Immunity through early development of coral larvae

C. V. Palmer [a] *, E. Graham [a], A. H. Baird [ab]

[a] School of Marine and Tropical Biology, James Cook University, Townsville, QLD, 4811, Australia.

[b] ARC Centre of Excellence for Coral Reef Studies, Townsville, QLD. 4811.

Abstract

As a determinant of survival, immunity is likely to be significant in enabling coral larvae to disperse and successfully recruit, however, whether reef-building coral larvae have immune defenses is unknown. We investigated the potential presence and variation in immunity in the lecithotrophic larvae of *Acropora tenuis* through larval development. Enzymes indicative of tyrosinase and laccase-type melanin-synthesis were quantified, and the concentration of three coral fluorescent proteins measured over six developmental stages; egg, embryo, motile planula, planula post-exposure to crustose coralline algae (CCA; settlement cue), settled, settled post-exposure to *Symbiodinium* (endosymbiont). Both types of melanin-synthesis pathways and the three fluorescent proteins were present in *A. tenuis* throughout development. Laccase-type activity and red fluorescence increased following exposure of planula to CCA, whereas tyrosinase-type activity and cyan fluorescence increased following settlement. No change was detected in the measured parameters following exposure to *Symbiodinium*. This study is the first to document the coral larval immune responses and suggests the melanin-synthesis pathways have disparate roles- the laccase-type potentially non-immunological and the tyrosinase-type in cytotoxic defense. Our results indicate that corals have the potential to resist infection from the earliest life history phase.

Key words: Prophenoloxidase, larvae, coral, fluorescent protein, melanin, laccase

1. Introduction

Lecithotrophic larvae represent a crucial life history stage of broadcast spawning corals, which significantly contribute to the formation and maintenance of tropical coral reefs. In a period of global reef decline, coral larvae are not only responsible for maintaining population genetic structure and diversity, but also for potentially reestablishing degraded reefs and expanding geographic ranges; all of which are highly dependent on larval dispersal potential (Connolly and Baird, 2010; Graham et al., 2008). Successful dispersal requires propagules to run the gauntlet of threats in the open ocean, such as predation (Thorson, 1950), environmental stress (Pechenk, 1987) and disease (Rumrill, 1990) on depleting energy reserves (Figueiredo et al., 2012; Strathmann, 1985). While the sheer numbers released during mass spawning events arguably reduces the risk of predation (Harrison et al., 1984; Oliver et al., 1988), and the effects of environmental conditions on coral larval development are well understood e.g. (Negri et al., 2007; Schnitzler et al., 2012), the potential for reef-building coral larvae to resist disease remains unexplored.

All organisms possess immune defenses that increase fitness by promoting survival, through disease resistance and the maintenance of tissue integrity. While the presence of immune responses within reef-building coral larvae has not been investigated, their biology suggests that disease-resisting defenses would be adaptive. For example, the lecithotrophic larvae of many corals are released directly into a microbe-rich water column (Sweet et al., 2011), at a time of year consistent with high coral disease prevalence (Sato et al., 2009), and where they may reside for up to several months (Graham et al., 2008; Harrison and Wallace, 1990). Exposure to potentially pathogenic microbes is also likely to occur when, upon detecting a settlement cue such as crustose coralline algae (CCA) (Heyward and Negri, 1999), coral larvae search the substrate for a suitable place to settle (Harrison & Wallace 1990). Furthermore, settlement is the point at which larvae most likely come into contact with the obligate algae endosymbiont (*Symbiodinium* spp.) (Adams et al., 2009; Cumbo et al., In

review). Although the mechanisms of symbiosis onset are not elucidated, they likely involve aspects of immunity (Palmer and Traylor-Knowles, In review a; Weis and Allemand, 2009). Coral larval survival, recruitment and the establishment of obligate symbiosis are therefore likely to require a functional immune response. However, due to the energetic costs of immune maintenance and up-regulation (Sadd and Schmid-Hempel, 2009), coupled with a finite amount of maternally provided, endogenous reserves in lecithotrophic larvae, defenses are likely to be tightly controlled and/or traded-off against longevity.

Coral immunology is an emerging field and, although putative immune genes have been identified within coral larval transcriptomes and a genome (Miller et al., 2007; Shinzato et al., 2011), functional studies of coral larval immunity are lacking (Palmer and Traylor-Knowles, In review a). However, functional studies of adult hard corals indicate the presence of many key components of invertebrate innate immunity, including wound healing processes and cells (Palmer et al., 2011b) and a coagulation enzyme (Palmer and Bythell, In review; Palmer et al., In press). Two melanin-synthesis pathways (tyrosinase type and laccase type) have also been identified within a suite of coral species (Mydlarz and Palmer, 2011; Palmer et al., In press; Palmer et al., 2008). Melanin synthesis is considered central to invertebrate immune responses, providing antimicrobial defense, *via* the release of cytotoxic pathway intermediates, such as oxygen radicals (Nappi and Christensen, 2005), and structural support and barrier formation (encapsulation) by the melanin (Cerenius et al., 2010). However, the tyrosinase-type melanin-synthesis pathway is more cytotoxic than the laccase-type, which functions in non-immunological cuticular tanning in arthropods (Arakane et al., 2005) and is hypothesized to assist in rapidly re-establishing tissue integrity during wound healing in corals (Palmer and Bythell, In review).

