

Interplay between proteases and protease inhibitors in the sea fan—*Aspergillus* pathosystem

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Abstract Environmental conditions greatly impact the dynamics of host–pathogen relationships, affecting outbreaks and emergence of new diseases. In corals, epizootics have been a main contributor to the decline of reef ecosystems and rising sea surface temperatures associated with climate change are thought to exacerbate virulence of pathogens and/or compromise the immune responses of coral hosts. Among pathogens strategies, protease secretion is a common virulence factor that promotes colonization of the host. As such, protease inhibitors play an invaluable role to the host for protection against pathogen invasion. This study describes changes in activity of proteases secreted by the fungal pathogen, *Aspergillus sydowii*, and protease inhibitors of *Gorgonia ventalina* under ambient and elevated temperatures and health conditions (disease and healthy). *G. ventalina* colonies were collected from Florida Keys, Florida (24° 34.138N, 81° 22.905W) or La Parguera, Puerto Rico (17° 56.091N, 67° 02.577W) in 2007 and 2012, respectively. At elevated temperatures, protease activity was significantly higher than at ambient temperatures in *A. sydowii*. Temperature stress did not induce a change in protease inhibitor activity, but in healthy *G. ventalina* colonies, inhibitor activity against proteases was higher than in diseased individuals. Healthy colonies appear capable of resisting proteolytic activity, but diseased individuals are still likely affected by pathogen infections. These data suggest that rising sea surface temperatures due to climate change may increase levels of virulence

(protease activity) of *A. sydowii*, while immunity (protease inhibitor) of *G. ventalina* may not be affected.

Introduction

The number of infectious diseases and outbreaks has been increasing across marine and terrestrial ecosystems (Altizer et al. 2013). Host–pathogen interactions and disease development are key factors to understand the dynamics of epizootics (Harvell 2004). In order to prevent disease, a host must be able to mount a successful immune response and success in evading disease is determined by both the pathogen virulence and host immunocompetence.

Pathogen-produced proteases aid in the colonization of a host (Lee et al. 2002; Monod et al. 2002) by benefiting the pathogen in two ways: nutrient acquisition through host tissue breakdown and/or interfering or disrupting host immune function (Hoge et al. 2010). Therefore, many hosts produce protease inhibitors as a response against pathogen proteases. In several plant and invertebrate species, protease inhibitors are key components of the innate immune system and can act directly against proteases produced by microbial pathogens, insects, and other pests (Jongsma and Bolter 1997; Faisal et al. 1998; Donpuksa et al. 2009).

Fungal pathogens, particularly *Aspergillus* spp., are prevalent in many disease systems including plants, arthropods, birds. (Kulshrestha and Pathak 1997; Asis et al. 2009; Rahim et al. 2013). Copious levels of proteases make pathogenic *Aspergillus* spp. especially virulent (Dunaevskii et al. 2006). In the past few decades, aspergillosis of the Caribbean sea fan coral, *Gorgonia ventalina* (Linnaeus: Geiser et al. (1998), has decimated sea fan populations throughout the Caribbean (Bruno et al. 2011). Aspergillosis infection can cause tissue necrosis that can spread through

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the entire coral colony leading to mortality. In some locations, over 50 % of the sea fan populations have been affected (Kim and Harvell 2004); and although there are signs of recovery in some areas, sea fan aspergillosis still persists in several Caribbean reefs (Flynn and Weil 2009).

Increase of disease and consequent population decline has been observed not only in sea fans, but in other corals worldwide (Ruiz-Moreno et al. 2012). In general, the outbreaks of coral diseases have been linked to changing environmental conditions such as elevated sea surface temperatures associated with global climate change (Hoegh-Guldberg and Bruno 2010). Chronic exposure to elevated temperatures for extended periods of time has adverse effects on the immunity of corals likely increasing disease susceptibility (Mydlarz et al. 2010).

