Corals through the light: phylogenetics, functional diversity and adaptive strategies of coral-symbiont associations over a large depth range

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Publication date
2009

Document Version
Final published version

Citation for published version (APA):
CHAPTER 7

Afterthoughts
**Main Findings of This Thesis and Their Relevance**

This thesis focuses on the phylogenetic and functional diversity of coral-symbiont associations and their adaptive strategies to cope with large light gradients. Light constitutes the main energy resource in the coral reef ecosystem and its availability varies acutely over large depth ranges on reef slopes (Jerlov 1966; Kirk 1994; Veron 1995). Understanding how coral holobionts thrive across large light gradients over reef slopes was the main objective of this study. Accomplishing it involved comprehending the nature and phylogenetic relationships within each of the symbiotic partners (Chapters 2 and 6), further addressing the distribution and ecological zonation of host-symbiont combinations (Chapters 2 and 4) and finally the functional mechanisms that regulate the interactions between partners and between the holobiont and the environment (Chapters 3, 4 and 5). The present chapter highlights the main findings and discusses their relevance in the context of coral reef research.

Among several acclimatisation and adaptation properties described in previous studies for the two symbiotic partners, it has been hypothesised that the most significant feature of holobiont response to light gradients is the occurrence of taxonomically and functionally diverse algal symbionts establishing more or less specific associations with the numerous coral species that form a reef (Iglesias-Prieto and Trench 1997a; Baker 2003; Coffroth and Santos 2005). Particularly, the photobiological variation involved in such diverse algal assemblages is hypothesised to play a role in controlling host vertical distribution patterns (Iglesias-Prieto and Trench 1994; Iglesias-Prieto et al. 2004) and in determining coral reef resilience (Rowan 2004; Abrego et al. 2008; Sampayo et al. 2008).

**Photoecology and Light Adaptive Strategies**

The present thesis confirms the applicability of the rDNA ITS2 region for studies on coral symbiont ecology and evolution, and strongly suggests ecological niche partitioning among the symbionts, including distinct levels of host specificity (Frade et al. 2008c; Frade et al. 2008a). This is consistent with accumulating evidence suggesting that *Symbiodinium* ITS types relate to distinct ecological, biogeographical and evolutionary lineages, likely corresponding to the species level (LaJeunesse and Trench 2000; LaJeunesse 2001; Sampayo et al. 2009).

The present research (Frade et al. 2008b; Frade et al. 2008a) also stresses the important role of irradiance on coral physiology and reveals that photobiological mechanisms are either regulated by the environment (e.g. photochemical efficiencies) or genetically constrained (e.g. symbiont cell sizes). Furthermore the research highlights the role of host properties in the adjustment of the internal environment available for the endosymbionts. Different holobiont strategies thought to relate to symbiont cell density in the host tissue vary in their optimization of light-harvesting (light collecting pigments) or photoprotective mechanisms (non-photochemical quenching) and are proposed to relate to host-species vertical distribution and dominance over the reef slope. Overall, symbiont functional diversity does not appear to explain host distribution patterns. Instead, this thesis highlights the importance of species-specific morphological and physiological properties of the host to the photobiology of the intact symbiosis. Such host properties can relate to mechanisms such as varying skeletal morphology (Anthony and Hoegh-Guldberg 2003; Enriquez et al. 2005), tissue and polyp behaviour (Brown et al. 2002a; Levy et al. 2006a) or host pigment composition (Dove et al. 2006; Field et al. 2006), all reported by past studies to have a role in modulating the intensity
and distribution of light reaching the endosymbionts.

In the context of light control by the host, this research shows symbiont distribution to be significantly colour morph- (besides depth-) dependent in *M. pharensis* (Frade et al. 2008a). The absence of symbiont variation over large light-gradients at fixed depths (i.e., varying light quantity but constant spectral quality) further suggests that symbiont ecological distribution may be at least partially regulated by spectral light niches (Frade et al. 2008c; Frade et al. 2008a). Although colour has no taxonomic property in corals, colours other than the symbiont-generated browns are usually attributed to the presence of fluorescent proteins in the host tissue (Oswald et al. 2007). These have been suggested to either shade (Salih et al. 2000) or amplify (Schlichter et al. 1994) the light reaching the endosymbionts.

The present study demonstrates that *M. pharensis* holobiont functional differences are attributable to either acclimatisation or adaptation of symbiont ITS2 types (Frade et al. 2008a). In the latter case, symbiont cell size is hypothesised to be a property playing a significant role in the adaptation of coral holobionts to the deeper reef. The large functional difference measured between two B-type symbionts confirms that the clade classification does not correspond to functional or ecological identity (LaJeunesse 2001; Tchernov et al. 2004; Sampayo et al. 2007). Although there is unambiguous *in situ* evidence for ecological (Frade et al. 2008c) and physiological (Frade et al. 2008b; Frade et al. 2008a) distinction of ITS2 symbiont lineages, symbiont functional diversity would be better understood if studied under controlled experiments, using isolated symbionts. Isolating and culturing algal symbionts of different lineages has allowed past studies to confirm the central role of irradiance in their physiology (Chang et al. 1983; Iglesias-Prieto and Trench 1994,1997b). Culturing algal isolates offers a detailed characterization of their photobiology (Goulet et al. 2005; Robison and Warner 2006; Hennige et al. 2009), as there is no influence of host tissue, host morphological adaptations or effects caused by different cell densities in the tissue. This topic will be addressed in the section on ongoing research (see below).