In addition to the classic innate immune components identified within corals, coral fluorescent proteins may also be involved in immunity. Coral fluorescent proteins are responsible for the bright colors of adult colonies, and occur in eggs (Roth et al., 2007), larvae

(Kenkel et al., 2011b) and recruits (Baird et al., 2006). Although these iconic proteins are responsible for up to 14% of an adult coral's total protein concentration (Oswald et al., 2007), their biological functions remain unresolved. Multiple hypotheses of coral fluorescent protein function currently exist, including an involvement in symbiosis maintenance (Salih et al., 2000) and establishment (Hollingsworth et al., 2005), calcification (D'Angelo et al., 2008) and radical quenching (Bou-Abdallah et al., 2006). While measurement of fluorescent protein gene expression is frequently used as a stress response indicator in coral studies e.g. (Kenkel et al., 2011a), functional studies are limited. Fluorescent proteins are, however, found in high abundance in compromised (Palmer et al., 2008), injured (Palmer and Bythell, In review) and infected (Palmer et al., 2009a) coral tissue. Fluorescent proteins also efficiently scavenge hydrogen peroxide (Palmer et al., 2009b), a highly cytotoxic by-product of melanin synthesis (Nappi and Christensen, 2005).

Here we use *Acropora tenuis* to investigate the presence and potential variation in immunity and fluorescence from the egg, through larval development, induction of settlement, metamorphosis and the introduction of symbionts to settled recruits.

2. Methods

2.1. Larval culturing and sampling

Prior to the anticipated mass-spawning event in October 2010, 6 fecund colonies of *Acropora tenuis* were collected from Magnetic Island, Queensland, Australia, and transferred to outdoor aquaria at James Cook University. Spawned gametes were collected from all colonies on October 28th, combined to maximize fertilization success, and placed into 10L indoor aquaria containing 0.2 micron filtered seawater (FSW). At three days post-fertilization larvae commenced swimming and were actively exploring the aquaria after 4 days. At day 6 post-fertilization, six pieces of crustose coralline algae (CCA), ~ 5mm in length, were introduced into the aquaria to induce larval settlement. *Symbiodinium* sp. freshly isolated

from adult *A. tenuis* colonies (following Yakovleva et al., 2009) were introduced into the aquaria at densities of 2.5 x 10^6 ml^{-1} on day 12 post-fertilization.

Three samples of 200 individuals were taken of unfertilized eggs, then at 12h, 24h, 36h post-fertilization and every 24h thereafter until 6d. On day 12 post-fertilization, three samples of 200 swimming larvae were taken, as well as three aliquots of 50 settled larvae. Three samples of 50 recruits were taken 12 and 48h post *Symbiodinium* inoculation (days 13 and 14). All samples, three for each of the 12 sampling points, were immediately snap-frozen in liquid nitrogen and stored at -80°C.

2.2. Sample processing and biochemical assays

Samples were freeze-dried overnight, then immediately rehydrated with 1ml extraction buffer (50 $mmol.l^{-1}$ phosphate buffer, pH 7.8 with 0.05 $mmol.l^{-1}$ dithiothreitol) on ice. Samples were then vortexed for 30 s with half a spatula of glass beads and left on ice for 5 min to allow for protein dissolution. Samples were then centrifuged for 3 min at x 1900*g*, and, avoiding the mucus layer, the supernatant was carefully removed and stored at -80°C.

Activities of the two prophenoloxidases (PPOs; tyrosine-type and laccase-type) were determined by adding 40 µl of phosphate buffer (50 $mmol.l^{-1}$ pH 7.8) and 25 µl of trypsin (0.1 mg ml^{-1} in deionized water) to 20 µl of each larval sample, in triplicate, in a 96-well microtitre plate. Plates were left to incubate at room temperature for 20 min, then 30 µl of a 10 $mmol.l^{-1}$ solution of either dopamine hydrochloride (Sigma-Aldrich H8502; for tyrosinase-type PPO activity) or hydroquinone (Sigma H9003; for laccase-type PPO activity) was added (Palmer et al. In press). The absorbance at 490 nm was recorded every 5 min for 25 min using a Spectramax M2 (Molecular Devices) spectrophotometer, and the change in absorbance for the linear portion of the reaction curve was used to determine PPO activities.

Fluorescence was measured as per Palmer et al. (2009b). Briefly, aliquots of 20 µl of each sample were added to triplicate wells of a black/clear 384-well microtitre plate, with

parallel aliquots of extraction buffer to control for potential background fluorescence. Each well was excited at 280 nm and the emission spectra were measured in 5 nm increments from 460 to 600 nm. The different colored fluorescence was calculated by summing the relative fluorescence units (RFUs) of the relevant wavelengths; cyan = 460–500 nm, green = 505–555 nm and red = 560-600 nm and dividing by the number of larva within the sample.

2.3. Statistics

Samples from the 12 time points were pooled into six larval developmental stages including egg (time 0), embryo (12h to 3 days post-fertilization), planula (days 4 and 5 post-fertilization), planula + CCA (day 6 and time point 1 on day 12), settled (time point 2 day 12) and recruits + *Symbiodinium* (days 13 and 14). One-way ANOVAs with Tukey's HSD *post-hoc* tests were used to compare response variables among larval developmental stage where log-transformed data met the assumption of normality and homogeneity of variance (tyrosinase-type *o*-diphenoloxidase activity and green fluorescence). Laccase-type PPO activity (*p*-diphenoloxidase), cyan and red fluorescence did not satisfy parametric assumptions, so a Kruskal-Wallis rank test was used to compare activity through development. Relationships among the response variables were investigated using Pearson's correlation coefficient using log-transformed data.

