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Apoptosis as a post-phagocytic winnowing mechanism in a coral–dinoflagellate mutualism

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Summary

This study was aimed at detecting apoptosis as a post-phagocytic mechanism of symbiont selection during the onset of symbiosis in larvae of the scleractinian coral *Fungia scutaria.* Larvae were infected with one of three *Symbiodinium* types: freshly isolated homologous ITS-type C1f from adult *F. scutaria*, heterologous C31 from adult *Montipora capitata*, known to be unable to successfully colonize *F. scutaria* larvae, and type B1 from the symbiotic sea anemone *Aiptasia* spp. Apoptosis was detected by the activation of caspases, enzymes specific to apoptosis. Caspase activity was measured *in situ* by cleavage of a specific fluorophore and detection with confocal microscopy. At 6 h post infection, there was a significant increase in caspase activation in gastrodermal cells in C31-infected larvae, compared with larvae infected with C1f or B1 types. Compared with control larvae infected with C31, which had decreased infection rates present by 24 h post infection, when C31-infected larvae were incubated with a broad-scale caspase inhibitor, the per cent of larvae infected with C31 did not significantly decrease over time. This indicates that the reduction in infection success observed in untreated C31-infected larvae can be rescued with inhibition of caspases and apoptosis. This suggests the presence of a post-phagocytic recognition mechanism. Larvae infected with freshly isolated B1 retained infection success over time compared with C31-infected larvae, suggesting that there is host discrimination between heterologous algae. Initiation of this post-phagocytic response may occur more readily with a highly specific heterologous symbiont type such as C31, compared with a generalist heterologous type such as clade B1.

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Introduction

Mutualistic endosymbioses between microbial symbionts and larger hosts are prevalent in all environments. The host acquires symbionts through vertical and/or horizontal transmission (Douglas, 1994). Vertical transmission is a closed system where symbionts are passed directly between host generations. Horizontal transmission is an open system, where symbionts are newly acquired from the environment by each host generation. An advantage to acquiring genetically varied symbionts is that they can be acclimatized to local conditions and therefore have the potential of increasing the fitness of the host and symbiont (Douglas, 1998). A disadvantage of horizontal transmission is that low symbiont abundance or unfavourable environmental conditions may reduce the availability of viable symbionts for host acquisition, thereby resulting in reduced fitness.

An additional complexity of horizontal transmission is that potential symbionts have to encounter and ultimately bypass host defences and immune responses to establish a new symbiosis. There is often a complex series of recognition interactions between the host and symbiont, all of which are necessary but none alone of which is sufficient to result in a stable association. This step-by-step process, recently termed 'the winnowing' (Nyholm and McFall-Ngai, 2004), has been described in detail in well-studied mutualisms, such as legume-rhizobial bacteria and squid-luminous bacteria associations (McFall-Ngai, 2000; Hirsch *et al*., 2001). Winnowing begins with initial cell surface contact and cellular signalling between partners. These processes are homologous to the cellular interactions between hosts and invading pathogens and parasites and involve the same pathways found in host–pathogen and host–parasite associations (Nyholm and McFall-Ngai, 2004). Winnowing can, however, extend far beyond cellular interactions to include longer-term ecological competition between symbionts for the host intracellular niche.

A common strategy for pathogens and parasites during host invasion is to evade the host immune system by controlling innate immune cellular pathways (Sacks and Sher, 2002; Vergne *et al*., 2003; Cossart and Sansonetti, 2004; Amer and Swanson, 2005; Gruenberg and van der Goot, 2006). If a pathogen fails to evade host innate immune defences, in some cases the host removes the

pathogen by apoptotic host cell death (James and Green, 2004). Conversely, some pathogens retard their removal via host apoptotic cell death by controlling host antiapoptotic signalling mechanisms (James and Green, 2004; Koul *et al*., 2004). It is likely that some of these same strategies of promotion of host-cell apoptosis by hosts and corresponding apoptosis inhibition by microbes are at play in the winnowing processes of mutualistic interactions.

Symbiosis between cnidarians, such as corals, and photosynthetic dinoflagellates in the genus *Symbiodinium* form the trophic and structural foundation of coral reef ecosystems. The cnidarian provides inorganic carbon, nitrogen, a high-light environment and refuge from herbivory to the symbiotic dinoflagellate. In return, the dinoflagellates translocate photoassimilated reduced organic carbon, lipids and amino acids to the host (Furla *et al*., 2005). An estimated 85% of corals acquire dinoflagellates through horizontal transmission during the host planula larva or juvenile polyp stages (Fadlallah, 1983; Harrison and Wallace, 1990). Hosts most often ingest and then phagocytize symbionts during feeding. The symbionts ultimately reside intracellularly within vacuoles in host gastrodermal cells. The majority of adult corals harbour only one symbiont type (Baker, 2003; Goulet, 2006), although there are many exceptions. For example, populations of symbiont types in some species of corals may change due to environmental conditions and changing physiological demands (Buddemeier and Fautin, 1993; Apprill and Gates, 2007; Baker and Romanski, 2007; Mieog *et al*., 2007). During the onset of symbiosis, both partners therefore ultimately proceed through a winnowing process that narrows the possible partners from many potential partners to one or a very few. Aspects of winnowing in cnidarian-algal symbiosis have been studied for over 30 years in a large variety of partnerships (reviewed in Rodriguez-Lanetty *et al*., 2006); however, still very little is understood about the early cellular events involved in the onset of symbiosis.