**Bleaching and protective mechanisms**

One key aspect of the symbiosis to which the physiological properties of the symbionts are believed to relate intimately is bleaching susceptibility (Berkelmans and van Oppen 2006; Ulstrup et al. 2006b; Sampayo et al. 2008). Bleaching, a loss or reduction of the symbiont populations or their photosynthetic pigments which commonly results in host mortality is one of the most important causes for worldwide coral reef decline (Donner et al. 2007; Lesser 2007; Carpenter et al. 2008). Bleaching is caused by synergistic effects of elevated seawater temperature and high light intensity (Brown 1997; Douglas 2003; Lesser and Farrell 2004) which are known to promote the distortion between light collection and light use at or downstream of PSII (Warner et al. 1999; Fitt et al. 2001; Jones and Hoegh-Guldberg 2001). This results in the production of reactive oxygen species which damage protein and membrane functions of both the photosymbiont and the host and which often culminate in bleaching (Brown et al. 1995; Franklin et al. 2004; Venn et al. 2008a). Resistance of algal symbionts to environmental stress has often been related to mechanisms such as photoprotective pathways involved in dissipating excessive excitation energy (NPQ: Warner et al. 1996; Brown et al. 1999; Muller et al. 2001) and a more stable lipid composition of their thylakoid membranes (Tchernov et al. 2004). In vascular plants and free-living algae, however, other protective agents have been reported (McNeil et al. 1999). There, betaines are well known metabolites with protein and
membrane stabilizing effects that act as protective agents of PSII (Papageorgiou and Murata 1995). These metabolites have been involved in successful genetic engineering attempts to ameliorate temperature and irradiance-related cellular stresses (Chen and Murata 2002). The present research suggests that betaines may have a defensive role against bleaching in reef-building corals, opening a new research line with potential future applicability (Chapter 5).

Host species boundaries

A crucial aspect to fully understand the diversity, specificity, photobiology and adaptations of coral-symbiont associations is to address the nature of the evolutionary units that constitute them. For the animal component in particular, many studies have shown incongruence between the morphologically described species and molecular phylogenetics (van Oppen et al. 2001b; Willis et al. 2006). Besides a broad inherent intraspecific morphological plasticity (Todd 2008), scleractinians have been associated with low reproductive barriers and with interspecific hybridization events (Szmant et al. 1997; Willis et al. 1997; van Oppen et al. 2002), which decouple morphology and DNA phylogenetic inferences. Although mass spawning events characteristic of broadcast spawning corals are known to maximize hybridization opportunities (Veron 1995; Vollmer and Palumbi 2002; Willis et al. 2006), introgressive hybridization has also been suggested for brooding corals, particularly in the genus Madracis (Diekmann et al. 2001). Previous research pointed out that the morphological species distinction only corresponds to monophyletic groups for M. senaria and M. mirabilis, while all the other morphospecies comprise a paraphyletic complex (Diekmann et al. 2001). The study on the Madracis phylogeny included in this thesis indicates the same overall patterns, with non-monophyly of most Madracis taxa (M. senaria is the exception) and high levels of shared polymorphism. These results, combined with evidence for relatively old fossil record of some Madracis species (Budd et al. 1994; Budd et al. 1995; Budd and Johnson 1999), suggest that introgressive hybridization is likely to have played a role in the evolution of the genus. Madracis consists of brooders that release planulae from April to December. Madracis senaria, however, has a lunar pattern of planulae release superimposed on the seasonal cycle and there again it constitutes an exception (Vermeij et al. 2003b) which can justify its relative isolation. Furthermore, hybridization appears to be spatially-mediated, as suggested for instance by a closer genetic distance of deep water M. pharensis to M. carmabi than to shallow water M. pharensis conspecifics (Chapter 6). The study suggests that Madracis morphospecies remain recognizable either because hybridization is non-pervasive or because disruptive selection prevails over the homogenizing effect of gene flow. Depth divergence within M. pharensis is an example suggesting that symbiont functional differences may play a role in depth-based disruptive selection of the coral host and holobiont niche diversification over reef slopes.

Revisiting coral-symbiont associations and their resilience to climate change

Unravelling the functioning of coral-algal associations under extreme environmental gradients contributes to a better understanding of holobiont responses to climatic changes, and offers new insights on coral reef conservation, by illustrating the nature of species, their genetic relationships and evolutionary patterns.
New developments on symbiont diversity

The suitability of the PCR-DGGE fingerprinting technique in detecting ecologically dominant rDNA symbiont populations has recently been questioned (van Oppen 2007). A study employing bacterial cloning to address the diversity of *Symbiodinium* ITS2 types in coral associations suggested that the existing DGGE protocols may not reliably detect the full symbiont diversity or the dominance of certain sequence types (Apprill and Gates 2007). A response study has shown that instead sequencing bacterially cloned rDNA genes substantially exaggerates the level of eukaryotic microbial diversity due to the existence of intragenomic variation, pseudogenes and PCR artefacts (Thornhill et al. 2007), indicating that the doubts posed are at least partially invalid. Nevertheless, the symbiotic population within a host may include several background symbiont types present at very low amounts, and these are sometimes below the detection level of DGGE (Thornhill et al. 2006). Recent technical developments (real-time PCR) have allowed very precise quantification of symbiont lineages and revealed a higher occurrence of mixed assemblages in single colonies than previously reported (Loram et al. 2007b; Mieog et al. 2007; Mieog et al. 2009).