3. Results and Discussion

Acropora tenuis larvae have a finite energetic resource, with total protein concentration depleting throughout development ($K\text{-}W_{(5)} = 25.59$, $P<0.001$; Fig. S1). However, costly activities of immune-related enzymes (Sadd and Schmid-Hempel, 2009) and fluorescence increased during development, suggesting they served key roles.

3.1. Melanin-synthesis pathways

Melanin-synthesis pathways are fundamental invertebrate immune defenses (Cerenius et al., 2010), and although widely documented within adult corals (e.g. Mydlarz and Palmer, 2011; Palmer et al., In press; Palmer et al., 2008), this study is the first documentation within the early developmental stages of a coral. Laccase-type activity increased within the planula with CCA stage, indicating a response to the addition of the CCA as a settlement cue ($K\text{-}W_{(5)} = 17.5$, $P = 0.004$; Fig. 1). This response may be non-immunological, as for arthropods, which use laccase for cuticle tanning (Sugumaran, 2002). As cuticle tanning is an unlikely function within corals, this laccase-type response may be related to the induced transition from one developmental stage to another, as documented for arthropod larvae (Benesova et al., 2009). Settlement responses to illumination cues within ascidian (Jiang et al., 2005; Sato and Yamamoto, 2001) and sponge larvae (Leys and Degnan, 2001) utilise pigment cells that contain melanin granules. Concomitantly, photosensory perception in all animals requires the presence of a photoreceptor coupled with a dark pigment, which is also documented to be melanin, as a pigment ring, within jellyfish (Cnidaria) (Kozmik et al., 2008). Therefore, as laccase-type activity increases just prior to settlement, it may have a photosensory function within coral planulae. In support of this hypothesis, a melanopsin-like gene, of a non-visual photoreceptor, was recently identified within *Acropora millepora* (Vize, 2009) with its homologues located within melanin-containing cells of vertebrates and invertebrates (Provencio et al., 1998). However, although melanin-containing cells appear ubiquitous among adult anthozoans (Palmer et al., 2010; Palmer et al., 2008) and coral larvae demonstrate spectral discrimination (Mason et al., 2011), the potential role of melanin-containing cells in phototaxis during larval development has yet to be tested. Similarly, the current study does not rule out the previously proposed roles of laccase-type melanin synthesis for structural support and barrier formation (Palmer and Bythell, In review; Palmer et al., In press).

The tyrosinase-type melanin-synthesis pathway, which, due to its cytotoxicity (Nappi and Christensen, 2005; Sugumaran, 2002) likely has a strict immunological function (Cerenius et al., 2010), increased upon settlement, when larvae were in direct contact with the substrate ($F_{(5, 35)} = 23.97$, $P<0.001$; Fig. 1). This increase is consistent with the up-regulation of a suite of immunity genes during ascidian metamorphosis (Roberts et al., 2007) and a role of phenoloxidases in mediating gastropod metamorphosis (Pires et al., 2000), as well as with a function of tyrosinase-type melanin synthesis in disease resistance e.g. (Butt and Raftos, 2008; Wang et al., 2012), including within corals (Palmer et al., 2011a; Palmer et al., 2011c). Therefore, a tyrosinase-type response upon settlement coincides with the likely necessity of clearing and defending against potentially pathogenic organisms on the selected settlement site, and likely promotes post-settlement survival. Although bacterial densities were not measured, the increased activities of this key immune component suggest that effective defenses are vital for successful larval settlement.

Melanin-synthesis pathways did not respond to the presence of the obligate symbiont, *Symbiodinium* sp. post-settlement (Fig. 1). However, as the activities of the melanin-synthesis pathways were both at their highest during this phase, it might indicate an energetic constraint on the use of costly immune responses. However, non-maternally derived obligate symbioses establishment and maintenance in many organisms occurs through parasitic behavior of the endosymbiont (Schmid-Hempel, 2008), and can include immunosuppression of melanin-synthesis pathways e.g. (Tian et al., 2010). The lack of change in melanin-synthesis activities upon exposure to endosymbionts (Fig. 1) is consistent with the limited differential gene expression observed in studies of coral-*Symbiodinium* symbiosis establishment (Schnitzler and Weis, 2010; Voolstra et al., 2009). This potentially suggests employment of avoidance mechanisms by the symbionts to circumvent the coral immune system. However, symbiosis establishment was not confirmed within every sampled recruit in the current study, and the

physiological mechanisms of both the coral host and *Symbiodinium* endosymbiont involved in symbiosis onset remain to be established.

3.2. Fluorescence

Consistent with previous documentation of fluorescence within coral larvae (Baird et al., 2006; Hollingsworth et al., 2006; Kenkel et al., 2011b), low concentrations of fluorescence were detected within the eggs of *A. tenuis* through to the planula stage (Fig. 2). Total fluorescence increased significantly post-settlement ($F_{(5, 35)} = 31.97$, $P<0.001$; Fig. S2) and was driven by cyan fluorescence (K-W $_{(5)} = 20.17$, $P = 0.001$; Fig. 2). However, red fluorescence increased 6-fold pre-settlement in the presence of CCA (K-W $_{(5)} = 23.89$, $P < 0.001$; Fig. 2). This suggests that different colored fluorescent proteins have different functions (Field et al. 2006) during coral larval development.