Winnowing processes in intracellular mutualisms can be grouped into pre- and post-phagocytic events. There is growing evidence that lectin–glycan interactions, which are prevalent in animal–microbe associations, are an important pre-phagocytic recognition step in cnidarian–dinoflagellate mutualisms (Lin *et al*., 2000; Wood-Charlson *et al*., 2006). There is also evidence of post-phagocytic recognition and winnowing, whereby appropriate symbionts appear to control host cell vesicular trafficking, thereby preventing phagosomal maturation (Fitt and Trench, 1983; Chen *et al*., 2003; 2004; 2005). There is also evidence of competition between symbiont types for the host niche (Coffroth *et al*., 2001; Belda-Baillie *et al*., 2002; Little *et al*., 2004; Thornhill *et al*., 2006). Despite progress in these areas, we are still far from a complete description and sequence of processes involved in winnowing.

In this study, we investigate the role of host cell apoptosis in the post-phagocytic winnowing process in coral–dinoflagellate symbiosis. Apoptosis is involved in many cellular processes in cnidarians (David *et al*., 2005); however, most notable here is its role in the removal of symbionts during cnidarian bleaching in response to environmental stress (Dunn *et al*., 2004; 2007; Ainsworth *et al*., 2007). We hypothesized that the entry of non-specific (heterologous) symbionts into hosts elicits host cell apoptosis as a means of removing inappropriate heterologous symbiont cells and that hosts challenged with specific (homologous) symbionts do not exhibit the same amount of apoptotic activity. This work is part of ongoing studies of the coral *Fungia scutaria*, which is an ideal model for examining the onset of symbiosis, as it produces large quantities of aposymbiotic larvae that can be challenged with a variety of symbiont types (Schwarz *et al*., 1999). In coral larvae, we used an *in vivo* assay for caspase activity, a key component of apoptosis. Caspases are highly conserved aspartate-specific cysteine proteases that operate in a proteolytic cascade which leads to disassembly of the apoptotic cell (for review see Fan *et al*., 2005). Apoptosis activation was compared in *F. scutaria* larvae challenged with either homologous or heterologous algal types. The results of this study are the first to suggest that apoptosis plays a role in post-phagocytic symbiont removal during the onset of symbiosis.

Results

In vivo *caspase activity is present in larvae infected with heterologous symbionts*

Figure 1 describes the types of activated fluorophore labelling that were observed in larvae. Untreated aposymbiotic larvae did not emit a detectable green fluorescence of the activated fluorophore (Fig. 1A). Incubation of aposymbiotic larvae in colchicine resulted in caspase activity indicated by the appearance of the green fluoroprobe emission in cells of both the epidermis and gastrodermis, showing that the fluoroprobe was successfully entering larvae and that the specific caspase activity was detected by inducing cell death (Fig. 1B and C). Rhodamine fluoroprobe activation in infected larvae displayed two distinct qualities that we describe here and then relate to symbiont type in the following paragraph. The fluoroprobe appeared as diffuse fluorescence (Fig. 1D) or as an intense, condensed punctate signal (Fig. 1E and F). The intense staining in the gastrodermis could appear in host cells immediately adjacent to symbionts (Fig. 1E′) or alone and not in direct contact with symbionts (Fig. 1F′).

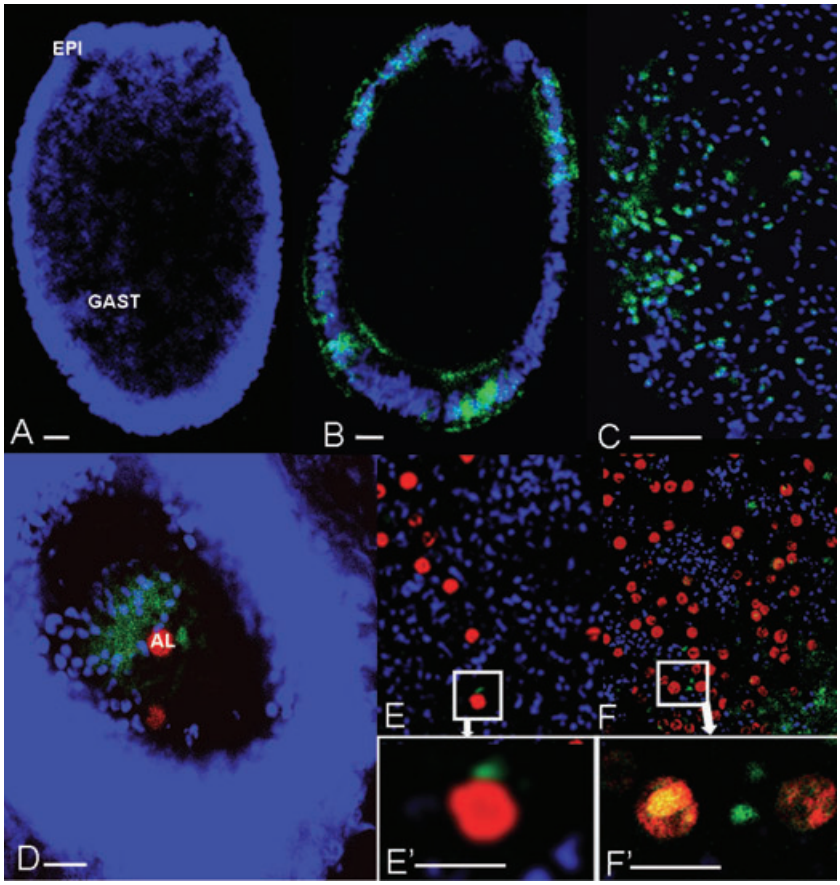

Fig. 1. Caspase activity in *F. scutaria* larvae indicated by cleavage of the Rhodamine 110 green fluorophore aspartic acid substrate.
A. Untreated aposymbiotic larva showing no signal (negative control).
B and C. Colchicine-induced caspase activation of fluorophore (positive control) in aposymbiotic larvae at low and high magnification respectively.
D. Diffuse rhodamine staining in the gastrodermis of an infected larva.
E and F. Condensed and intense caspase signal present in host cells.
E′. High magnification of a host cell immediately adjacent to a symbiont.
F′. High magnification of a host cell not directly associated with a symbiont.
Red = algal chlorophyll autofluorescence (488/600 nm); green = Rhodamine 110 fluoroprobe (498/521 nm); blue = Hoescht DNA stain (405/450 nm). AL, alga; EPI, epidermis; GAST, gastrodermis. Scale bars: 10 μm.