These quantitative approaches have important repercussions for understanding the potential response of coral symbioses to environmental stresses. In fact, an important issue when addressing the adaptive potential of bleaching events is whether the new symbionts are acquired from the surrounding medium (switching) or rather proliferate from background symbiont cells (shuffling) already present in the host. These recent studies (e.g. Mieog et al. 2007) suggest that the chance for new lineages to become dominant through symbiont shuffling is larger than previously thought.

Potential evolution of bleaching resistance

Actually, more crucial than symbiont switching or shuffling in single colonies during bleaching, a key issue underpinning the idea of adaptive bleaching is the method of symbiont transmission between coral generations. Also, whether there is gene flow from free-living symbionts into the holobiont populations. Day et al. (2008) present an innovative but preliminary theoretical model on coevolutionary interactions between symbiotic partners to explore factors affecting the potential evolution of bleaching resistance in coral holobionts. Their model showed that the mode of sexual reproduction strongly influences the spread of resistance alleles. Overall, sexual reproduction with vertical symbiont transmission more often contributes to enhancing the rate of spread of resistance alleles, although this is dependent on how the genotypes of host and symbiont combine to give rise to phenotypic resistance by the holobiont. Intuitively, a closed transmission mode seems to be the key for the prevalence of adapted symbiont populations in the coral descendents, as it is likely to allow the long isolation periods necessary for an evolutionary split to occur. Moreover, the model of Day et al. (2008) suggests that bleaching resistance alleles spread more quickly when bleaching results from holobiont death than from dissociation of symbiosis with ejection of competent symbiont cells (Ralph et al. 2001). This is because the death of the holobiont is more efficient at removing the disfavoured allele from the population. The authors emphasize that understanding the evolutionary adaptation in corals and their symbionts involves fully unravelling processes such as the genetic control of bleaching, but also addressing factors of ecological dynamics like gene flow between free-
living and in hospite symbionts or trade-offs among fitness components.

The presence of *Symbiodinium* clade D in several coral species (Mieog *et al.* 2007) has become a recent focus of research, as this clade is usually considered a more thermotolerant symbiont (Baker *et al.* 2004; Rowan 2004; Tchernov *et al.* 2004; but see Abrego *et al.* 2008). Adaptive bleaching involving emergence of clade D symbionts has been hypothesised to have happened over large geographical reef areas affected by recent climate change (Baker *et al.* 2004). A different study suggests that the prevalence of clade D over another geographical region is the result of long-term ecological and evolutionary mechanisms rather than due to recent coral bleaching (LaJeunesse *et al.* 2008). Another question relevant to the adaptive character of changes caused by bleaching is whether affected reefs revert to their original symbiont communities over time in case they do not experience repeated warming episodes (Baker *et al.* 2004). There is substantial geographical evidence suggesting that individual colonies revert back to their original symbionts postbleaching (Thornhill *et al.* 2006; Sampayo *et al.* 2008).

**Addressing coevolution of symbiotic partners**

New evidence accumulates indicating that the host has a more important role in determining holobiont physiology than previously assumed (Goulet *et al.* 2005; Abrego *et al.* 2008; Frade *et al.* 2008b), even in relation to bleaching susceptibility. An example is the role of heterotrophy in coral survival during bleaching (Grottoli *et al.* 2006; Rodrigues and Grottoli 2007; Palardy *et al.* 2008). During temperature and light stress, Papina *et al.* (2007) found most changes in fatty acid response of both host and symbionts to occur in the first, suggesting that hosts may be more susceptible to environmental change or that they may be shielding the symbionts. These examples highlight the complexity of the interaction between partners.