Concomitant with their potential different functions, cyan fluorescence correlated most strongly with tyrosinase-type activity ($C= 0.82$, $P<0.001$; Fig. S3) whereas red fluorescence correlated most strongly with laccase-type melanin synthesis ($C = 0.63$, $P<0.001$; Fig. S3). These correlations suggest that melanin-synthesis pathways and fluorescent proteins are either responding to the same activators, are independently involved in cue detection and settlement, or that their functions are intrinsically linked. Co-variation in coral melanin-synthesis pathways and fluorescence is common (Palmer and Bythell, In review; Palmer et al., 2010; Palmer et al., 2011c; Palmer et al., 2008; Palmer et al., 2009a), and functional links between them have been hypothesized, as FPs are able to scavenge the cytotoxic by-products of melanin-synthesis (Palmer et al., 2009b). However, co-variation of red fluorescence with laccase-type activity may indicate a sensory function (Bogdanov et al., 2009), in addition to antioxidant capabilities. Confirming co-localization of laccase activity and red fluorescence would help to test this hypothesis.

Green fluorescence within larval corals has been proposed as a *Symbiodinium* spp. attractant (Hollingsworth et al., 2005) and was supported by the "greening" of *A. millepora* larvae (both planulae and recruits) post-induction with CCA (Kenkel et al., 2011b). However, in the current study, there was no change in the relatively low levels of green fluorescence throughout larval development ($F_{(5, 35)} = 0.82$, $P = 0.55$; Fig. 2). This suggests that *A. tenuis* larvae do not use fluorescence to attract symbionts, or that they use a different fluorescence, most likely cyan. Fluorescence did not increase following the addition of *Symbiodinium* (Fig. 2 and Fig. S2), indicating that if fluorescence plays a role in symbiosis establishment then the presence of the symbiont does not trigger it.

Overall, we document for the first time the presence of classic invertebrate immune mechanisms within coral larvae, indicating disease resistance capabilities that likely increase larval survivorship. The differential regulation of the two melanin-synthesis pathways suggests that they have multiple functions during development, which is also the case for the various fluorescent proteins. Future work should aim to explore the potential links between melanin synthesis and fluorescent proteins.

Acknowledgements

The authors thank Chia-miin Chua for adult coral collection and Line Bay for isolating the *Symbiodinium*. Research was funded by the Australian Research Council.

References

Adams, L.M., Cumbo, V.R., Takabayashi, M., 2009. Exposure to sediment enhances primary acquisition of Symbiodinium by asymbiotic coral larvae. Mar. Ecol. Prog. Ser. 377, 149-156.
Arakane, Y., Muthukrishnan, S., Beeman, R.W., Kanost, M.R., Kramer, K.J., 2005. Laccase 2 is the phenoloxidase gene required for beetle cuticle tanning. PNAS 102, 11337-11342.
Baird, A.H., Salih, A., Trevor-Jones, A., 2006. Fluorescence census techniques for the early detection of coral recruits. Coral Reefs 25, 73-76.
Benesova, J., Dobes, P., Hyrsl, P., 2009. Developmental changes in phenol-oxidizing activity in the greater wax moth *Galleria mellonella*. Bulletin of Insectology 62, 237-243.