Caspase activity through time in populations of larvae infected with the three different algal types, freshly isolated homologous C1f and heterologous C31 and cultured heterologous cultB, displayed markedly different patterns depending on symbiont type (Fig. 2A and B). In C1f-infected larvae, the label was restricted to rare, very small punctate dots of intense signal in the epidermis and gastrodermis (data not shown) in just two out of 20 larvae at 2 h (Fig. 2B). The percentage of C1f-infected larvae with label decreased to 0 at 6 h and increased back to 10% at 14 h (Fig. 2B). In C31-infected larvae showing labelling, diffuse labelling was present in the gastrodermis (Fig. 2A). C31-infected larvae started with no rhodamine fluorescence at 2 h, increased to 80% at 6 h and remained high at 50% at 14 h (significantly higher than 0 h, $P < 0.001$), suggesting that caspase activity was not part of the initial response to infection but was present from 6 h on (Fig. 2B). Larval populations infected with cultB displayed patchy localized rhodamine signal in both epidermis and gastrodermis (Fig. 2A). Percentages of cultB-infected larvae displaying the rhodamine fluorescence were low at 2 and 6 h, 5% and 0%, respectively, but increased to 40% by 14 h (different from 2 and 6 h, $P < 0.001$) (Fig. 2B). Infection success of the different algal types was not quantified during confocal microscopy. However, qualitative observations of algal populations within larvae suggested that C31 was not as successful as C1f or cultB in uptake or retention in the gastrodermis over time (Fig. 2A and B).

Apoptosis inhibition increases infection success in larvae infected with heterologous C31 symbionts

To examine the role of apoptosis in recognition and specificity during symbiosis onset, larvae infected with C1f and C31 were incubated in the presence of the caspase inhibitor Z-Val-Ala-Asp-fluoromethylketone (ZVAD-fmk) and infection success was compared with controls. Larval infection success, as measured by per cent of larvae infected and algal density in larvae, repeated patterns shown many times previously (Weis *et al.*, 2001; Rodriguez-Lanetty *et al.*, 2004). The patterns were characterized by high infection success with C1f that persisted after 24 h compared with lower initial infection with heterologous strains and, in particular, C31.

In this study, the per cent of larvae infected with C1f and C1f density per larva were not significantly different between 0 h (93.5 ± 4.6% and 78 ± 5.2 respectively) and 24 h (92.7 ± 3.3% and 63 ± 4.7) (Fig. 3A and B). In contrast, per cent of larvae infected with C31 significantly decreased by 15% from 86 ± 2.9% at 0 h to 72.5 ± 6.7%

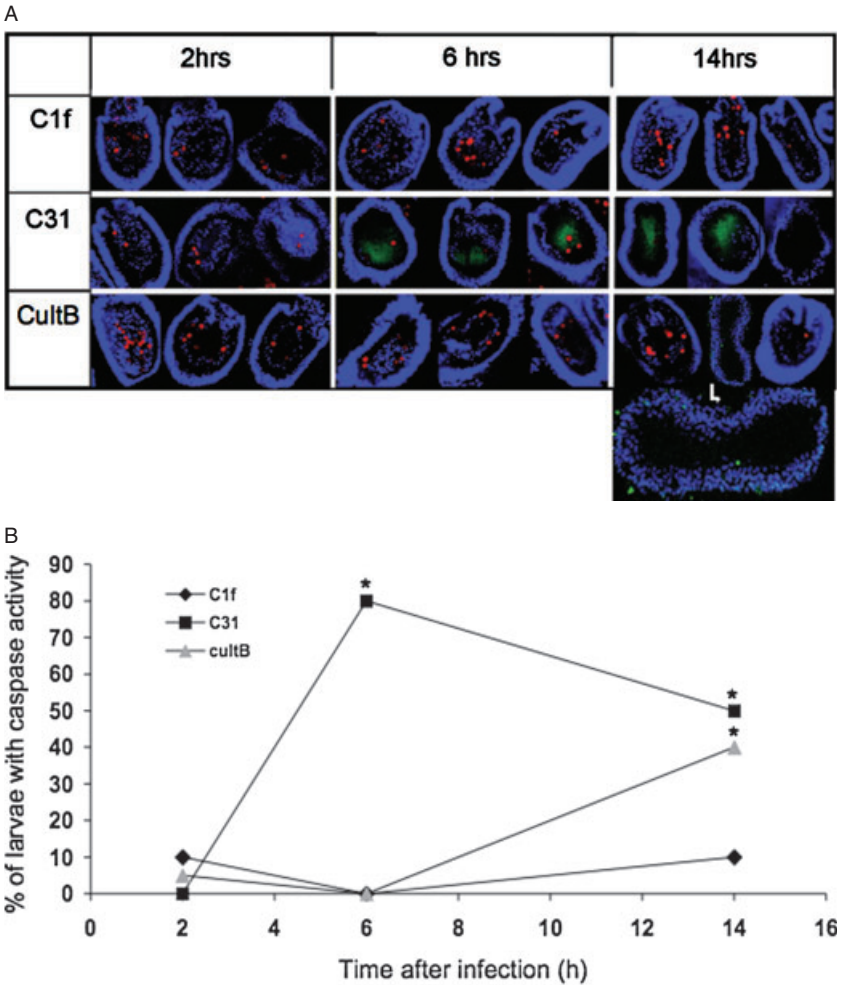

Fig. 2. Caspase activity over time following infection of larvae with different algal types.
A. Rhodamine 110 green fluorescence indicating caspase activity in several representative *F. scutaria* larvae infected with different algal types. Red fluorescence = algal chlorophyll; blue fluorescence = Hoescht 33342 DNA stain. Inset shows enlarged image of larvae infected with cultB at 14 h to illustrate cells with caspase activity.
B. The percentage of larvae displaying Rhodamine 110 fluorescence when infected with C1f, C31 or cultB algae over time. Binomial distribution test results of caspase signal comparison in larvae infected with the same algal type over time (*$P < 0.05$, $n = 20$ larvae for 0–2 h and $n = 10$ larvae for 6 and 14 h).