Given the fact that both symbiotic partners have a part in defining holobiont survival, it is reasonable to predict that if thermal resistance occurs, it is likely to be acquired by adaptation from one of the partners. Functional mutations in one of the symbiotic partners may have evolutionary consequences for the other partner, generating a set of co-evolving symbiotic partners (Vermeij 1994). A classical example is the divergence of two coral populations through reproductive isolation (Palumbi 1994), which ultimately could contribute to a genetic split in the symbiont populations they are hosting. Again, this would depend on mode of reproduction and rates of gene flow (Day *et al.* 2008). Conversely, whenever the symbiont population present in coral planulae plays a role in differential habitat-based host survival (Abrego *et al.* 2008; Sampayo *et al.* 2008), the animal population may hypothetically become spatially structured and eventually experience genetic divergence. An extreme case would be when distinct functional performances of symbionts shape the divergence of the coral lineages hosting them, which could, for instance, constitute a driving force leading to differential coral light niche occupation (Iglesias-Prieto *et al.* 2004). Depth divergence in *M. pharensis* populations may well represent such an example of disruptive selection mediated by symbiont physiological performance (Chapter 6 of this thesis). Similarly to what is predicted in terms of the adaptive potential for bleaching resistance (Day *et al.* 2008), one should expect that the potential for coevolution is more common among brooding corals (where larval development occurs inside the mother colony after internal fertilization) than among spawning corals. This should be particularly true for brooding corals with symbiont vertical transmission, for which it is legitimate to assume less recurrent gene flow or host-
symbiont recombination events. In contrast, broadcast spawners, which release gametes to the water column, have probably more open or flexible symbiotic associations and more chances of recombination among symbionts (Little et al. 2004). Besides, the larger dispersal ranges offered by broadcast spawning (Harrison et al. 1984; Szmant 1986) probably reduce chances for genetic divergence, due to more prevalent gene flow between populations (Palumbi 1994; Bohonak 1999). A recent study has, however, contradicted such prediction (Miller and Ayre 2008b). Still, there is no substantiated evidence of coevolution among host-symbiont systems in corals, and many studies reporting on the high degree of host and symbiont flexibility (Baker 2003). The advance of finer resolution genetic markers (Pettay and Lajeunesse 2007) may further contribute to this discussion (Coffroth and Santos 2005).

**The Madracis lessons**

Several studies conclude that corals occupy restricted ecological niches to which they have adapted, together with their symbionts (Goulet 2006; Sampayo et al. 2007). Other studies, applying higher-resolution techniques, have shown that most corals may establish flexible associations with their symbionts (Mieog et al. 2007). The *Madracis* holobionts studied here appear to establish very specific associations (LaJeunesse 2002; Diekmann et al. 2003; Frade et al. 2008c), although the complete extent of symbiont populations has not yet been fully explored. However, they are an elucidative example on how the clarification of the existing evolutionary lineages constituting the symbioses may help understanding the potential adaptation of corals to climate change. A depth generalist species such as *M. pharensis*, that initially appeared to show some flexibility with respect to the symbionts it hosts, is now unravelled as a highly structured taxon, with fine genetic differentiation between two distinct depth populations (Chapter 6). These two host populations exactly match the symbiont type harboured, suggesting a very sensible tuning between genetic strains of the two partners. Such evidence of high specificity and niche specialization do not support optimistic interpretations of a widespread role of symbiont shuffling in coping with rapid climate change. At least, a considerable part of the genetic diversity may be lost even if some coral populations are to cope with the new environmental conditions. Perhaps the most important issue refers to the scale at which corals can adapt in the short term, which may well be narrower than the predicted timescales for increases in seawater temperature (Middlebrook et al. 2008). Finally, it is not always clear whether the relative dominance of a new resistant symbiont is caused by a change occurring within individual colonies or actually by differential mortality of colonies hosting the non-competent lineage (Sampayo et al. 2008). If the last case occurs more often, then large parts of the coral reef may disappear before the stress-resistant lineages become dominant.

**The *Symbiodinium* “species” problem**

Understanding the diversity in the genus *Symbiodinium* is critical to address the ecology and biogeography of coral-algal associations and their capacity to acclimatise and adapt to environmental change. Specifically, enhancing the resolution of *Symbiodinium* systematics depends on using multiple independent markers (Coffroth and Santos 2005). In a recent study seeking to institute widely accepted taxonomic classification within the genus, Sampayo et al. (2009) compared thirteen distinct molecular genetic analyses providing evidence of consistency
between methods and widely confirming the phylogenetic resolution of the *Symbiodinium* ITS rDNA “type” classification. The strong correlation between phylogenetically independent lineages and distinct ecological and physiological attributes lead the authors to conclude that the present available molecular methods can be used to assign species designations, if used in combination with ecological data.

**Species concepts**

Ultimately, defining *Symbiodinium* “species”, relates to the concept of species itself, a problem that is recurrent also in host taxonomy (this thesis). The complications in recognizing “real” species in the genus are related with difficulties in applying the morphological and biological species concepts. Defining “true” symbiont species (according to the biological species concept) depends on assuming that sexual recombination occurs in *Symbiodinium* and that there is reproductive isolation delimitating species boundaries among these organisms. Since most eukaryotes have a sexual phase in their life history, genetic analyses could allow confirmation or rejection of sexual recombination between different symbiont types. So far, little evidence for genetic recombination has been found between types (Santos *et al.* 2004; Pettay and Lajeunesse 2007), suggesting that these lineages may be reproductively isolated (Sampayo *et al.* 2009). However, evidence for sexual recombination occurring at all within *Symbiodinium* is limited (Baillie *et al.* 2000; Rodriguez-Lanetty 2003; Santos and Coffroth 2003; Santos *et al.* 2003b; Santos *et al.* 2004) and as such the utility of the biological species concept may be compromised (Correa and Baker 2009).

Moving to the phylogenetic species concept is also not a straight-forward approach, as datasets with too little or too much variation, and therefore with an insufficient number of phylogenetically informative characters, will reduce the resolution of the tree.

