, until approximately 18 h PTI when many individuals started going through metamorphosis, discard their shells and extend their elongated body, resulting in a swift increase in size.

The change in chl-a content in *Mussisimilia hispida* planulae was influenced by all three factors investigated, *Symbiodinium* clade (three-way ANOVA, $F = 47.5$, $df = 5$, $p < 0.001$), temperature (three-way ANOVA, $F = 242.3$, $df = 2$, $p < 0.001$) and time (three-way ANOVA, $F = 251.5$, $df = 3$, $p < 0.001$). All the *Symbiodinium* strains acquired by planulae presented similar trends, with a slight increase in chl-a at 26ºC, followed by stabilization at 29ºC and a decrease at 32ºC (Fig. 4). One-way ANOVA at 26ºC showed no significant differences in the relative change of chl-a content between clades, but the results for the 29ºC treatment show that larvae harboring clade E had a significantly higher chl-a loss when compared to larvae associated with the other clades (one-way ANOVA, $F = 251.5$, $df = 3$, $p < 0.001$). At 32ºC, larvae associated with clade A had a significantly lower bleaching rate than larvae associated with all the other clades (one-way ANOVA, $F = 20.3$, $df = 5$, $p < 0.01$).

Table 1 Acquisition of *Symbiodinium* clades A-F by *Mussismilia hispida* (scleractinian coral), *Berghia stephanieae* (nudibranch) and *Tridacna crocea* (giant clam) larvae after 12 hours of exposure. Symbionts were not offered to control larvae.

Symbiodinium clade	*Symbiodinium* cells acquired		
	M. hispida	*B. stephanieae*	*T. crocea*
Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
A	77.1 ± 20.1	18.1 ± 2.0	19.1 ± 5.5
B	40.0 ± 9.9	14.5 ± 2.3	17.7 ± 6.7
C	33.4 ± 7.8	15.4 ± 1.1	20.0 ± 4.5
D	29.1 ± 11.1	14.4 ± 2.9	22.5 ± 8.7
E	21.1 ± 5.5	13.9 ± 2.4	15.6 ± 8.8
F	30.1 ± 9.0	15.0 ± 3.0	13.3 ± 5.7

In the case of *B. stephanieae*, time (three-way ANOVA, $F = 220.2$, $df = 3$, $p < 0.01$) and temperature (three-way ANOVA, $F = 164.7$, $df = 2$, $p < 0.01$) influenced the change in chl-a content in larvae and juveniles, but no significant effect by *Symbiodinium* clade was detected. Furthermore, it was detected during the experiments that the majority of *Symbiodinium* cells inside larvae and juvenile tissues, independently of clade, were partially digested. At 72 h PTI for the 32ºC treatment, nearly zero *Symbiodinium* cells were found in juvenile *B. stephanieae*.

Changes in the chl-a content in *T. crocea* veliger larvae was, likewise for *M. hispida*, affected by all three factors: *Symbiodinium* clade (three-way ANOVA, $F = 76.6$, $df = 5$, $p < 0.01$), temperature (three-way ANOVA, $F = 112.7$, $df = 2$, $p < 0.01$) and time (three-way ANOVA, $F = 187.6$, $df = 3$, $p < 0.01$). Again similar to *M. hispida*, all the *Symbiodinium* clades presented similar trends, with no change in chl-a content at 26ºC, a slight bleaching at 29ºC and a stronger bleaching followed by mortality at 32ºC (Figs. 3 and 4). At 29ºC, larvae associated with *Symbiodinium* clade A were the only ones not to present a decrease in chl-a, statistically different from larvae harboring other clades (one-way ANOVA, $F = 11.2$, $df = 5$, $p < 0.01$). At 32ºC, larvae containing *Symbiodinium* clade A also had a significantly lower loss of chl-a when compared to larvae associated with all other clades, while clades C and D presented lower chl-a loss when compared to clades B, E and F (one-way ANOVA, $F = 90.7$, $df = 5$, $p < 0.01$).