Bogdanov, A.M., Mishin, A.S., Yampolsky, I.V., Belousov, V.V., Chudakov, D.M., Subach, F.V., Verkhusha, V.V., Lukyanov, S., Lukyanov, K.A., 2009. Green fluorescent proteins are light-induced electron donors. Nature Chemical Biology 5, 459-461.
Bou-Abdallah, F., D. N. Chasteen, Lesser., M., 2006. Quenching of superoxide radicals by green fluorescent protein. . Biochim. Biophys. Acta 1760, 1690-1695.
Butt, D., Raftos, D., 2008. Phenoloxidase-associated cellular defence in the Sydney rock oyster, Saccostrea glomerata, provides resistance against QX disease infections. Developmental and Comparative Immunology 32, 299-306.
Cerenius, L., Kawabata, S.I., Lee, B.L., Nonaka, M., Soderhall, K., 2010. Proteolytic cascades and their involvement in invertebrate immunity. Trends in Biochemical Sciences 35, 575-583.
Connolly, S.R., Baird, A.H., 2010. Estimating dispersal potential for marine larvae: dynamic models applied to scleractinian corals. Ecology 91, 3572-3583.
Cumbo, V.R., Baird, A.H., van Oppen, M.J.H., In review. The promiscuous larvae: flexibility in the establishment of symbiosis in corals. Coral Reefs.
D'Angelo, C., Denzel, A., Vogt, A., Matz, M.V., Oswald, F., Salih, A., Nienhaus, G.U., Wiedenmann, J., 2008. Blue light regulation of host pigment in reef-building corals. Marine Ecology-Progress Series 364, 97-106.
Field, S.F., Bulina, M.Y., Kelmanson, I.V., Bielawski, J.P., Matz, M.V., 2006. Adaptive evolution of multicolored fluorescent proteins in reef-building corals. Journal of Molecular Evolution 62, 332-U315.
Figueiredo, J., Baird, A.H., Cohen, M.F., Flot, J.F., Kamiki, T., Meziane, T., Tsuchiya, M., Yamasaki, H., 2012. Ontogenetic change in the lipid and fatty acid composition of scleractinian coral larvae. Coral Reefs 31, 613-619.
Graham, E.M., Baird, A.H., Connolly, S.R., 2008. Survival dynamics of scleractinian coral larvae and implications for dispersal. Coral Reefs 27, 529-539.
Harrison, P.L., Babcock, R.C., Bull, G.D., Oliver, J.K., Wallace, C.C., Willis, B.L., 1984. Mass spawning in tropical reef corals. Science 223, 1186-1189.
Harrison, P.L., Wallace, C.C., 1990. Reproduction, dispersal and recruitment of scleractinian corals, in: Dubinsky, Z. (Ed.), Ecosystems of the world 25: Coral Reefs. Elsevier, New York, pp. 133-207.
Heyward, A.J., Negri, A.P., 1999. Natural inducers for coral larval metamorphosis. Coral Reefs 18, 273-279.
Hollingsworth, L.L., Kinzie, R.A., Lewis, T.D., Krupp, D.A., Leong, J.A.C., 2005. Phototaxis of motile zooxanthellae to green light may facilitate symbiont capture by coral larvae. Coral Reefs 24, 523-523.
Hollingsworth, L.L., Lewis, T.D., Krupp, D.A., Leong, J.A.C., 2006. Early onset and expression of green fluorescent proteins (GFPs) in the larvae of the mushroom coral, Fungia scutaria. Proc. 10th Int Coral Reef Symp., 99-105.
Jiang, D., Tresser, J.W., Horie, T., Tsuda, M., Smith, W.C., 2005. Pigmentation in the sensory organs of the ascidian larva is essential for normal behavior. Journal of Experimental Biology 208, 433-438.
Kenkel, C., Aglyamova, G., Alamaru, A., Bhagooli, R., Capper, R., Cunning, J.R., deVillers, A., Haslun, J.A., Hédouin, L., Keshavmurthy, S., Kuehl, K.A., Mahmoud, H., Mcginty, E., Montoya-Maya, P.H., Muñoz, J.A.S., Palmer, C.V., Pantile, R., Schils, T., Silverstein, R.N., Squiers, L.B., Tang, P.C., Goulet, T.L., Matz, M.V., 2011a. Diagnostic gene expression markers of acute heat-light stress in *Porites* spp. PloS One 10.1371/journal.pone.0026914.
Kenkel, C.D., Traylor, M.R., Wiedenmann, J., Salih, A., Matz, M.V., 2011b. Fluorescence of coral larvae predicts their settlement response to crustose coralline algae and reflects stress. Proceedings of the Royal Society B-Biological Sciences 278, 2691-2697.
Kozmik, Z., Ruzickova, J., Jonasova, K., Matsumoto, Y., Vopalensky, P., Kozmikova, I., Strnad, H., Kawamura, S., Piatigorsky, J., Paces, V., Vlcek, C., 2008. Assembly of the

cnidarian camera-type eye from vertebrate-like components. Proceedings of the National Academy of Sciences of the United States of America 105, 8989-8993.
Leys, S.P., Degnan, B.M., 2001. Cytological basis of photoresponsive behavior in a sponge larva. Biological Bulletin 201, 323-338.
Mason, B., Beard, M., Miller, M.W., 2011. Coral larvae settle at a higher frequency on red surfaces. Coral Reefs 30, 667-676.
Miller, D.J., Hemmrich, G., Ball, E.E., Hayward, D.C., Khalturin, K., Funayama, N., Agata, K., Bosch, T.C.G., 2007. The innate immune repertoire in Cnidaria - ancestral complexity and stochastic gene loss. Genome Biology 8, R59.
Mydlarz, L.D., Palmer, C.V., 2011. The presence of multiple phenoloxidases in Caribbean reef-building corals. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology 159, 372-378.
Nappi, A.J., Christensen, B.M., 2005. Melanogenesis and associated cytotoxic reactions: Applications to insect innate immunity. Insect Biochemistry and Molecular Biology 35, 443-459.
Negri, A.P., Marshall, P.A., Heyward, A.J., 2007. Differing effects of thermal stress on coral fertilization and early embryogenesis in four Indo Pacific species. Coral Reefs 26, 759-763.
Oliver, J.K., Babcock, R.C., Harrison, P.L., Willis, B.L., 1988. Geographic extent of mass coral spawning: Clues to ultimate causal factors. Proc. 6th Int Coral Reef Symp. 2, 803-810.
Oswald, F., Schmitt, F., Leutenegger, A., Ivanchenko, S., D'Angelo, C., Salih, A., Maslakova, S., Bulina, M., Schirmbeck, R., Nienhaus, G.U., Matz, M.V., Wiedenmann, J., 2007. Contributions of host and symbiont pigments to the coloration of reef corals. Febs Journal 274, 1102-1109.
Palmer, C.V., Bythell, J.C., In review. Functional differences in immunity among coral colour morphs. Functional Ecology.
Palmer, C.V., Bythell, J.C., Willis, B.L., 2010. Immunity parameters of reef corals underpin bleaching and disease susceptibility. The Federation of American Societies for Experimental Biology 24, 1935-1946.
Palmer, C.V., Bythell, J.C., Willis, B.L., 2011a. A comparative study of phenoloxidase activity in diseased and bleached colonies of the coral *Acropora millepora*. Dev. Comp. Immunol.
Palmer, C.V., Bythell, J.C., Willis, B.L., In press. Enzyme activity demonstrates multiple pathways of innate immunity in Indo-Pacific corals. Proceedings of the Royal Society B-Biological Sciences.
Palmer, C.V., McGinty, E.S., Cummings, D., Bartels, E., Mydlarz, L.D., 2011c. Patterns of coral ecological immunology: Variation in the responses of Caribbean corals to elevated temperature and a pathogen elicitor. Journal of Experimental Biology.
Palmer, C.V., Modi, C.K., Mydlarz, L.D., 2009b. Coral fluorescent proteins as antioxidants. PLoS One 4, e7298.
Palmer, C.V., Mydlarz, L.D., Willis, B.L., 2008. Evidence of an inflammatory-like response in non-normally pigmented tissues of two scleractinian corals. Proc. R. Soc. Lond. B. Biol. Sci. 275, 2687-2693.
Palmer, C.V., Roth, M.S., Gates, R.D., 2009a. Red fluorescent protein responsible for pigmentation in trematode-infected *Porites compressa* tissues. Biological Bulletin 215, 68-74.
Palmer, C.V., Traylor-Knowles, N., In review a. Towards an integrated network of coral immune mechanisms. Proceedings of the Royal Society of London Series B-Biological Sciences.
Palmer, C.V., Traylor-Knowles, N.G., Willis, B.L., Bythell, J.C., 2011b. Corals use similar immune cells and wound-healing processes as those of higher organisms. PLoS One. 6(8): e23992.

