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NOTE

Coral tumor-like growth anomalies induce an immune response and reduce fecundity

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ABSTRACT: Coral growth anomalies (GAs) are chronic diseases that adversely affect organism health and fitness. We investigated immunity and fecundity within and among GA-affected and visually healthy control colonies of the reef-building coral *Acropora hyacinthus*. Compared to controls, GAs had higher activity of the key immunity enzyme phenoloxidase (PO), suggesting a localised immune response within the GA. Both GAs and healthy tissue of GA-affected colonies had significantly greater total potential PO (tpPO) — PO activity inclusive of the activated latent PO, prophenoloxidase — than control colonies. Higher tpPO activity in GA-affected corals suggests elevated constitutive immunity compared to visually healthy controls. Additionally, fewer GA-affected colonies produced gametes, fewer polyps had oocytes ($p < 0.001$) and the number of oocytes per polyp was lower. Therefore, GAs in *A. hyacinthus* might induce, or represent a shift in resource investment towards immunity and away from reproduction. While the effect on population growth is likely to be small, reduced fecundity in GA-affected colonies does suggest a selective pressure against GAs.

KEY WORDS: Coral reefs · Fecundity · Growth anomaly · Prophenoloxidase · Phenoloxidase · Reproduction · Tumor · Immunity

INTRODUCTION

Tumors, or neoplasias, throughout the metazoa result from uncontrolled proliferation of genetically altered cells and can be benign or malignant (Robert 2010). Causes include physical (e.g. radiation), chemical (carcinogens) or infectious agents (Robert 2010, Newton & Lewbart 2017) and the presence of a tumor can increase mortality risk and reduce fecundity (e.g. in the softshell clam *Mya arenaria*; Brousseau & Baglivo 1991), indicating a fitness cost. Immune responses mitigate and eliminate tumorous tissue (Pastor-Pareja et al. 2008, Hauling et al. 2014), but are costly to up-regulate and maintain (Sadd & Schmid-Hempel 2009). Given a finite access to energy, investment into immunity is likely to involve a reduced investment in other vital processes such as growth and reproduction (Sadd & Schmid-Hempel 2009, Palmer et al. 2010).

Tumor-like growths occur in numerous coral species (e.g. Bak 1983, Peters et al. 1986, Gateño et al. 2003, Domart-Coulon et al. 2006, Aeby et al. 2011). These tumor-like growths are often referred to as growth anomalies (GAs) because it is not always possible to distinguish between hyperplasia (a normal proliferation response to stimulus) and genetically altered cell growth of neoplasia (Work et al. 2008). GAs affect coral fitness by reducing growth (Bak 1983) and fecundity (Peters et al. 1986, Yamashiro et al. 2000, Work et al. 2008, Burns & Takabayashi 2011)

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and causing partial mortality (Peters et al. 1986, Work et al. 2008). GAs can also alter immune activity (Kelly et al. 2016).

Corals possess a suite of innate immune mechanisms that respond to infection and tissue disruption (Palmer & Traylor-Knowles 2012), including multiple melanin-synthesis pathways (Mydlarz & Palmer 2011, Palmer et al. 2012), which are considered the mainstay of invertebrate immunity (Cerenius et al. 2010). Anthozoans likely have multiple types of phenoloxidases (POs) — the activators of melanin-synthesis — including mono-phenoloxidase, *para*-diphenoloxidase (laccase-type) and *ortho*-diphenoloxidase (tyrosinase-type), as well as 2 prophenoloxidases (PPOs; laccase and tyrosinase types; Mydlarz & Palmer 2011, Palmer et al. 2012). In arthropods, PPOs are the inactive, stored zymogen form of PO, and indicate constitutive levels of immunity, however the storage system for coral phenoloxidases is unknown. PPO is inactive, and therefore it cannot be measured enzymatically. The standard immunological measurement of this latent PO is referred to as total potential PO (tpPO; e.g. van de Water et al. 2015).

Highly cytotoxic tyrosinase-type PO activity and PPO (Cerenius et al. 2010) are negatively correlated with disease susceptibility of healthy corals, demonstrating an advantage of investment in constitutive immunity (Palmer et al. 2010). Of 22 Indo-Pacific anthozoan species investigated, *Acropora hyacinthus* has amongst the lowest PO activity and PPO levels, suggesting low investment into immunity under healthy conditions, and thus a high susceptibility to disease (Palmer et al. 2010). The colonial nature of corals offers an ideal system with which to investigate the possible localized (within-colony) and whole-organism regulation of immunity, and fitness, in response to GAs.