at 24 h (Fig. 3A; $P_{adj} < 0.05$). The algal density in C31-infected larvae decreased by 40% from 19 ± 7.8 at 0 h to 11.4 ± 3.5 algae per larvae at 24 h, although the decrease was not significant (Fig. 3B). The algal density in C31-infected larvae remained significantly lower than in C1f-infected larvae across all treatments (Fig. 3B; $P_{adj} < 0.05$).

In C1f-infected larvae, caspase inhibition had no effect on infection success; both per cent of larvae infected and algal density in larvae at 24 h were high, 92.2 ± 2.7% and 57.8 ± 1.7 algae per larva, respectively, and not different from controls (Fig. 3A and B). There was also no significant difference between untreated C1f-infected larvae and those in vehicle solvent controls (data not shown). These data suggest that apoptosis was not active during the first 24 h after infection in C1f-infected larvae. In contrast, caspase inhibition did affect infection success in C31-infected larvae. After 24 h, the percentage of C31-infected larvae incubated with the caspase inhibitor did not decrease as it did in controls (by 40%) but instead remained stable at 87.7 ± 4.1% and was not significantly different from controls at 0 h (Fig. 3A). These data suggest that inhibition of caspase prevented loss of C31 algae during the first 24 h after infection and therefore that caspase activity plays a role in post-phagocytic mechanisms of specificity. Density of algae in C31-infected larvae in caspase inhibitor did decrease less than controls at 24 h but the difference was not significant (Fig. 3B).

Infection success through time of heterologous clade B1 symbionts is comparable to homologous C1f symbionts and higher than heterologous C31 symbionts

To follow up on the unexpected qualitative observation that cultB algae persisted in host larvae after 24 h (Fig. 2A and B), we quantified infection success in larvae infected with freshly isolated clade B1 from *Aiptasia pulchella* and compared them with C1f-infected larvae. The percentage of larvae infected with B1 was not different from C1f-infected larvae at 0 h (90.7 ± 3.7%) or after 24 h (92.5 ± 4.2%; Fig. 3A). B1 density in larvae was lower than C1f density in larvae at 0 h (41.2 ± 3.8 algae per

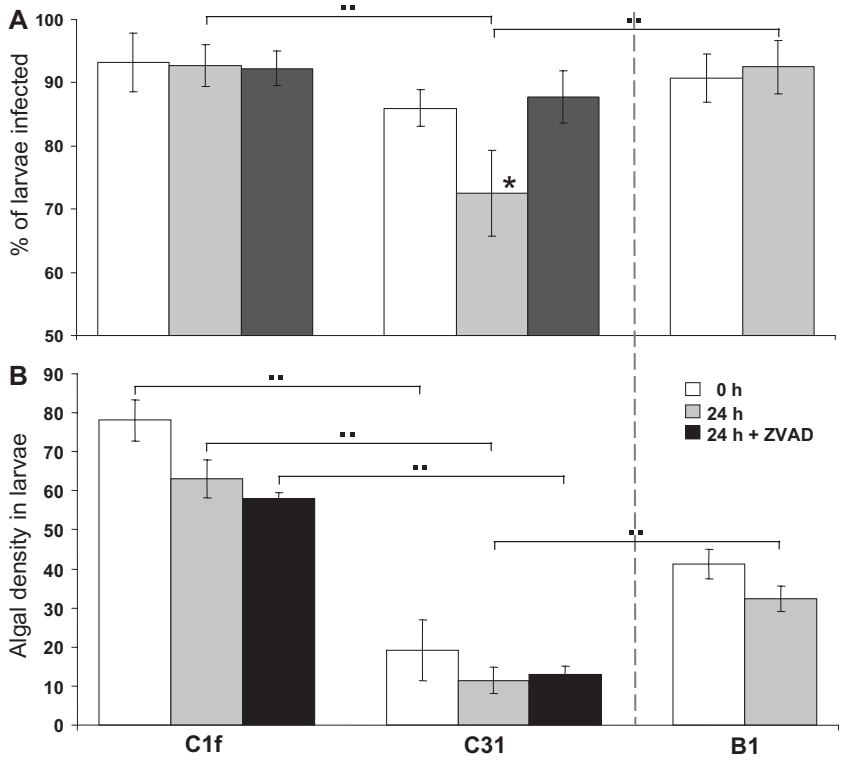

Fig. 3. A. The percentage of larvae infected with clade C1f, C31 or B1 algae over time, and following treatment with caspase inhibitor ZVAD-fmk over 24 h (within-clade infection multiple comparison, one-way ANOVA, $*P_{adj} < 0.05$ and between clades at the same time points, $■■P_{adj} < 0.05$).
B. The density of C1f, C31 and B1 algae in larvae, and following treatment with caspase inhibitor ZVAD-fmk over 24 h (Kruskall–Wallis multiple comparison between clades at the same time points, $■■P_{adj} < 0.05$).
Bars represent means ± SD [*n* (wells) = 4].

larva), and higher than densities in C31-infected larvae, but neither difference was significant ($P_{adj} > 0.05$) (see Fig. 3B). In all larvae, the density of dinoflagellates was reduced after 24 h. The density of B1 in larvae decreased at 24 h by 20% to 32.5 ± 3.3 algae per larva, but this was not significantly lower than B1-infected larvae at 0 h or C1f-infected larvae ($P_{adj} > 0.05$) at 24 h. However, the density of algae in B1-infected larvae remained significantly higher ($P_{adj} < 0.05$) than that in C31-infected larvae, indicating that B1 was retained more successfully than C31.