**A new approach and the Madracis example**

Alternatively, Correa and Baker (2009) suggest a new, imperfect but interesting, cluster-based approach in which many closely related *Symbiodinium* ITS2 types rather represent genetic variants of the same species. This statistical parsimony approach would reduce diversity from c. 175 down to 35 “species”. Species would be clusters of closely related sequences diverging from ancestral variants that are typically ecologically dominant. As such, *Symbiodinium* ITS2 types would bridge inter- and intraspecific variation. This approach is in agreement with the cohesive species concept (Templeton 1989,2001), for which “a cohesion species is an evolutionary lineage, whose boundaries arise from the genetic and ecological forces that create cohesive reproductive communities”. The cluster-based interpretation of *Madracis* specific ITS2 types, B7, B13 and B15 (LaJeunesse 2002; Diekmann *et al.* 2003; Pettay and Lajeunesse 2007), would support the interpretation of types B7 and B13 as intraspecific variants of a single species (within the B1 cluster, which would include other diverse ITS2 types), and B15 as a member of a separate cluster or species, also including several distinct types (Correa and Baker 2009). Data presented in this thesis (Frade *et al.* 2008b; Frade *et al.* 2008c; Frade *et al.* 2008a) offer clear ecological and functional background to this species classification. To date, type B13 has only been assigned to *M. mirabilis*, and in this respect its distribution differs from that of type B7, considered a depth generalist with low host specificity (Frade *et al.* 2008c). However, this variation in host specificity is the only difference known
between the two sequence types, that show no functional difference over a wide range of functional parameters (Frade et al. 2008b). Due to the lack of clear niche diversification, B13 could instead represent a derived variant of the ancestral B7 type that arose through mutation and became dominant as a result of genetic drift (Correa and Baker 2009). The typically vertical symbiont transmission mode of *M. mirabilis* (Vermeij et al. 2003b) and the species’ great success in dominating great patches of the shallow reef induced by recurrent colony fragmentation (Nagelkerken et al. 2000) may cause *Symbiodinium* type B13 to remain restricted to that host species. The relative genetic isolation of *M. mirabilis* (Diekmann et al. 2001 and this thesis) supports this high host specificity.

Although attractive, this cluster-based interpretation of ITS2 diversity is not always backed-up by ecological zonation or physiological measurements. In fact, several of the clusters suggested include ITS2 types which have already been isolated and proved to have significantly functional differences (Robison and Warner 2006; Hennige et al. 2009). For instance, types B1 and B7, which fall in the major B1 cluster, have been shown to differ significantly in traits of light response when cultured under the same conditions (this thesis, see “Ongoing research”). The unresolved question of *Symbiodinium* species identity has necessary consequences for comparing levels of diversity and addressing issues of host specificity and coevolution, as well as on our understanding and testing of coral adaptation to climate change (Coffroth and Santos 2005).

**ONGOING RESEARCH**

The following paragraphs summarize preliminary results of ongoing research, comprising topics such as coral reproductive traits, transplantation experiments to address acclimation potential of coral-algal associations, intracolonial symbiont variation or isolation and further culturing of the dinoflagellate component of coral holobionts. This section is not intended to give a detailed and exhaustive description of methods and results, but rather presents general statements and hints to future directions in our research.

**Variation in morphological traits of *Madracis* planulae**

Reef-building corals vary in their reproductive strategies (Szmant 1986) and some of these traits, such as spawning time, can sometimes be used to distinguish between closely related, morphologically similar species (Knowlton et al. 1997; Levitan et al. 2004). *Madracis* species are simultaneous hermaphrodites and show no temporal reproductive isolation, releasing planulae from April to December. *Madracis senaria*, however, has a lunar pattern of planulae release superimposed on the seasonal cycle and it spawns more planulae than all other species. There are also relevant differences in planula or oocyte size, with *M. mirabilis* and *M. senaria* showing large yolk reserves (Vermeij et al. 2003b, 2004).

This study re-addressed the reproductive behaviour of four members of the coral genus *Madracis* (*M. mirabilis*, *M. carmabi*, *M. decactis* and *M. senaria*). Cone-shaped net traps made of plankton-gauze were attached over colonies with the aid of rubber bands. Planulae were collected every morning from plastic tubes previously fixed at the upper end of the nets. The study showed that there is less interspecific variation in planula morphology than previously reported (Vermeij et al. 2003b). The presence or absence of visible symbiont assemblages in the planulae (as a conspicuous brown tip or ring on their oral end) was characterised by high
intraspecific variation and should therefore not be used as a species-specific character. Planula size showed large intraspecific variation and was variable even within individual colonies. Besides, shape and development level of planulae (oocyte-like spheroid or developed planulae) did not match previous results by Vermeij et al. (2003b). Large intraspecific variation suggests that there are environmentally induced mechanisms regulating planula morphology.