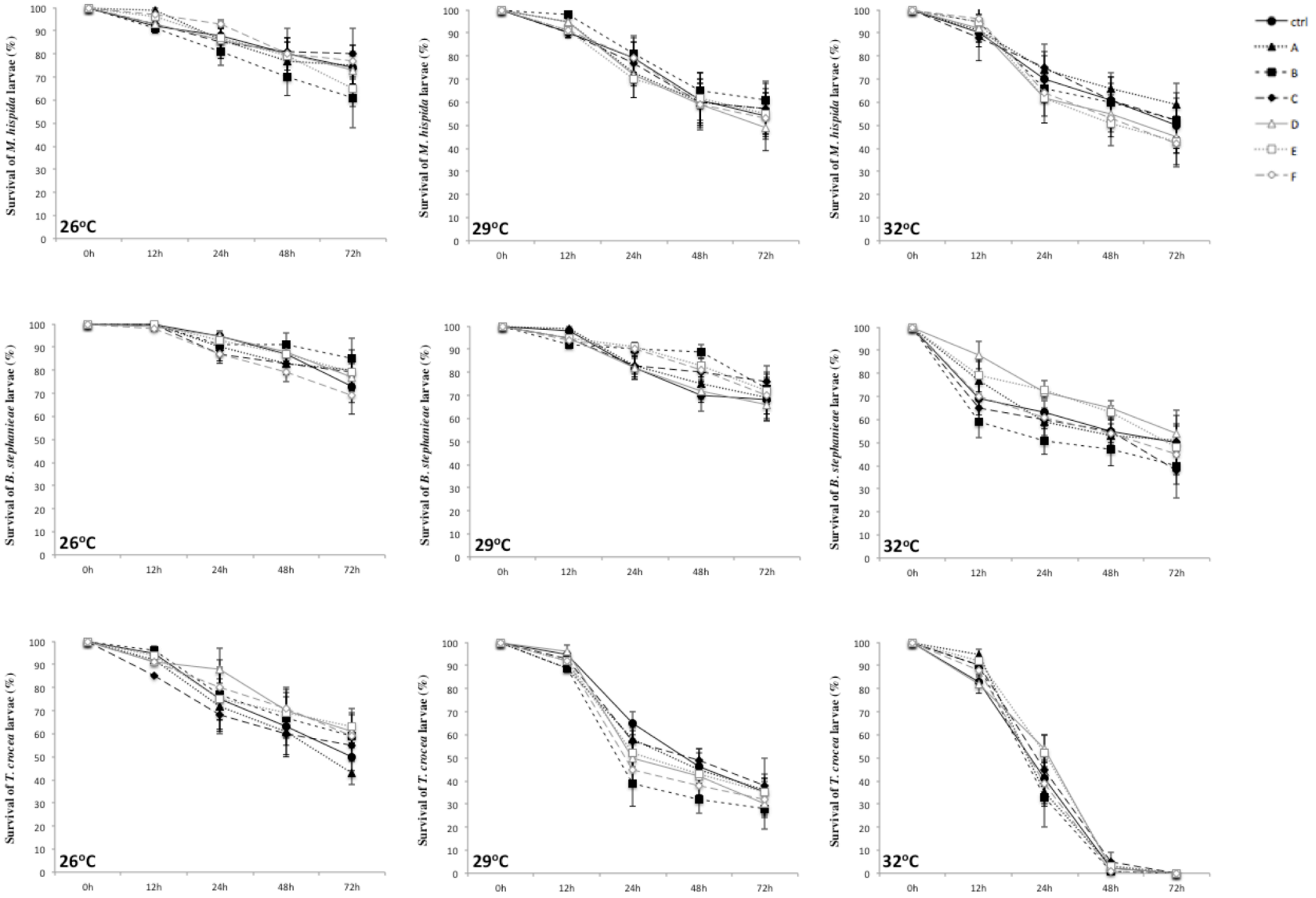

Fig. 2 Survival curves for *Mussimilia hispida* (scleractinian coral), *Berghia stephanieae* (nudibranch) and *Tridacna crocea* (giant clam) larvae associated with *Symbiodinium* clades A-F during a 72-h exposure to 26, 29 and 32ºC. Symbionts were not offered to the control larvae.