Discussion

The role of apoptosis in post-phagocytic recognition

Fungia scutaria larvae infected with different algal types displayed differing amounts of caspase activation that corresponded with infection success in the host larvae. Larvae infected with homologous C1f algae maintain stable populations of symbionts over time, whereas algal numbers in C31-infected larvae decline and disappear altogether within 21 h post infection (Rodriguez-Lanetty *et al*., 2004). The absence of caspase activity in C1f-infected larvae and contrasting presence of activity in C31-infected larvae (Fig. 2) suggest that host-cell apoptosis participates in a post-phagocytic winnowing process that recognizes and eliminates inappropriate symbionts from host tissues. A role for apoptosis in recognition is further supported by the persistence of C31 in larvae when caspase activity is inhibited (Fig. 3).

Activation of apoptosis in host cells infected with invading microbes is a common innate immune response in higher metazoans (Navarre and Zychlinsky, 2000). Likewise, multiple pathogens and parasites actively manipulate host apoptotic pathways to avoid immune detection and promote invasion (James and Green, 2004). There is increasing evidence that mutualistic host–microbe interactions regulate their partnerships with pathways homologous to those employed by parasitic or pathogenic interactions. Two studies have demonstrated that mutualistic symbionts can modulate host developmental programmes through manipulation of host-cell apoptosis (Foster and McFall-Ngai, 1998; Pannebakker *et al*., 2007). Our study appears to be the first from a cnidarian–dinoflagellate mutualism to demonstrate the involvement of apoptosis directly in recognition and the apparent modulation of this response by an appropriate symbiont. Although apoptosis has not been previously shown to function during onset of symbiosis, there is now increasing evidence that it plays a role in symbiosis dysfunction and breakdown during cnidarian bleaching (Dunn *et al*., 2004; 2007). Taken together, these data and those from this study suggest that apoptosis is a fundamental cellular process involved in the regulation of the symbiosis.

The small amounts of caspase activity detected in the epidermis and gastrodermis of both C1f- and B1-infected larvae (Fig. 2) could be due to programmed cell death as a part of host development and not related to onset of symbiosis. Caspase activity as a part of programmed cell death during animal development and morphogenesis is prevalent in metazoans. Other studies have shown evidence of cell degradation in embryos of *F. scutaria* (Marlow and Martindale, 2007) and programmed cell death in larvae of the hydroid *Hydractinia echinata* (Seipp *et al*., 2006).

The success of clade B1 in colonizing F. scutaria *larvae*

The success of heterologous clade B1 in colonizing *F. scutaria* larvae to a much stronger degree than another heterologous clade C31 was unexpected, given that B1 is phylogenetically more distant from homologous C1f than is C31. There are several possible scenarios that could act alone or in concert to explain this result. One explanation is that B1 is able to navigate the winnowing process in its early stages more successfully than C31 only to be weeded out during later winnowing events. Our data could suggest that the stage in the winnowing process at which an inappropriate symbiont is removed might vary between symbionts and/or host-symbiont combinations, i.e. C31 appears to be removed by pre-phagocytic (Rodriguez-Lanetty *et al*., 2006) and early post-phagocytic recognition processes (this study), akin to a host innate immune response, while B1 is not but is removed later instead. Other studies of a variety of cnidarian host taxa have shown that hosts early in ontogeny harbour a variety of symbiont types but ultimately end up with one or a very few types as adults (Coffroth *et al*., 2001; Belda-Baillie *et al*., 2002; Thornhill *et al*., 2006). Our experimentally generated snapshot from this study is taken early on in this process, likely before the final sorting has taken place.

Another explanation is that clade B1 is fully able to navigate the winnowing process but that other factors, such as low abundance due to limited ecological distribution, prevent its occurrence in *F. scutaria* in nature. Clade B1's phylogenetically ancestral position relative to clade C (Baker, 2003; Pochon *et al*., 2006) could mean that B1 retains generalist characteristics that allow it to survive winnowing. Clade B1, however, is not prevalent in scleractinians in the Indo-Pacific; it is limited in Hawaii to the anemone *A. pulchella* (LaJeunesse *et al*., 2004). Therefore it is possible that although B1 is fully capable of colonizing *F. scutaria*, its biogeographic, evolutionary and ecological history restricting its distribution in the Indo-Pacific prevents it from occurring in *F. scutaria* in nature.

Consistent with this argument is the correlation between C31's derived position within the C21 branch of clade C (LaJeunesse *et al*., 2004) and its failure to colonize *F. scutaria*. The resulting increased number of genomic changes from the ancestral clade could reflect changes away from qualities that enable a broader range of host colonization and therefore in the case of C31, an inability to colonize *F. scutaria*. This genetic distance suggests the derived C31 has co-evolved tight specificity with its host to survive the winnowing process only in its homologous host *Montipora capitata* and not in other heterologous hosts. This proposed relationship between phylogenetic placement and ability to navigate winnowing remains to be determined empirically.

A final scenario that could explain the apparent successful colonization by B1 in *F. scutaria* larvae but its absence from adults is that B1 is present in adults at very low abundance. Recent studies are finding previously undetected symbiont diversity at a very low abundance (Apprill and Gates, 2007; Mieog *et al*., 2007; Sampayo *et al*., 2007). In the case of *F. scutaria* then, B1 could be successfully entering hosts, along with C1f and persisting in adults at very low levels.