**Depth-transplantations reveal large acclimation potential in Madracis holobionts**

Understanding the photobiology of coral holobionts may contribute to a better prediction of coral resilience under a rapidly changing climate (Hoegh-Guldberg et al. 2007). Previous research has shown that there is broad acclimatisation potential in Madracis-symbiont associations and that the attenuation of light with depth plays an important role in coral ecology (Frade et al. 2008b). Transplantation experiments of the whole holobiont over the reef slope provide important information on how the intact symbiosis reacts to environmental change. Besides, these experimental procedures may address to what extent the host-symbiont associations are the result of prevailing environmental conditions or genetically constrained (Iglesias-Prieto et al. 2004). In this study, colony fragments of *M. mirabilis* and *M. senaria* were reciprocally transplanted between 10 and 25 m of depth (n = 10 for each species and depth) and firmly attached to racks (Figure 7.1). Fragments of the same transplanted colonies were transplanted to the same original depth as controls for non-depth related effects. Acclimation was followed by measuring the maximum light excitation pressure over PSII ($Q_m$, see Frade et al. 2008b) after 1, 5, 30, 60 days and 1 year. Symbiont densities, pigment contents and photosynthetic rates, as well as the genetic identity of the dominant ITS2 *Symbiodinium* populations, were monitored after 30 days and after 1 year.

In general, most physiological properties showed significant acclimation changes after transplantation, with the new acclimated state mimicking the functioning of the conspecific control colonies originally from that same depth. Dominant symbiont populations remained practically unchanged, reflecting non-variable symbiont populations along the reef slope for these same host species (Frade et al. 2008c). The study highlights broad acclimation potential for Madracis holobionts hosting B-type symbionts.

**Comparing methods for the isolation of Symbiodinium from host tissue**

Previous work on isolated symbionts has provided important contributions to the field of photobiology of corals (Chang et al. 1983; Iglesias-Prieto and Trench 1994; Robison and Warner 2006). Obtaining mono-algal *Symbiodinium* cultures is crucial to fully understand the light response of distinct symbiont lineages and it allows extensive comparative research. However, isolation methods are complicated, time-consuming and most of the times success is a matter of chance (Trench 1971a; Polnefuller 1991; Santos et al. 2001). Although most methods aim to achieve a completely pure axenic culture, this is not always necessary, especially for short-term experiments. In this study the objective was to establish a relatively easy and fast method to provide clean *Symbiodinium* cultures for further research on light response mechanisms. Tissue was separated from skeleton into two distinct main media, FSW (filtered seawater, 0.2 µm) or ASP-8A+ (a variation of ASP-8A, Blank 1987). After homogenization, filtering and centrifuging, the obtained algal pellet was resuspended in the isolation media, consisting of one of the main mediums mentioned, with or without the addition of antibiotics and GeO$_2$, to
hinder bacterial and diatom growth, respectively. Antibiotics consisted of either Rifampicin (transcription inhibitor) or a mixture of 10 other antibiotics (Polnefuller 1991) with several modes of action, including translation inhibition or bacterial cell membrane deterioration. Several combinations of the above mentioned treatments were applied to freshly isolated symbiont cells originating from several Madracis coral hosts (M. pharensis, M. decactis, M. senaria and M. mirabilis). Samples were examined after five days and medium replaced with ASP-8A⁺. Of the available isolation media, treatment with GeO₂ and the antibiotic mixture of Polnefuller (1991) appeared to be the most effective based on low bacterial abundance and the absence of other organisms such as diatoms. This isolation method does not yield an axenic culture and does not guarantee a unialgal culture either. However, this method offers an easy and fast alternative to achieve cultures in which Symbiodinium predominates, which will be useful for many applications.

**Photoacclimation of isolated Symbiodinium types in culture**

Reef-building corals inhabiting the photic zone host distinct symbiont lineages with distributions suggesting ecological niche partitioning (Iglesias-Prieto and Trench 1997a; Sampayo *et al.* 2007). In the coral genus *Madracis*, three Symbiodinium ITS2 types, known as B7, B13 and B15, have been described (Diekmann *et al.* 2003). Their distributions suggest depth-based ecological function and host specificity (Frade *et al.* 2008c). Type B7 is a generalist, occurring in all host species at all depths. Type B13 is restricted to the shallow-water specialist *M. mirabilis*. Type B15 is typical of deep reef environments. Furthermore, in
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situ functional evidence indicates that type B15 is significantly different from the B7 and B13 types, based on parameters such as cell size and photosynthetic pigment contents (Frade et al. 2008b; Frade et al. 2008a).

Initially, this study aimed to study the photoacclimation of all three existing Madracis symbionts by means of continuous and batch cultures, following isolation of the symbionts from their hosts in the field. Studying isolated symbionts allows a better understanding of their photosynthetic activity as there is no influence of host tissue environment, host morphological adaptations or differential packaging effects of symbiont cells. The use of continuous cultures constitutes a novel approach to the study of Symbiodinium. Running in technically advanced chemostats, continuous cultures are specifically designed for studies of light-limited growth and have the advantage that organisms can be maintained at a constant growth rate under steady-state light conditions, thus allowing perfectly controlled culture conditions (Huisman et al. 2002; Passarge et al. 2006). Due to unsuccessful isolation attempts and recurrent contamination, we report here on a single experiment using batch cultures. This study compared the photoacclimation responses of two Symbiodinium ITS2 types over a range of light intensities (20, 40, 80, 160 and 240 μmol photon m⁻² s⁻¹) by means of photosynthesis-irradiance curves based on PSII fluorescence. Symbionts included were type B7 isolated from a M. senaria colony in 2005 in Curaçao and type B1 isolated from the octocoral Pseudoterogorgia sp. in 2001 in Jamaica. The latter is part of the collection initiated by Dr. Robert Trench (collection clone number 367) and was provided by Dr. Roberto Iglesias-Prieto. Type B7 was able to cope with a broad range of irradiances, reflecting its generalist nature already characterised in situ. However, acclimation potential of type B1 was even broader, especially into the upper irradiance level.