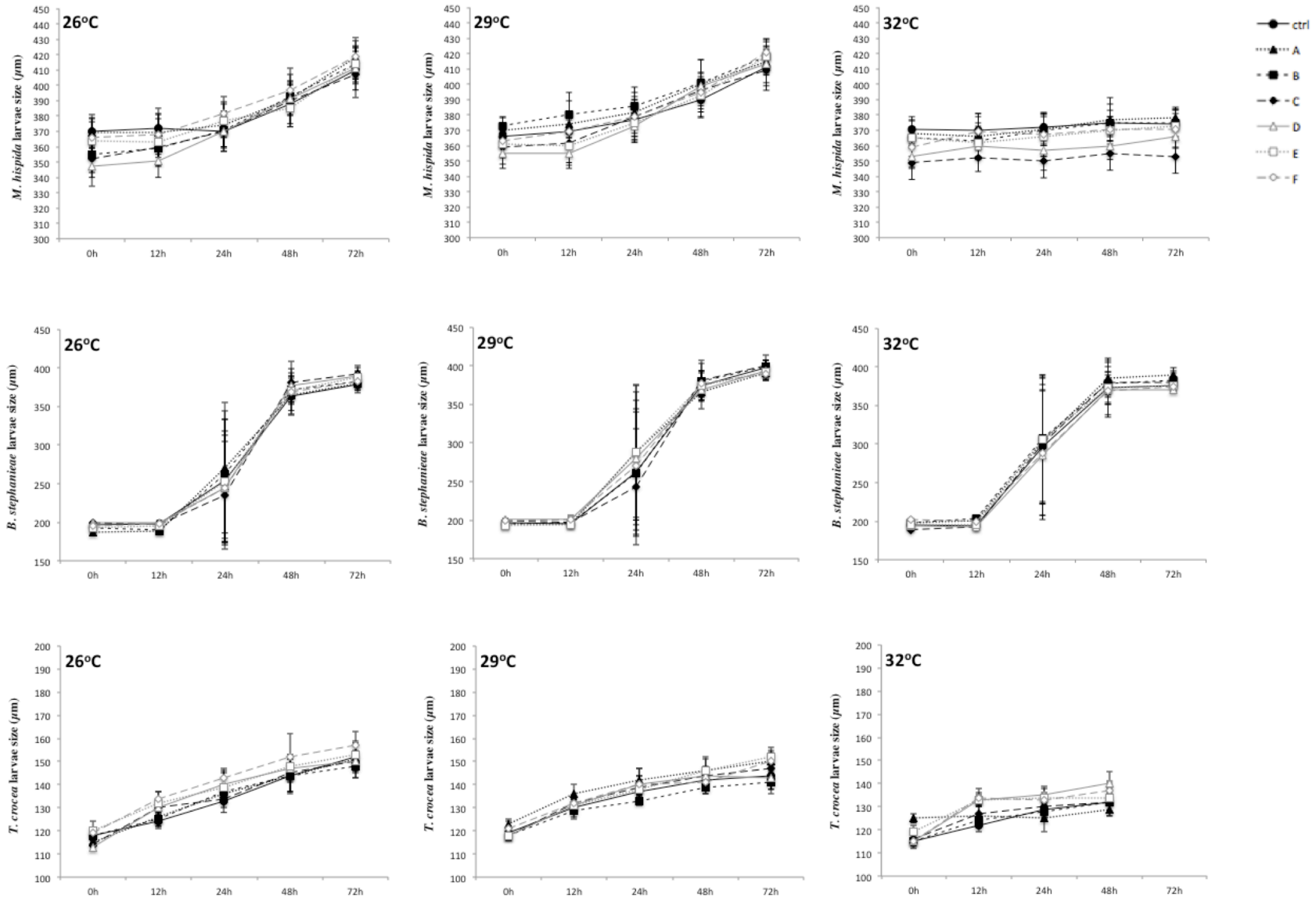

Fig. 3 Growth curves for *Mussimilia hispida* (scleractinian coral), *Berghia stephanieae* (nudibranch) and *Tridacna crocea* (giant clam) larvae associated with *Symbiodinium* clades A-F during a 72-h exposure to 26, 29 and 32ºC. Data were not collected for *T*. crocea at 72 h at 32ºC because larvae died. Symbionts were not offered to the control larvae.