Conclusion

This study suggests that the host innate immune response and symbiont modulation of this process play a role in the onset of cnidarian–dinoflagellate symbiosis. Our work demonstrates the involvement of just one component of the innate immune response, apoptosis, and highlights the need for future studies to examine the influence of innate immunity on recognition, specificity and physiological variability between algal symbionts, cnidarian hosts and symbiosis stability.

Experimental procedures

Collection and maintenance of coral larvae, preparation of Symbiodinium *isolates and infection experiments*

Fungia scutaria spawns 2–3 days after the summer full moon (Krupp, 1983). Adult corals were collected from Kaneohe Bay, and placed in running seawater tables at the Hawaii Institute of Marine Biology (HIMB). Gametes were collected and fertilized to produce larvae free of symbionts (aposymbiotic) as previously described (Schwarz *et al*., 1999). Freshly isolated symbionts from the tissues of adult *F. scutaria* (homologous clade C1f), the coral *M. capitata* (heterologous clade C31) (Rodriguez-Lanetty *et al*., 2004) and the anemone *A. pulchella* (heterologous clade B1) (LaJeunesse *et al*., 2004) were prepared as previously described. Host tissue contamination was minimized by repeated rinses of algal pellets in accordance with Rodriguez-Lanetty and colleagues (2006). Furthermore, host contamination has previously been shown to play little or not role in the infection dynamics of *F. scutaria* larvae (Rodriguez-Lanetty *et al*., 2006). For some experiments, heterologous clade B1 symbionts were obtained from

a culture (cultB) originally isolated from the sea anemone *Aiptasia pallida* (Santos *et al*., 2002).

Infections of larvae with symbionts were performed after development of a mouth, 3–4 days after fertilization (Schwarz *et al*., 1999). Larvae were concentrated using a 60 μm mesh filter. Concentrated larvae were placed in six-well culture plates (Costar®, Corning) in 4 ml aliquots of filter sterilized seawater (FSW) or caspase inhibitors dissolved in FSW (see below). One millilitre of FSW containing 1.6×10^6 algae was added to each well. A few drops of the supernatant from homogenized *Artemia* sp. were added to the algal suspension, prior to inoculation, to stimulate larval feeding. Culture plates were placed in seawater tables to maintain an ambient seawater temperature. After 4 h, larvae were washed on a 60 μm mesh filter to remove any un-ingested algae and then returned to clean plates with FSW or inhibitor treatments.

In some experiments, infection success was quantified. One-millilitre aliquots of larvae were removed from treatments at 0 and 24 h post infection and processed according to Rodriguez-Lanetty and colleagues (2004). Aliquots were rinsed in FSW, fixed in 4% paraformaldehyde-PBS (48 μM NaH_2PO_4, 0.85 mM Na_2HPO_4, 0.12 M NaCl) and stored at 4°C until processing. Larvae were then rinsed in 1× PBS and visualized with a compound microscope. For each treatment replicate [n (wells) = 4], infection success was determined as per cent of larvae infected ($n = 100$) and average density of algae in larvae ($n = 100$).

Confocal microscopy of in vivo *caspase activity in infected larvae*

The fluoroprobe Rhodamine 110 aspartic acid caspase substrate (Molecular Probes) was used to detect host cell caspase activity and apoptosis following infection of larvae with different algal types. The fluorophore is activated upon substrate cleavage by a specific family of apoptotic proteases known as caspases and detectable using confocal microscopy. Larvae infected with homologous C1f, heterologous C31 or heterologous cultB were removed from culture and rinsed in FSW at 2, 6 and 12 h post infection. Following removal and rinsing, larvae were incubated in 100 μM Rhodamine 110 caspase substrate (dissolved in 0.2% DMSO) in FSW for 30 min. Hoescht 33342 DNA stain (200 μg ml^{-1}) was also added to visualize host nuclei. As a positive control for apoptosis, aposymbiotic larvae were incubated in the above-mentioned stains in the presence of 0.5% colchicine (Sigma Aldrich), a known inducer of apoptosis.

Following incubation, samples were rinsed and mounted in well slides with glycerol (50%) and observed under a Zeiss LSM 510 metahead confocal microscope. Samples were scanned with excitations of UV (405 nm) and green (498 and 543 nm) light, and emissions were collected at 450 nm to visualize stained host cells, 521 nm for caspase activity and 600 nm for the autofluorescence of algal symbionts. Ten larvae per infection type were randomly selected for scoring of caspase activity. This activity was scored as either absent or present, regardless of intensity.

Effect of caspase inhibition on infection success

To determine the role of apoptosis in post-phagocytic symbiont release from larvae, groups of larvae were incubated in a broad-scale non-reversible caspase inhibitor ZVAD-fmk (Enzyme Systems Products) during and following infection with homologous C1f and heterologous C31 symbionts. Infections with both algal types included control, vehicle control (0.2% DMSO in FSW) and experimental (25 μM ZVAD-fmk in 0.2% DMSO in FSW) treatments, with four replicates of each, for a total of 24 wells of larvae. Infection success was quantified in control and experimental treatments as described above after 24 h.

Statistical analysis

In the caspase activity assay, the amounts of larvae displaying Rhodamine 110 fluorescence over time were compared across treatments using a binomial probability distribution test using SSPS 9.0 software. In the experiment quantifying infections success, larval infection percentages were tested for normality and heteroscedasticity. Following an arcsine transformation, a one-way ANOVA was performed with *post hoc* Tukey pair-wise comparison. The density of algae in larvae data was tested for normality and heteroscedasticity, and found to be abnormally distributed. The density data were then tested using Kruskall–Wallis multiple test comparison with probability of $P \leq 0.05$ corrected for multiple tests using the Bonferroni procedure with Minitab (v15) software.