METHODS AND MATERIALS

Sampling design

Five *Acropora hyacinthus* colonies with GAs (Fig. 1) were located at between 1 and 2 m depth on the reef crest of Trimodal Reef, Lizard Island, Great Barrier Reef (14.6996° S, 145.4482° E), Australia, in November 2010. One GA was collected from each affected colony plus a 2nd fragment (between 10 and 80 cm^2) from an apparently healthy area of the same colony at least 20 cm distance from the GA (hereafter 'healthy'). Similar sized fragments were collected in the same locality from 5 visibly healthy *A. hyacinthus* colonies (control). GAs tended to form in the centre of colonies (Fig. 1), and control fragments were therefore also taken from the centre of healthy colonies. All samples were immediately halved and fixed in either 10% seawater formalin for histology or liquid nitrogen for biochemical analyses. All colonies sampled were larger than the ~300 cm^2 size at first reproduction of *A. hyacinthus*, above which per-polyp fecundity does not significantly vary with size (Álvarez Noriega et al. 2016). GAs covered less than 1% of the surface area of all affected colonies.

Biochemistry

Tissue was airbrushed from frozen samples over ice with extraction buffer containing 50 mM phosphate buffer with 0.05 mM dithiothreitol, at pH 7.8. This low concentration of dithiothreitol has been found to minimally affect phenoloxidase activity, while helping to stabilise total protein, and is used as standard in coral immunity enzyme assays (e.g. Palmer et al. 2010). Tissue slurries (i.e. tissue–buffer mixture) were homogenized for 30 s (Power Gen 12; Fisher Scientific) and left on ice for 5 min before centrifuging at 4°C, 1900 × *g* for 5 min. The supernatant was stored in liquid nitrogen for transport to the mainland. To determine tyrosinase-type PO activity and tpPO (activity of both PO and latent PO, i.e. PPO), 40 µl of phosphate buffer (50 mmol l^{-1}, pH 7.8) was added to 20 µl of sample, in triplicate, in a 96 well microtiter plate. For the PO assay, 25 µl of double distilled water was added and 25 µl trypsin

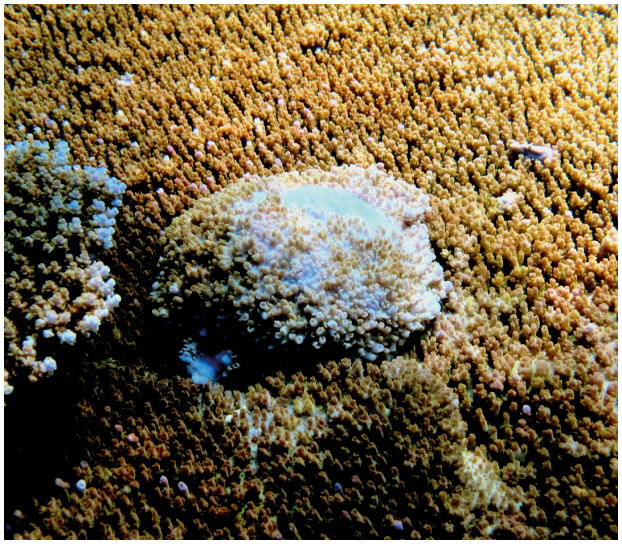

Fig. 1. A growth anomaly on a tabular *Acropora* colony typical of that used in this study

(0.1 mg ml^{-1} in deionized water) for the tpPO assay and incubated at room temperature for 20 min (Palmer et al. 2011a, Kelly et al. 2016). Then, 30 µl of a 10 mmol l^{-1} solution of dopamine hydrochloride (Sigma-Aldrich H8502) was added and absorbance at 490 nm recorded every 5 min for 25 min (Palmer et al. 2011a). Peroxidase activity was determined as per Palmer et al. (2011a); briefly, 10 µl sample extract with 35 µl phosphate buffer (10 mmol l^{-1}, pH 6.0) was aliquoted for each sample in triplicate. Then, 40 µl of guaiacol (Sigma G5502; 25 mmol l^{-1}) was added, and 25 µl of hydrogen peroxide (H_2O_2; 20 mmol l^{-1}) used to initiate the reaction. Absorbance at 470 nm was recorded over 45 min. For both tyrosinase-type PO and tpPO activities and peroxidase, a Spectramax M2 (Molecular Devices) spectrophotometer was used to measure absorbance. The change in absorbance for the linear portion of the reaction curve was used to determine enzyme activities. All enzyme activities were normalised to sample total protein (mg^{-1}) as determined using Red 660 total protein assay, as per manufacturer's instructions (G-Biosciences).