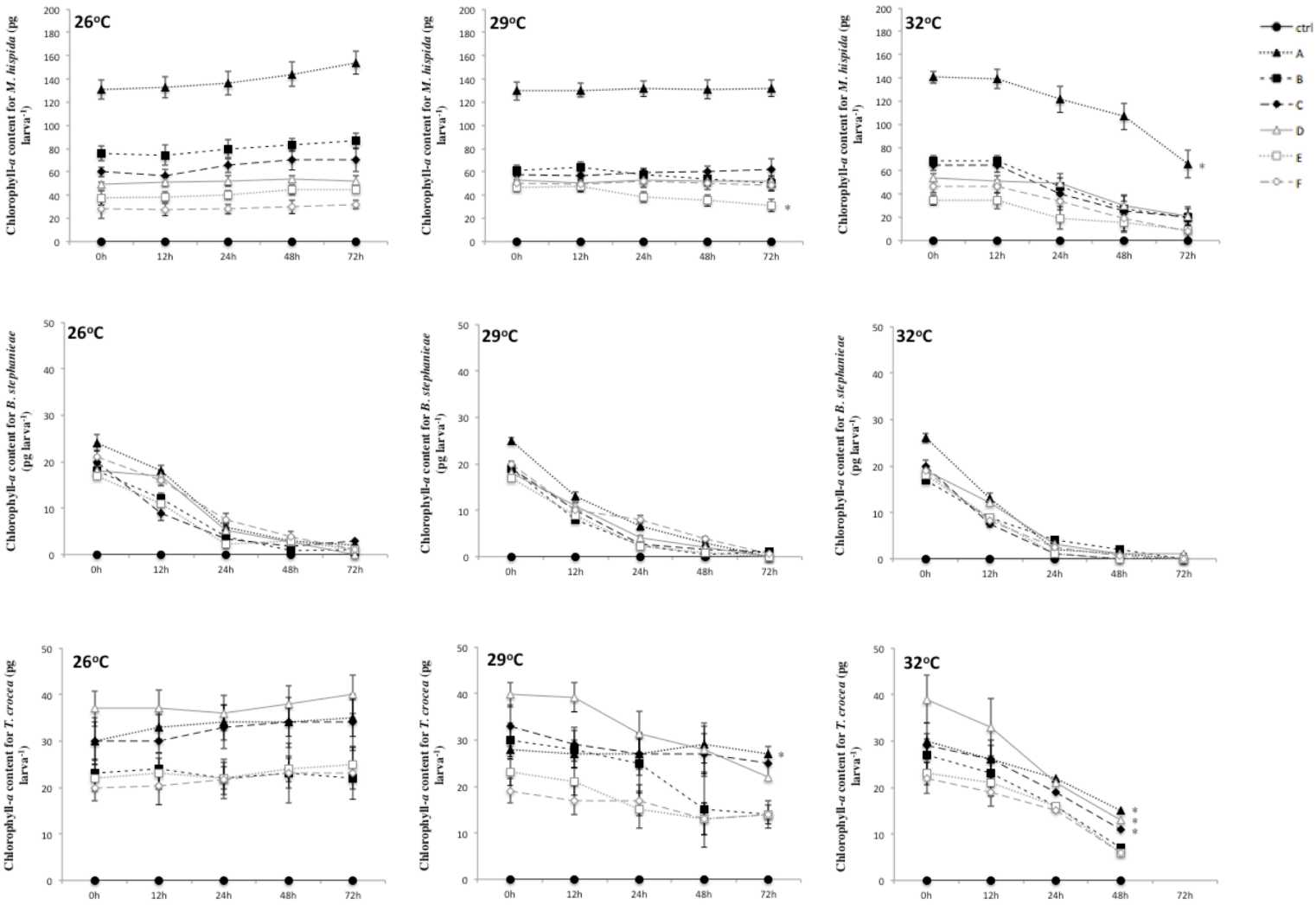

Fig. 4 Chlorophyll-*a* content in *Mussimilia hispida* (scleractinian coral), *Berghia stephanieae* (nudibranch) and *Tridacna crocea* (giant clam) larvae associated with *Symbiodinium* clades A-F during a 72-h exposure to 26, 29 and 32ºC. Data were not collected for *T*. crocea at 72 h at 32ºC because larvae died. Symbionts were not offered to the control larvae. Asterisks denote groups statistically different for relative change in chlorophyll-*a* content.