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References

Ainsworth, T., Kvennefors, E., Blackall, L., Fine, M., and Hoegh-Guldberg, O. (2007) Disease and cell death in white syndrome of Acroporid corals on the Great Barrier Reef. *Mar Biol* **151:** 19.

Amer, A.O., and Swanson, M.S. (2005) Autophagy is an immediate macrophage response to *Legionella pneumophila*. *Cell Microbiol* **7:** 765–778.

Apprill, A.M., and Gates, R.D. (2007) Recognizing diversity in coral symbiotic dinoflagellate communities. *Mol Ecol* **16:** 1127–1134.

Baker, A.C. (2003) Flexibility and specificity in coral-algal symbiosis: diversity, ecology, and biogeography of *Symbiodinium*. *Ann Rev Ecol Evol Syst* **34:** 661–689.

Baker, A.C., and Romanski, A.M. (2007) Multiple symbiotic partnerships are common in scleractinian corals, but not in octocorals: comment on Goulet (2006). *Mar Ecol Prog Ser* **335:** 237–242.

Belda-Baillie, C.A., Baillie, B.K., and Maruyama, T. (2002) Specificity of a model cnidarian–dinoflagellate symbiosis. *Biol Bull* **202:** 74–85.

Buddemeier, R.W., and Fautin, D.G. (1993) Coral bleaching as an adaptive mechanism. *Bioscience* **43:** 320–326.

Chen, M.-C., Cheng, Y.-M., Sung, P.-J., Kuo, C.-E., and Fang, L.-S. (2003) Molecular identification of Rab7 (ApRab7) in *Aiptasia pulchella* and its exclusion from phagosomes harboring zooxanthellae. *Biochem Biophys Res Comm* **308:** 586–595.

Chen, M.-C., Cheng, Y.-M., Hong, M.-C., and Fang, L.-S. (2004) Molecular cloning of Rab5 (ApRab5) in *Aiptasia pulchella* and its retention in phagosomes harboring live zooxanthellae. *Biochem Biophys Res Comm* **324:** 1024–1033.

Chen, M.-C., Hong, M.-C., Huang, Y.-S., Liu, M.-C., Cheng, Y.-M., and Fang, L.-S. (2005) ApRab11, a cnidarian homologue of the recycling regulatory protein Rab11, is involved in the establishment and maintenance of the *Aiptasia–Symbiodinium* endosymbiosis. *Biochem Biophys Res Comm* **338:** 1607–1616.

Coffroth, M.A., Santos, S.R., and Goulet, T.L. (2001) Early ontogenetic expression of specificity in a cnidarian–algal symbiosis. *Mar Ecol Prog Series* **222:** 85–96.

Cossart, P., and Sansonetti, P.J. (2004) Bacterial invasion: the paradigm of enteroinvasive pathogens. *Science* **304:** 242–248.

David, C.N., Schmidt, N., Schade, M., Pauly, B., Alexandrova, O., and Bottger, A. (2005) *Hydra* and the evolution of apoptosis. *Integr Comp Biol* **45:** 631–638.

Douglas, A.E. (1994) *Symbiotic Interactions*. Oxford, UK: Oxford Science Publications.

Douglas, A.E. (1998) Host benefit and the evolution of specialization in symbiosis. *Heredity* **81:** 599–603.

Dunn, S.R., Thomason, J.C., Le Tissier, M.D.A., and Bythell, J.C. (2004) Heat stress induces different forms of cell death in sea anemones and their endosymbiotic algae depending on temperature and duration. *Cell Death Differ* **11:** 7213–7222.

Dunn, S.R., Schnitzler, C.E., and Weis, V.M. (2007) Apoptosis and autophagy as mechanisms of dinoflagellate symbiont release during cnidarian bleaching: every which way you lose. *Proc R Soc Lond B* **274:** 3079–3085.

Fadlallah, T.H. (1983) Sexual reproduction, development and larval biology in scleractinian corals. A review. *Coral Reefs* **2:** 129–150.

Fan, T.-J., Han, L.-H., Cong, R.-S., and Liang, J. (2005) Caspase family proteases and apoptosis. *Acta Biochim Biophys Sinica* **37:** 719–727.

Fitt, W.K., and Trench, R.K. (1983) Endocytosis of the symbiotic dinoflagellate *Symbiodinium microadriaticum* Freudenthal by endodermal cells of the scyphistomae of *Cassiopeia xamachana* and resistance of the algae to host digestion. *J Cell Sci* **64:** 195–212.

Foster, J.S., and McFall-Ngai, M.J. (1998) Induction of apoptosis by cooperative bacteria in the morphogenesis of host epithelial tissues. *Dev Gen Evol* **208:** 295.

Furla, P., Allemand, D., Shick, J.M., Ferrier-Pages, C., Richier, S., Plantivaux, A., *et al.* (2005) The symbiotic anthozoan: a physiological chimera between alga and animal. *Integr Comp Biol* **45:** 595–604.

Goulet, T.L. (2006) Most corals may not change their symbionts. *Mar Ecol Prog Ser* **321:** 1–7.

Gruenberg, J., and van der Goot, F.G. (2006) Mechanisms of pathogen entry through the endosomal compartments. *Nat Rev Mol Cell Biol* **7:** 495.

Harrison, P.L., and Wallace, C.C. (1990) Reproduction, dispersal and recruitment of scleractinian corals. In *Ecosystems of the World: Coral Reefs*. Dubinsky, Z. (ed.). Amsterdam, the Netherlands: Elsevier, pp. 133–207.

Hirsch, A.M., Lum, M.R., and Downie, J.A. (2001) What makes the rhizobia-legume symbiosis so special? *Plant Physiol* **127:** 1484–1492.