Due to the sensitivity of corals toward temperature stress (Pandolfi et al. 2011), the ability to easily identify and collect diseased sea fans, and the existence of several isolates of the pathogen (*Aspergillus sydowii*) from diseased corals (Rypien et al. 2008), the sea fan—*Aspergillus* pathosystem—is an ideal model for investigating questions about the effects of environmental stressors on host–pathogen dynamics. In this study, we investigate how environmental stress (e.g., elevated temperature) affects the relationship between the putative pathogen *A. sydowii* and the sea fan. Specifically, protease activity of *A. sydowii* was measured and the antagonistic interactions between protease inhibitor activity of the sea fan and *A. sydowii*-derived and commercial proteases were evaluated.

Materials and methods

Aspergillus sydowii culturing and maintenance

Five strains of *A. sydowii* from various sites and origins were used in this project: three strains were isolated from sea fans from reefs in the Florida Keys, Florida; San Salvador, Bahamas; and Saba, the Netherland Antilles, one from a mangrove (NRRL250), and one from a human (NRRL254). Stock spore solutions of the sea fan *A. sydowii* strains were obtained from the Harvell lab at Cornell University, while the other two spore stocks were acquired from the USDA Agricultural Research Service Culture Collection. Delineation between the sea fan-isolated strains of *A. sydowii* was first determined by metabolic profiles in Alker et al. (2001). Phylogeny was further established between strains of multiple origins (sea fan, mangrove, and human) in Rypien et al. (2008) and Rypien and Andras (2008).

Cultures of *A. sydowii* from the spore collections were grown on peptone–yeast–glucose agar (PYG: 1.25 g peptone, 1.25 g yeast, 3.0 g glucose, 30 g Instant Ocean salt

mix L⁻¹). The human *A. sydowii* strain was grown with no added Instant Ocean salt mix. Spores were harvested from germinated cultures on PYG agar, strained through a 40- μ m cell strainer (BD Falcon, San Jose, California, USA), and stored in quarter-strength PYG until use. Spore concentrations were quantified using a Bright-Line hemocytometer before use in the assays described below (Sigma-Aldrich, St. Louis, Missouri, USA).

Aspergillus sydowii protease assays

Protease activity was quantified using two culture methods: growth on casein-enriched PYG agar plates (3 % casein) and growth in PYG broth (0.1 % peptone, 0.1 % yeast, 3 % glucose, 3 % Instant Ocean salt mix L⁻¹). In the first protease assay, the sea fan *A. sydowii* strains were inoculated on the center of agar plates (3 per strain) from approximately 1×10^3 spores and incubated either at 25 or 30 °C for 48 h. The radial colony growth and the protease activity (the zone of clearing, indicating cleavage of the casein protein) were quantified with ImageJ (National Institutes of Health, Bethesda, Maryland, USA). Total protease activity was measured by calculating the ratio of radial colony growth to the radial zone of clearing around colony.

For the second protease assay, spores were grown from approximately 5×10^7 spores per 100 mL of PYG broth in vented Erlenmeyer flasks (VWR, Radnor, Pennsylvania, USA). Three flask replicates per strain (two sea fans (San Salvador and Saba), mangrove, and human-isolated strains) were incubated at either 25, 28, 30, or 32 °C for 48 h and shaken at 100 rpm. After incubation, hyphal bodies were separated using 40- μ m cell strainers (BD Biosciences, San Jose, California, USA) and centrifuged at $2,880 \times g$ using an Eppendorf 5810R (Eppendorf, Hauppauge, New York, USA) for 20 min at room temperature. Proteases are typically produced with high hyphal body mass and minimal sporulation (Krull et al. 2010). Lack of spore production in the cultures was confirmed with visual inspection. In total, 1 mL of the supernatant was collected as extracellular proteases. Both the hyphal bodies and supernatant samples were flash frozen with liquid N₂ and stored at –80 °C until processing for protein extractions.