Discussion

Coral reefs are critically threatened by the increase in surface seawater temperature. It has been predicted that coral-dominated ecosystems are likely to disappear if the current trends in impact, mainly bleaching, are not mitigated immediately (Hoegh-Guldberg, 1999; Hoegh-Guldberg *et al*., 2007). While the bleaching phenomenon has been widely investigated in adult coral reef organisms, it is still very poorly understood for larval stages of development. Therefore, our experiment investigated if bleaching occurs in larval stages and if associated *Symbiodinium* clades respond differently to thermal stress. We used loss of chlorophyll-*a* as a proxy for *Symbiodinum* loss, in similar fashion to Tanaka *et al*. (2014).

Our results confirmed that bleaching does occur not only in scleractinian coral larvae, but also in giant clam larvae. The bleaching threshold for *Mussismilia hispida* larvae seems to be at 29ºC, while for *Tridacna crocea* it may be even lower (Fig. 4). This is in between the thresholds of 28 and 30ºC reported for the larvae of *Porites astreoides* (Edmunds *et al*., 2005) and *Acropora millepora* (Mieog *et al*., 2009), respectively. In the case of the nudibranch *Berghia stephanieae*, while our results show temperature-dependent decrease in chlorophyll-*a* content, it is not possible to determine if this is related to bleaching, symbiont digestion or the combination of both. In fact it is questionable if a mutualistic symbiosis is at work between this species and *Symbiodinium* (Mies *et al*., this thesis chapter 2).

The major finding in this study is that *Symbiodinium* clades A-F present different bleaching rates when associated with coral reef larvae (Fig. 4). Clade A presented the lowest bleaching rates in both coral and giant clam larvae, with clades C and D following as the most resistant types. This differs from the several reports for adult hosts that clade D is the most thermo-tolerant strain, that not only endures higher temperatures for longer periods, but also behave as opportunistic in the reinfection of bleached organisms (Chen *et al*., 2003; van Oppen *et al*., 2005; Abrego *et al*., 2009; Mieog *et al*., 2009; Ladner *et al*., 2012). Differences were presented by *Symbiodinium* clades already at initial larval infection. Our results show that *M. hispida* acquired *Symbiodinium* A in much higher quantities (Table 1). While there is debate on whether is there symbiont-specificity at initial infection (Belda-Baillie *et al*., 1999; Little *et al*.,

2004; Cumbo *et al*., 2012), our results seem to point to a preference by *M. hispida*. The better performance by *Symbiodinium* clade A and C in this study may be related to homology, as both clades are naturally found in *M. hispida* (Picciani *et al*., 2016) and *T. crocea* (DeBoer *et al*., 2012). This is in contrast with the results we obtained for the less-abundant clades E and F, which usually presented higher bleaching rates and lower concentrations in host larvae (Fig. 4). Furthermore, *Symbiodinium* clade A associated with *M. hispida* and *T. crocea* has been shown to differentially express a symbiosis-specific gene and produce higher amount of fatty acids that are typically translocated to the host (Mies *et al*., this thesis chapter 3).