James, E.R., and Green, D.R. (2004) Manipulation of apoptosis in the host–parasite interaction. *Trends Parasitol* **20:** 2004.

Koul, A., Herget, T., Klebl, B., and Ullrich, A. (2004) Interplay between mycobacteria and host signalling pathways. *Nat Rev Microbiol* **2:** 189.

Krupp, D.A. (1983) Sexual reproduction and early development of the solitary coral *Fungia scutaria* (Anthozoa: Scleractinia). *Coral Reefs* **2:** 159–164.

LaJeunesse, T.C., Thornhill, D.J., Cox, E.F., Stanton, F.G., Fitt, W.K., and Schmidt, G.W. (2004) High diversity and host specificity observed among symbiotic dinoflagellates in reef coral communities from Hawaii. *Coral Reefs* **23:** 596–603.

Lin, K.L., Wang, J.T., and Fang, L.S. (2000) Participation of glycoproteins on zooxanthellalcell walls in the establishment of a symbiotic relationship with the sea anemone *Aiptasia pulchella*. *Zool Stud* **39:** 172–178.

Little, A.F., Oppen, M.J.H.V., and Willis, B.L. (2004) Flexibility in algal endosymbioses shapes growth in reef corals. *Science* **304:** 1492–1494.

McFall-Ngai, M.J. (2000) Negotiations between animals and bacteria: the 'diplomacy' of the squid–vibrio symbiosis. *Comp Biochem Physiol A* **126:** 471–480.

Marlow, H.Q., and Martindale, M.Q. (2007) Embryonic development in two species of scleractinian coral embryos: *Symbiodinium* localization and mode of gastrulation. *Evol Dev* **9:** 355–367.

Mieog, J.C., van Oppen, M.J.H., Cantin, N.E., Stam, W.T., and Olsen, J.L. (2007) Real-time PCR reveals a high incidence of *Symbiodinium* clade D at low levels in four scleractinian corals across the Great Barrier Reef: implications for symbiont shuffling. *Coral Reefs* **26:** 449–457.

Navarre, W.W., and Zychlinsky, A. (2000) Pathogen-induced apoptosis of macrophages: a common end for different pathogenic strategies (Microreview). *Cell Microbiol* **2:** 265–273.

Nyholm, S.V., and McFall-Ngai, M.J. (2004) The winnowing: establishing the squid–*Vibrio* symbiosis. *Nat Rev Microbiol* **2:** 632–642.

Pannebakker, B.A., Loppin, B., Elemans, C.P.H., Humblot, L., and Vavre, F. (2007) Parasitic inhibition of cell death facilitates symbiosis. *Proc Natl Acad Sci USA* **104:** 213–215.

Pochon, X., Montoya-Burgos, J.I., Stadelmann, B., and Pawlowski, J. (2006) Molecular phylogeny, evolutionary rates, and divergence timing of the symbiotic dinoflagellate genus *Symbiodinium*. *Mol Phylogenet Evol* **38:** 20–30.

Rodriguez-Lanetty, M., Krupp, D.A., and Weis, V.M. (2004) Distinct ITS types of *Symbiodinium* in clade C correlate with cnidarian/dinoflagellate specificity during onset of symbiosis. *Mar Ecol Prog Ser* **275:** 97–102.

Rodriguez-Lanetty, M., Wood-Charlson, E.M., Hollingsworth, L.L., Krupp, D.A., and Weis, V.M. (2006) Temporal and spatial infection dynamics indicate recognition events in the early hours of a dinoflagellate/coral symbiosis. *Mar Biol* **149:** 713–719.

Sacks, D., and Sher, A. (2002) Evasion of innate immunity by parasitic protozoa. *Nat Immunol* **3:** 1041–1047.

Sampayo, E.M., Francescinis, L., Hoegh-Guldberg, O., and Dove, S. (2007) Niche partitioning of closely related symbiotic dinoflagellates. *Mol Ecol* **16:** 3721–3733.

Santos, S.R., Taylor, D.J., Kinzie, R.A., III, Hidaka, M., Sakai, K., and Coffroth, M.A. (2002) Molecular phylogeny of symbiotic dinoflagellates inferred from partial chloroplast large subunit (23S)-rDNA sequences. *Mol Phylogenet Evol* **23:** 97.

Schwarz, J.A., Krupp, D.A., and Weis, V.M. (1999) Late larval development and onset of symbiosis in the scleractinian coral *Fungia scutaria*. *Biol Bull* **196:** 70–79.

Seipp, S., Wittig, K., Stiening, B., Bottger, A., and Leitz, T. (2006) Metamorphosis of *Hydractinia echinata* (Cnidaria) is caspase-dependent. *Int J Dev Biol* **50:** 63–70.

Thornhill, D.J., Daniel, M.W., LaJeunesse, T.C., Schmidt, G.W., and Fitt, W.K. (2006) Natural infections of aposymbiotic *Cassiopea xamachana* scyphistomae from environmental pools of *Symbiodinium*. *J Exp Mar Biol Ecol* **338:** 50–56.

Vergne, I., Chua, J., and Deretic, V. (2003) *Mycobacterium tuberculosis* phagosome maturation arrest: selective targeting of PI3P-dependent membrane trafficking. *Traffic* **4:** 600–606.

Weis, V.M., Reynolds, W.S., deBoer, M.D., and Krupp, D.A. (2001) Host-symbiont specificity during onset of symbiosis between the dinoflagellate *Symbiodinium* spp. and the planula larvae of the scleractinian coral *Fungia scutaria*. *Coral Reefs* **20:** 301–308.

Wood-Charlson, E.M., Hollingsworth, L.H., Krupp, D.A., and Weis, V.M. (2006) Lectin/glycan interactions play a role in recognition in a coral/dinoflagellate symbiosis. *Cell Micro* **8:** 1985–1994.