Crude protein extracts from fungal hyphal bodies were prepared by grinding the samples in liquid N₂ with a mortar and pestle. Proteins including the proteases were extracted from the powder using 100 mM sodium phosphate-buffered solution (PBS), pH 7.8 for approximately 45 min on ice. Protein extract was recovered after centrifugation at $2,205 \times g$ at 4 °C for 10 min into 1.5-mL tubes. Total protein content was quantified using the Red660 protein assay (G Biosciences, St. Louis, Missouri, USA). Extracellular proteases were quantified from the media supernatant,

while intracellular were quantified from protein extracts of the hyphal bodies.

Extracellular and intracellular fungal proteins were assayed for general serine protease activity in 96-well black microplates (Greiner Bio One, Monroe, North Carolina, USA), generally following the protocol of (Twinning 1984). In total, 10 μL of sample extract was incubated with 20 μL 2.5 % fluorescein isothiocyanate (FITC) casein substrate (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 20 mM PBS with 150 mM NaCl, pH 7.6 for 30 min at 37 °C. The reaction was stopped with 10 % trichloroacetic acid to precipitate remaining proteins. Proteins were pelleted at $5,590\times g$ (Baxter Scientific Products, Deerfield, Illinois, USA), and the fluorescence of the supernatant was observed with excitation at 485 nm and emission wavelength at 535 nm on a Synergy 2 Microplate Reader (BioTek Instruments, Winooski, Vermont, USA). Units (U) of protease in the intracellular and extracellular samples were quantified using a standard curve from a serial dilution of 1,000 U bovine trypsin (Sigma-Aldrich, St. Louis, MO, USA). Intracellular and extracellular protease activity was standardized to the total protein content of the extract to account for potential biomass differences between growth temperatures. PYG broth and extraction buffers were used as negative controls.

Gorgonia ventalina sample collections

Protease inhibitor activity against *A. sydowii* proteases in natural populations of *G. ventalina* was examined from ten healthy and ten diseased colonies collected from Looe Key Research Reef (24° 34.138N, 81° 22.905W), Florida Keys, USA, using SCUBA equipment. The depth for these colonies ranged from 3 to 6 m (Florida Keys National Marine Sanctuary Collections Permit No. 2004-092). Samples consisted of approximately 15 cm^2 of tissue cut with scissors from each colony. Diseased colonies were sampled from the lesion site (as determined by purple coloration and lesion morphology—Mydlarz and Harvell (2007)) and from a visually healthy area at least 10 cm away from the lesion site. All samples were immediately flash frozen in liquid N_2 and sent on dry ice to the University of Texas at Arlington (UTA).

To examine the effect of temperature on protease inhibitor activity in healthy sea fans (no sign of infection, necrosis, or other injury), we experimentally exposed twelve sea fan colonies to elevated temperatures. Using SCUBA, sea fans were collected from Media Luna reef (17° 56.091N–67° 02.577W) in La Parguera, Puerto Rico. Samples (~20 cm^2) were brought back to the station and divided into two fragments (3 \times 5 cm), one for temperature treatment and the other as control. Fragments for each treatment were evenly divided across three indoor tanks for a total

of six tanks. All tanks were maintained as closed systems with 20 % daily water changes and aerated using aquarium water pumps (TAAM Inc. USA). Artificial lighting with full-spectrum bulbs was set to a 12-h day/night cycle, and temperature was maintained with aquarium heaters (Hydor, Sacramento, California, USA). After a 2-day acclimation period, temperatures were increased over 2 h to 30–32 °C and held for 14 days. Controls were held at 26–28 °C. At the end of the experiment, no aspergilloles lesions or otherwise were observed. Samples were immediately flash frozen in liquid N_2 and shipped on dry ice to UTA.