The different response by some clades and the fact that no differences were found in survival for larvae associated with clades A-F may also contribute to the debate on the effect of reactive oxygen species (ROS) in coral reef larvae. It has been reported that, under increased temperature, the presence of symbionts may reduce larval survival due to the relatively higher production of ROS (Yakovleva *et al*., 2009; Schnitzler *et al*., 2012). This event reduces larval survival as its consequences include DNA damage and high rates of enzymatic antioxidant activity (Yakovleva *et al*., 2009; Nesa *et al*., 2012). Therefore, it has been suggested that bleaching may be important for larval survival at higher temperatures (Weis, 2008; Yakovleva *et al*., 2009), as symbiont presence, while may increase host fitness (Weis *et al*., 2001), is not necessary for successful settlement and metamorphosis for many coral species (Morse *et al*., 1996). However, it has been shown that symbiont presence is an important stimulating factor for settlement and metamorphosis (Vermeij *et al*., 2013). The scenario that symbiont presence decreases survival would lead to a higher mortality of *M. hispida* larvae associated with *Symbiodinium* clade A1, which in this study presented statistically significant lower bleaching rates. However, such mortality was not found and relates to the few studies that have reported that, associated with homologous clades (including A1), both coral adults and larvae under thermal stress (as high as 32ºC) did not show an increase in ROS production or higher mortality (McGinty *et al*., 2012; Haryanti *et al*., 2015). Therefore, it is relevant to further investigate the relationship between coral reef larvae and *Symbiodinium* type, especially since it may have an important role in recruitment.

This study has shown that temperature, time and *Symbiodinium* strain are critical elements that directly influence on the bleaching phenomenon in coral reef larvae. Our

major contribution is to perhaps raise awareness that bleaching events affect not only the adult hosts, but also the larval forms, which are crucial for the successful recruitment and maintenance of coral reef biodiversity.

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Author contributions

M.M. designed the experiment, M.M. performed the experiment, C.B.C., D.O.P. E.N.C. and M.P. contributed with infrastructure/material/technical support, M.M. and A.Z.G. analyzed the data and M.M. and P.Y.G.S. wrote the manuscript.

CONCLUSIONS

The symbiotic relationship between *Symbiodinium* and their host organisms has been widely investigated (Stat *et al*., 2006; Venn *et al*., 2008; Weis *et al*., 2008; Davy *et al*., 2012). However, very little is known about this association for hosts still in their larval development (Mies *et al*., this thesis chapter 1). This lack of information, especially on metabolite exchange and differential gene expression, questions if there is a mutualistic relationship between *Symbiodinium* and coral reef larvae. In order to improve our understanding of the association between these organisms, this thesis performed molecular, biochemical and climate change-related experiments on the association between six different *Symbiodinium* clades (A-F) and coral (*Mussismilia hispida*), gastropod (*Berghia stephanieae*) and giant clam (*Tridacna crocea*) larvae, while also performing a review on the subject and placing our findings in perspective.

Our major findings show that a symbiosis-specific gene is expressed by *Symbiodinium* clade A1 associated with coral and giant clam larvae, and that this gene, the H^+-ATPase, is a relevant and perhaps the only currently available marker for the symbiotic state of *Symbiodinium* (Mies *et al*., this thesis chapter 2). We have also shown that homologous *Symbiodinium* clades associated with coral and giant clam larvae produce a higher amount of a fatty acid that is usually translocated from symbiont to host (Mies *et al*., this thesis chapter 3), while also being more resistant to bleaching (Mies *et al*., this thesis chapter 4). Finally, we show compelling evidence that there is no mutualism between *Symbiodinium* and the aeolid nudibranch *B. stephanieae* (Mies *et al*., this thesis chapters 2-4).

The implications of our findings are relevant for coral reef ecology, as the current knowledge on the mechanisms involved in symbiosis establishment has been strengthened. Furthermore, we have identified associations between certain larval hosts and certain *Symbiodinium* clades that confer higher larval fitness, which can be crucial for successful settlement and recruitment. Aquaculture and restocking programs are currently being employed for coral reef restoration (Pomeroy *et al*., 2006; Guest *et al*., 2010), and our results can also provide insights on culture techniques, as well as suggest

when and which symbionts may be offered to larvae. The results presented in the thermal stress experiment (chapter 4) also confirm that not only adult reef inhabitants suffer bleaching, but larvae do as well. This has implications for the current climate change scenario, as coral-dominated reefs may disappear in the coming decades due to ocean acidification and global warming (Hoegh-Guldberg, 1999; Hughes *et al*., 2003; Pandolfi *et al*., 2003; Hoegh-Guldberg *et al*., 2007; Baker *et al*., 2008). The more robust associations reported in chapter 4 may perhaps aid in the mitigation of climate change impacts and also on the predictions and assessments of future scenarios.

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