Determination of fecundity

After 24 h, samples were transferred from 10% seawater formalin into 25% ethanol for transport and decalcified in 10% formic acid, then stored in 10% seawater formalin.

To estimate per-polyp fecundity in the control and apparently healthy treatments, 6 polyps were chosen at random from below the sterile zone (Wallace 1985) in 4 randomly selected branches from each fragment. Decalcified branches were placed under a dissecting microscope at 10× magnification. Individual polyps were cut out of branches and split open with a needle to reveal the oocytes (following Tan et al. 2016). Oocytes were then teased out of the polyp and the number per polyp was counted. Since there was no branch structure in the GAs, 6 to 24 polyps were randomly selected and dissected. Oocyte size was measured using an eyepiece micrometre at 20× resolution, calibrated to a stage micrometre, on a dissecting microscope. A total of 20 oocytes from each treatment group were selected at random from oocytes dissected out of the polyps.

Statistical analysis

One-way ANOVA was used to test for differences in mean PO and tpPO activity among samples using log-transformed data. A non-parametric Kruskal-Wallis test was used to compare peroxidase activity among health categories. The association between health status and the number of polyps with oocytes was tested using a chi-squared contingency test. We used Bonferroni adjustment, giving a revised p-value of 0.01 to indicate statistical significance.

RESULTS AND DISCUSSION

Growth anomalies of *Acropora hyacinthus* from Trimodal Reef, Lizard Island, Great Barrier Reef, had higher immunity-associated enzyme activities than healthy tissue, both within and among colonies. Tyrosinase-type PO activity varied with health status, with the lowest activity in control colonies and the highest, by 1.5-fold, in GAs (Fig. 2a). Concomitant with the cytotoxicity of the tyrosinase-type melanin synthesis pathway, the mean activity of the antioxidant enzyme peroxidase was more than 20-fold higher in the GA than in control tissues. Increased PO activity in GA-affected *A. hyacinthus* tissue compared to the controls is consistent with findings of white syndrome-diseased *A. millepora* (Palmer et al. 2011a). However, investigations of plaque-like GAs in *A. muricata* found no difference in PO activity among healthy controls and GA tissue (Kelly et al. 2016). The increased PO activity in *A. hyacinthus* GAs suggests an increase of melanin-synthesis pathways in GAs compared to healthy tissue, which is consistent with studies of compromised coral tissue (Palmer et al. 2008). These disparate immune activities among corals likely indicate variable pathologies or specific responses to GAs.

The tpPO (PPO-activated to measurable PO with the addition of trypsin) was highest in both GA and apparently healthy tissue of GA-affected colonies, and lowest in control colonies ($F_{2,24} = 18.33$, $p < 0.001$; Fig. 2b). These differential levels of tpPO activity further suggest a different use of the coral immune repertoire by GA-affected colonies. Generalised heightened constitutive immunity in corals with GAs raises the intriguing question of what is activating the immune system, and whether immunity was heightened before or after the formation of the GA. These questions may be addressed by long-term immunological monitoring of corals

Only 60% (n = 5) of GA-affected coral colonies produced gametes, compared to 100% of control colonies. All polyps in the control colonies produced oocytes (n = 120) compared to 75% (n = 72) in