Pechenk, J.A., 1987. Environmental influences on larval survival and development, in: Giese, A.C., Pearse, J.S. (Eds.), Reproduction in marine invertebrates. Academic Press, New York, pp. 551-608.
Pires, A., Croll, R.P., Hadfield, M.G., 2000. Catecholamines modulate metamorphosis in the opisthobranch gastropod Phestilla sibogae. Biological Bulletin 198, 319-331.
Provencio, I., Jiang, G.S., De Grip, W.J., Hayes, W.P., Rollag, M.D., 1998. Melanopsin: An opsin in melanophores, brain, and eye. Proceedings of the National Academy of Sciences of the United States of America 95, 340-345.
Roberts, B., Davidson, B., MacMaster, G., Lockhart, V., Ma, E., Wallace, S.S., Swalla, B.J., 2007. A complement response may activate metamorphosis in the ascidian Boltenia villosa. Development Genes and Evolution 217, 449-458.
Roth MS, Alamaru A, Padilla-Gamino JL, Gates RD (2007) Fluorescence in eggs of the coral *Montipora capitata*. In: Gates RD, editor. The biology of corals: Developing a fundamental understanding of the coral stress response Final report of the Edwin W Pauley Summer Program in Marine Biology. Kaneohe, Hawaii. pp. 95.
Rumrill, S.S., 1990. Natural mortality of marine invertebrate larvae. Ophelia 32, 163-198.
Sadd, B.M., Schmid-Hempel, P., 2009. Principles of ecological immunology. Evolutionary Applications 2, 113-121.
Salih, A., Larkum, A., Cox, G., Kuhl, M., Hoegh-Guldberg, O., 2000. Fluorescent pigments in corals are photoprotective. Nature 408, 850-853.
Sato, S., Yamamoto, H., 2001. Development of pigment cells in the brain of ascidian tadpole larvae: Insights into the origins of vertebrate pigment cells. Pigment Cell Research 14, 428-436.
Sato, Y., Bourne, D.G., Willis, B.L., 2009. Dynamics of seasonal outbreaks of black band disease in an assemblage of Montipora species at Pelorus Island (Great Barrier Reef, Australia). Proceedings of the Royal Society B-Biological Sciences 276, 2795-2803.
Schmid-Hempel, P., 2008. Parasite immune evasion: a momentous molecular war. Trends in Ecology & Evolution 23, 318-326.
Schnitzler, C.E., Hollingsworth, L.L., Krupp, D.A., Weis, V.M., 2012. Elevated temperature impairs onset of symbiosis and reduces survivorship in larvae of the Hawaiian coral, Fungia scutaria. Marine Biology 159, 633-642.
Schnitzler, C.E., Weis, V.M., 2010. Coral larvae exhibit few measurable transcriptional changes during the onset of coral-dinoflagellate endosymbiosis. Marine Genomics 3, 107-116.
Shinzato, C., Shoguchi, E., Kawashima, T., Hamada, M., Hisata, K., Tanaka, M., Fujie, M., Fujiwara, M., Koyanagi, R., Ikuta, T., Fujiyama, A., Miller, D.J., Satoh, N., 2011. Using the Acropora digitifera genome to understand coral responses to environmental change. Nature advance online publication.
Strathmann, R.R., 1985. Feeding and non-feeding larval development and life-history evolution in marine invertebrates. Annual Review of Ecology and Systematics 16, 339-361.
Sugumaran, M., 2002. Comparative biochemistry of eumelanogenesis and the protective roles of phenoloxidase and melanin in insects. Pigment Cell Res 15, 2-9.
Sweet, M.J., Croquer, A., Bythell, J.C., 2011. Development of Bacterial Biofilms on Artificial Corals in Comparison to Surface-Associated Microbes of Hard Corals. PloS One 6.
Thorson, G., 1950. Reproductive and larval ecology of marine bottom invertebrates. Biol Rev Camb Philos Soc 25, 1-45.
Tian, C.H., Wang, L., Ye, G.Y., Zhu, S.Y., 2010. Inhibition of melanization by a Nasonia defensin-like peptide Implications for host immune suppression. Journal of Insect Physiology 56, 1857-1862.
Vize, P.D., 2009. Transcriptome Analysis of the Circadian Regulatory Network in the Coral Acropora millepora. Biological Bulletin 216, 131-137.