Assessing intracolony variation in M. mirabilis

Occurrence of intracolony variation including heterologous pools of symbiont lineages has been reported for several hosts (Rowan and Knowlton 1995; van Oppen et al. 2001a; Thornhill et al. 2006). For instance, Rowan et al. (1997) found light-dependent distributions of symbiont lineages within colonies of Montastreae spp. Mixed assemblages within single colonies may be a result of biotic and abiotic factors, including host specificity, microenvironments or mode of symbiont acquisition (Stat et al. 2006).

The coral M. mirabilis forms colonies composed of many narrow branches whose spacing varies across habitats (Sebens et al. 1997). Adult colonies can grow either as large aggregations covering vast areas of the shallow reef or as small isolated hemispherical patches. Potential shading depending on branch position and light scattering may generate a complex gradient of light microhabitats within individual colonies (Anthony and Hoegh-Guldberg 2003; Enriquez et al. 2005; Kaniewska et al. 2008). A previous study on M. mirabilis showed occurrence of mixed Symbiodinium assemblages of ITS2 types B7 and B13 throughout a studied depth range of 2-25 m (Frade et al. 2008c). However, the sampling strategy did not allow clear interpretation on intracolony variation patterns.

The present study aimed to address whether M. mirabilis colony landscape (branch position) has an effect on symbiont variation. This was tested by choosing eight separate hemispherical colonies at 10 m depth and characterizing the symbiont populations of outer and inner branches, at the tip and bottom of each branch, for each colony. Colonies were either exclusively associated with type B13 or with a mixed assemblage of B13 and B7 ITS2
types. There was no detectable symbiont variation within single colonies, indicating that the assumed micro-environmental variation generated by the colony landscape does not have an effect on symbiont presence. In *M. mirabilis*, the absence of symbiont variation within single colonies finds a match in the invariant populations previously described for the depth-gradient (Frade *et al.* 2008c). Results suggest little environmental influence in *M. mirabilis* symbiont composition and differences between colonies may relate to the original symbiont pool present in the planulae originating such colonies. Vertical symbiont transmission coupled to a strong role of colony fragmentation on the dispersion of the host can explain the differences in symbiont composition between colonies. Type B7 and B13 do not show distinct ecological distributions within *M. mirabilis*.

**FUTURE RESEARCH**

The aim of this section is to present and discuss some ideas for future research. Whilst some of the topics represent major research avenues that are top priority for most coral reef research groups, other side topics may be just not that obvious. Although the objective of this thesis was not to test new techniques but rather to apply existing tools on a comprehensive scale to the study of coral-algal symbiosis, it is worthwhile paying some attention to future technical developments. In the scope of photosynthesis estimates using PAM fluorometry, relevant new research has uplifted some important limitations of techniques used. For instance, Ulstrup *et al.* (2006a) showed that relative ETR measurements are probably underestimations of the real photosynthesis rates (oxygen evolution rates). A possible reason is that the vertical heterogeneity of symbionts in the tissue causes the displacement between measurements. Another explanation is that there are alternative electron pathways such as cyclic electron flow around PSII that are not accounted by fluorometry techniques. On a different topic, Hill and Ralph (2008) have shown that there are active pathways causing dark-reduction of the plastoquinone pool and as such the true maximum quantum yield of PSII may not be correctly estimated unless pre-exposure to far-red light is employed. These are just two examples of how the field of photosynthesis research in corals and their symbionts is expected to develop. As new cellular photobiology mechanisms are unravelled, research apparatuses are expected to become increasingly accurate in approximating real photosynthetic production and to include new tools to overcome recently recognised limitations.

Another unclear topic in coral-algal symbiosis research refers to the relative contribution of different combinations of hosts and symbionts to the holobiont. More specifically, an almost unexplored issue respects host-symbiont cellular processes such as transfer of photosynthetically-fixed carbon and how different symbiont lineages contribute to the growth and calcification of the host partner. Although different studies have explored correlations between holobiont phenotypic properties and the symbiont assemblages they harbour (Abrego *et al.* 2008; Frade *et al.* 2008a; Sampayo *et al.* 2008), and others accurately quantified differential contributions of heterotrophic feeding, even during bleaching events (Grottoli *et al.* 2006; Rodrigues and Grottoli 2006; Palardy *et al.* 2008), the actual direct quantification of the carbon input of distinct symbiont lineages into growth and calcification metabolism has not been explored, but is feasible (Muscatine *et al.* 2005). The research of Loram *et al.* (2007a) with giant sea anenomes represents a provisional exception. As much as different hosts can be more or less heterotrophic, different host-algal associations can be more or less
autotrophic depending on the photosynthetic properties of their symbionts. In the future, this line of research should include high resolution algal identification techniques below the cladal level. Quantifying the phenotypic variability coupled to *Symbiodinium* genetic diversity and the differential enhancement for coral fitness given by such variability is crucial to predict coral holobiont acclimatisation.