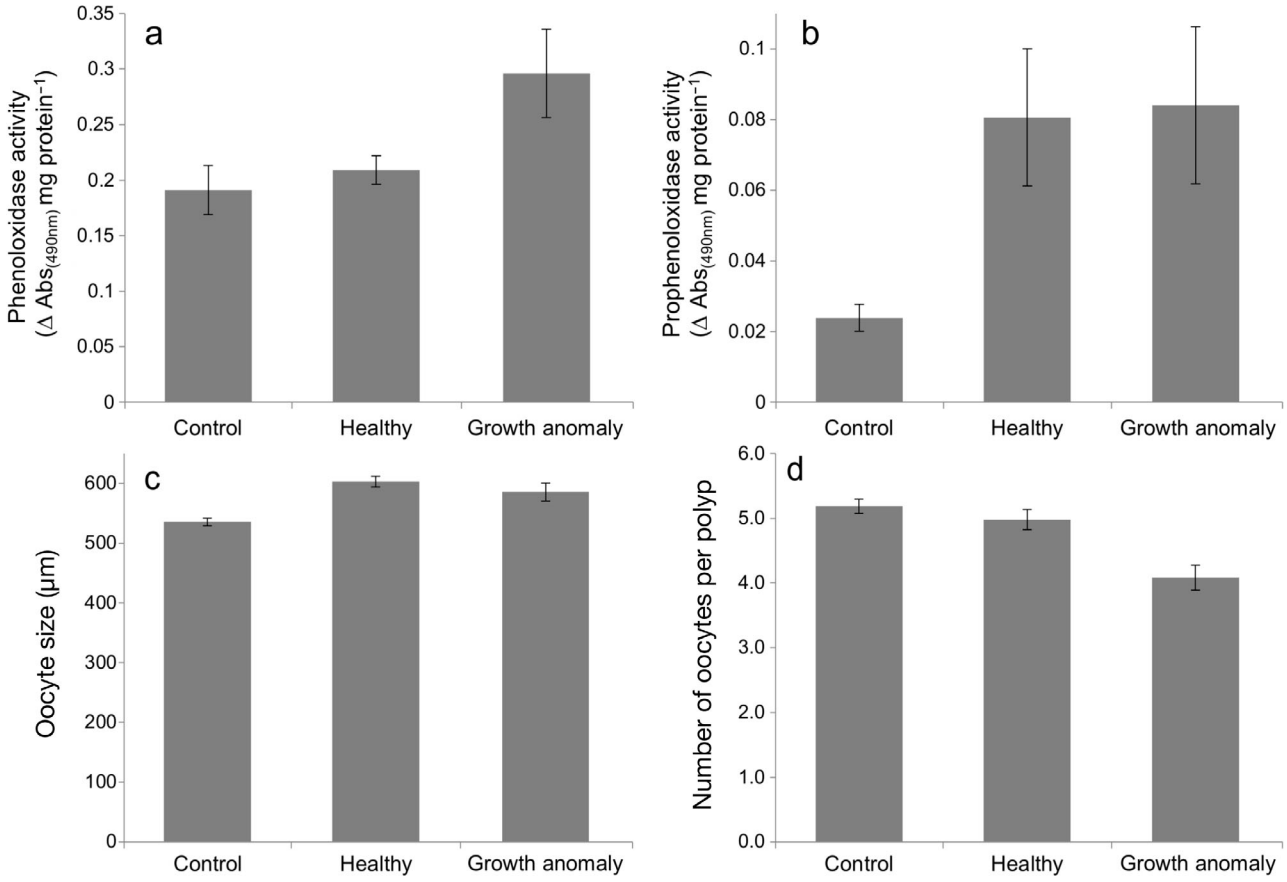

Fig. 2. Tyrosinase-type melanin synthesis pathway activity in the coral *Acropora hyacinthus* as demonstrated by mean (±SE) (a) phenoloxidase (PO) and (b) activated prophenoloxidase (PPO) activity, as well as (c) oocyte size and (d) number of oocytes per polyp in control, healthy and growth anomaly coral tissues. Note that in (a) and (b), the *y*-axes of PO and PPO are not comparable as assays were conducted at different times

the healthy branches of GA-affected colonies, and 33% of polyps in the GAs (n = 36) (χ^2 = 84.9, p < 0.001). In addition, the number of oocytes in polyps that did have gametes was lower in GAs than in healthy polyps or controls (Fig. 2c). Indicators of reduced fecundity in tumorous tissue is consistent with most previous research (Yamashiro et al. 2000, Work et al. 2008, Burns & Takabayashi 2011, Kelly et al. 2016).

Despite these effects on reproductive output at the colony level, the effect on the population is likely to be minor. We estimate that less than 1% of *A. hyacinthus* colonies at Lizard Island had GAs, so even if none of these colonies developed gametes, the effects on population growth is unlikely to be significant. Our results further suggest that the proportion of the population producing gametes, and the proportion of polyps contributing to colony fecundity are important variables to consider when evaluating the effect of stressors on coral reproductive output.

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