Voolstra, C.R., Schwarz, J.A., Schnetzer, J., Sunagawa, S., Desalvo, M.K., Szmant, A.M., Coffroth, M.A., Medina, M., 2009. The host transcriptome remains unaltered during the establishment of coral-algal symbioses. Molecular Ecology 18, 1823-1833.
Wang, D.L., Zuo, D., Wang, L.M., Sun, T., Wang, Q., Zhao, Y.L., 2012. Effects of white spot syndrome virus infection on immuno-enzyme activities and ultrastructure in gills of Cherax quadricarinatus. Fish & Shellfish Immunology 32, 645-650.
Weis, V.M., Allemand, D., 2009. What Determines Coral Health? Science 324, 1153-1155.
Yakovleva, I.M., Baird, A.H., Yamamoto, H.H., Bhagooli, R., Nonaka, M., Hidaka, M., 2009. Algal symbionts increase oxidative damage and death in coral larvae at high temperatures. Marine Ecology-Progress Series 378, 105-112.

Figure legends

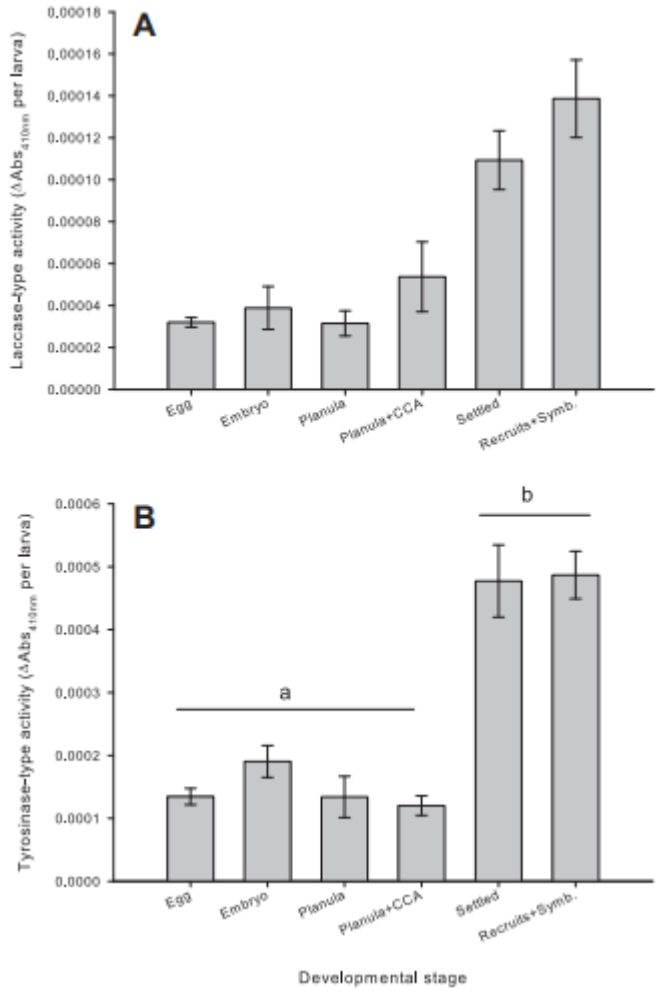


Fig. 1: Mean *o*-diphenoloxidase and *p*-diphenoloxidase activities (±SE) in the inactive form (prophenoloxidase) at each developmental stage.

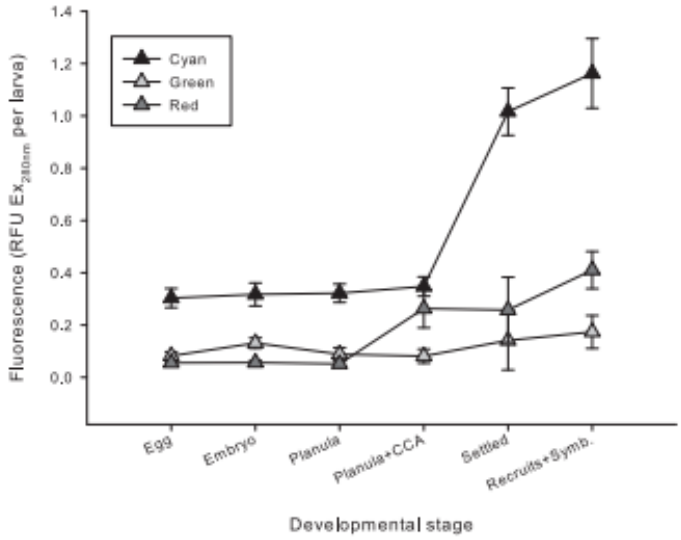


Fig. 2: Mean fluorescence in relative fluorescence units (RFU) per larva (±SE) for cyan, green and red fluorescent proteins at each developmental stage.

Supplementary material

Total protein and fluorescence per larva and the correlations among fluorescent proteins and melanin-synthesis pathway activity.

Methods

Total protein per larva was determined using the Bradford Quick-start assay (Bio-Rad). Briefly, 20 µl of each sample and of BSA protein standards (0.0015 – 1 mg protein) were placed in triplicate into a 96-well microtitre plate. Then, 180 µl of Bradford reagent was added to each well and the plate left to react for 5 min at room temperature. The plate was then read at 660 nm. Total protein was calculated per larva and compared among stage using a Kruskal-Wallis test as parametric assumptions were not met.