The previous topic relates to the degree of contribution from symbiont and host to the total energetic needs of the holobiont, which has been associated to the need of considering a continuum running from 100 % photoautotrophy to 100 % heterotrophy (Palardy *et al.* 2008). Recent studies on biological interactions have shown that the same pair of species can be mutualists, commensals or even parasites, depending on environmental conditions, and that the “positive” interaction is just one case of the continuum (Stachowicz 2001). Recent literature hypothesize that some specific coral-symbiont combinations may be actually closer to parasitism (Stat *et al.* 2008a). Pathogenic infection systems seem to provide new ideas for understanding the intracellular niche in the host-symbiont association (Schwarz 2008). In fact, a new challenge is to understand how shifting environmental conditions, such as predicted with climate change, will cause changes along the interaction continuum. Eventually, as environmental stress increases, a decisive step is to recognize habitat-ameliorating properties, which can be arguments for conservation decisions. Facing the widespread degradation of the last “pristine” reefs on Earth may force new conservation approaches, such as securing the future of habitats usually considered as marginal for coral reef development but within which some important genetic relics may survive (Vermeij *et al.* 2007b).

Light-dependent deep reefs may just constitute such kind of habitat. It is known that ecological changes on coral reefs do not affect the whole reef in the same manner (Bak *et al.* 2005; Nugues and Bak 2008). The recent hypothesis on a potential ecological refuge function of the deep reef (Hinderstein *et al.* in press) has brought growing interest to these mesophotic habitats (Venn *et al.* 2009). As the shallow reef is under a stronger influence of disturbance due to accelerated climate change scientists have asked themselves whether the deep reef represents an extension of the shallow community or, on the contrary, hosts distinct genetic assemblages. This thesis hints that the deep reef may hold host (Chapter 6) and symbiont genetic assemblages (Frade *et al.* 2008c) distinct from those present in the shallow reef. Understanding the processes and the genetic diversity involved with deep reefs is a key subject to predict whether these reefs can be a source for future shallow coral reef regeneration.

Still in the conservation context, an important issue is the recently recognised role of hybridization in the evolution of corals and its potential function as providing new adaptive tools under a changing environment (Willis *et al.* 2006). An alternative approach to the current focus on the conservation of species, is to direct efforts into the conservation of evolutionary processes that generate the biodiversity comprised in taxonomically complex groups such as corals (Ennos *et al.* 2005).

Another interesting question brought up by the present thesis is whether light spectral distribution has a role in defining symbiont niches (Frade *et al.* 2008a). Although acclimation to spectral quality constitutes an important factor determining variation in algal photosynthetic responses and growth rates (Falkowski and Laroche 1991), application of such knowledge to the ecology of coral-algal associations is speculative. Recent research has given new insights on the physical mechanisms generating the available spectral niches for phototrophic microorganisms (Stomp *et al.* 2007). As the light spectrum offers an axis for niche partitioning
Afterthoughts

(Falkowski et al. 2004; Stomp et al. 2004) it is reasonable to hypothesize that this possibility has also offered evolutionary opportunities in the context of coral-algal niche occupation. Testing this hypothesis involves performing field and lab experiments with either intact holobiont or isolated symbionts.

As bleaching disturbances are likely to become chronic in many reef areas in the coming decades (Baker et al. 2008), this topic will remain crucial. Perhaps one interesting idea for research is to explore coral-symbiont associations that are usually non-bleaching. Madracis constitutes such an association, for which bleaching is only reported after experimental handling of colonies (Frade et al. 2008a). In the field there are no reported observations of bleached Madracis (pers. comm. R.P.M. Bak). The prevalence of the intact association may be related to the high specificity between symbiotic partners (LaJeunesse 2002; Frade et al. 2008c). Eventually there is incapacity of the host to establish stable associations with any other new symbiont lineage. Hypothetically, these associations may have developed tools to cope with chronic stressful cellular conditions that go beyond the tolerance threshold of other more flexible holobionts (Ulstrup et al. 2008). Properties such as increased photoprotective mechanisms (Frade et al. 2008b) or even increased betaine production (Chapter 6) can help explain the absence of a bleaching trigger. Understanding such examples may provide key knowledge for bioengineering approaches. The ethics and applicability of genetic manipulation, as the last resort available to help saving some (limited) components of the threatened diversity of coral reefs, will most likely one day need to be addressed. Obviously, before those times arrive, much more need to be learned of coral functional genomics, particularly with respect to crucial processes controlling bleaching (Desalvo et al. 2008). Determining the relative importance of different cellular mechanisms (and their genomic basis) that interact to mediate the bleaching response needs further attention (Venn et al. 2008a).

Acknowledgements

I would like to thank Rolf Bak and Jef Huisman for valuable comments on this chapter.