The total fluorescence per larva was determined as per the methods within the manuscript, and for each sample was calculated as the sum of standardised relative fluorescence units (RFUs) between 460 and 600 nm, which was then divided by the number of larva within the sample. One-way ANOVAs with Tukey's HSD *post-hoc* tests was used to compare total fluorescence among larval developmental stage as log-transformed data met the assumption of normality and homogeneity of variance.

Relationships among the response variables were investigated using Pearson's correlation coefficient using log-transformed data.

Results

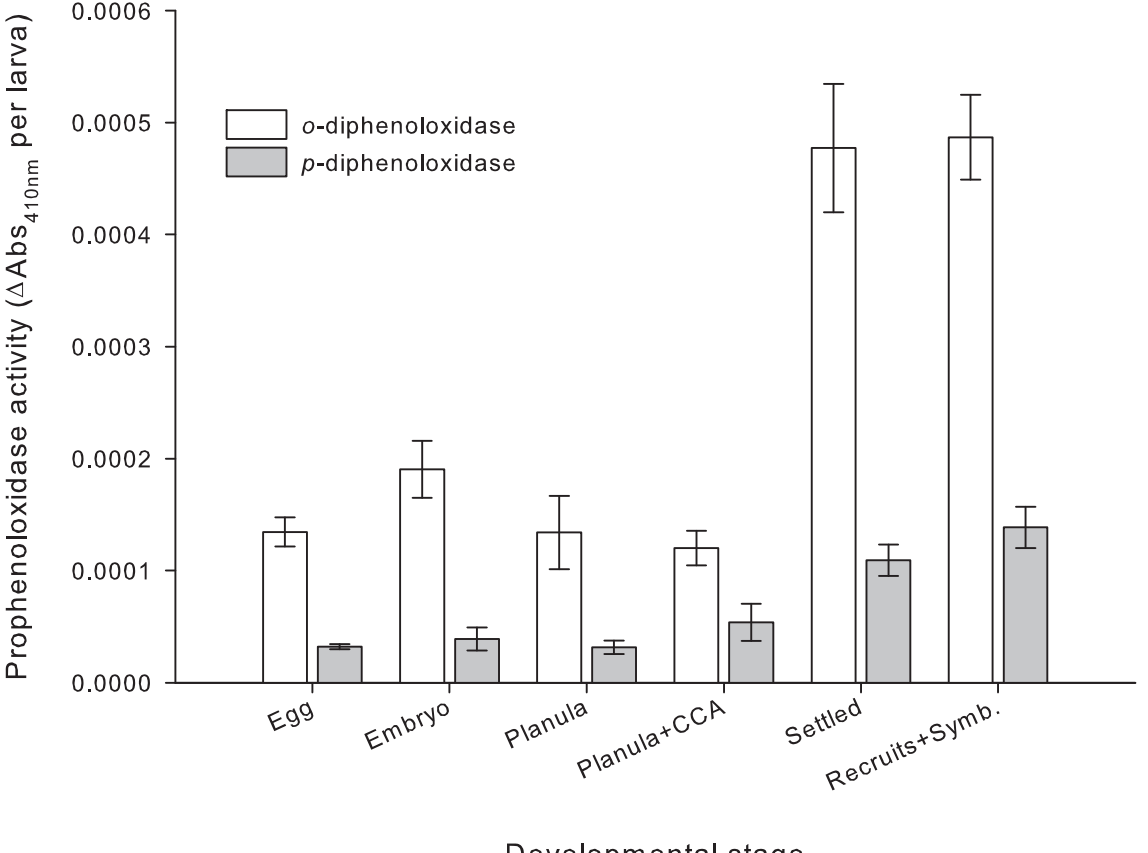
Total protein per larva varied significantly with developmental stage ($K\text{-}W_{(5)} = 25.59$, $P<0.001$), driven by the 70% decrease between embryo and planula, consistent with the use of finite maternally-derived resources (Fig. S1). Total fluorescence varied significantly through larval development ($F_{(5, 35)} = 31.97$, $P<0.001$), which was primarily driven by an increase of approximately 2-fold post-settlement (Fig. S2). Both *o*-diphenoloxidase and *p*-diphenoloxidase activities were significantly correlated with total, cyan and red fluorescence,

but not with green (Fig. S3). The relationship between *o*-diphenoloxidase activity and both total fluorescence and cyan, were the strongest at 0.82 ($P<0.001$). *p*-diphenoloxidase activity correlated the strongest with total fluorescence ($C =0.68$, $P<0.001$), and the correlation with red fluorescence ($C = 0.63$, $P<0.001$) was slightly stronger than that with the cyan ($C = 0.63$, $P<0.001$).

Fig. S1: Mean total protein per larva (±SE) at each developmental stage.

Fig. S2: Mean total fluorescence in relative fluorescence units (RFU) per larva (±SE) at each developmental stage. Lettered bars indicate Tukey's HSD *post –hoc* test groupings.

Fig. S3: The relationships among the two melanin-synthesis pathway activities (*o*-diphenoloxidase and *p*-diphenoloxidase) and total fluorescence (total FP), cyan and red.


0.0006
0.0005
0.0004
0.0003
0.0002
0.0001
0.0000
Prophenoloxidase activity (ΔAbs410nm per larva)
o-diphenoloxidase
p-diphenoloxidase
Egg
Embryo
Planula
Planula+CCA
Settled
Recruits+Symb.
Developmental stage