Crude protein extracts from sea fan fragments were prepared by grinding the entire sea fan sample (tissue and skeleton) in liquid N_2 with a mortar and pestle. Proteins including the proteases were extracted from the powder using 100 mM sodium PBS, pH 7.8 for approximately 45 min on ice. Protein extract was recovered after centrifugation at $2,205\times g$ at 4 °C for 10 min to remove cellular debris. Total protein content of the serum was quantified using the Red660 protein assay (G Biosciences, St. Louis, Missouri, USA).

Protease inhibitor assay

A protease inhibitor assay was developed to quantify activity of the sea fan protein extracts against commercial proteases and fungal-derived proteases from *A. sydowii*. The assay modifies the previously described protease activity assay by calculating decrease in proteolytic cleavage of a given protease. In total, 10 μL of sea fan extract was incubated with either 30 μL of fungal extracellular protease extract (derived from the San Salvador sea fan strain, grown at 30 °C, described above), 10 μL trypsin (0.1 mg mL^{-1}) (Sigma-Aldrich, St. Louis, MO, USA), or 10 μL α -chymotrypsin (1 mg mL^{-1}) (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at room temperature. The sea fan extract/protease mixture was then added to 40 μL 2.5 % FITC casein substrate (in 20 mM PBS, 150 mM NaCl, pH 7.6) and cleavage of fluorescently bound casein measured as described above. Protease inhibitor activity was calculated as a decrease in proteolytic cleavage by the coral extract–protease mixture compared to the protease alone. Activity was standardized to the total protein of the coral extract.

Controls for the assay included the protease, to determine the maximum protease activity in each assay, and sample blanks with coral protein extraction buffer alone and combined with PYG broth as control for the fungal protease. As reference, standard curves of commercial protease inhibitors (aprotinin and leupeptin, Sigma-Aldrich, St. Louis, MO, USA) were analyzed to obtain a reference of activity for the coral protease inhibitors. A minimum of 0.6 mM aprotinin was required to inhibit trypsin and

chymotrypsin 50–100 %, while trypsin was inhibited completely by 0.01 mM leupeptin. The extraction buffer, PYG broth, and coral extracts did not affect or quench the fluorescence component of the FITC substrate by themselves. The assay was also validated by testing the heat-inactivated coral extracts to ensure the inhibitor activities were from a protein/enzyme source.

Data analysis

Homoscedasticity of all data sets was confirmed with the Brown–Forsythe test, and normality was determined with the Shapiro–Wilk test. Any data sets that were non-normally distributed or of unequal variance were successfully transformed using the Box–Cox method. A two-way analysis of variance (ANOVA) of protease activity of *A. sydowii* was performed with temperature and strain as factors. A one-way ANOVA of sea fan protease inhibitor activity was performed for each type of protease inhibitor with either disease or temperature as an effect. In both cases, Tukey–Kramer post hoc analyses were used to detect differences between factors and effects. Statistical analysis was performed using JMP 10.0 software (SAS, Cary, North Carolina).

Results

Aspergillus sydowii protease activity

All fungal strains showed measurable levels of protease activity as validated by biochemical assays. There were significant overall changes in protease activity for the three sea fan-isolated *A. sydowii* strains grown on casein-enriched agar (two-way ANOVA, $F_{(2,5)} = 16.9230$, $P = 0.003$, Fig. 1). There were also significant differences in protease activity between the three strains (strain effect, ANOVA, $F_{(2,5)} = 230.2$, $P < 0.001$). Elevated temperature significantly increased extracellular protease activity for all three fungal strains (temperature effect, ANOVA, $F_{(1,5)} = 221.8$, $P < 0.0001$). Sea fan fungal strains from San Salvador exhibited the highest overall protease activity at ambient (25 °C) and elevated temperatures (30 °C), while the Florida Keys strain had the lowest overall activity, with essentially no protease activity detectable at 25 °C in this assay.

All *A. sydowii* strains had detectable amounts of intracellular and extracellular protease activity when grown in PYG broth media. Both the strain identity and temperature had significant effect on intracellular protease activity (Two-way ANOVA, $F_{(9,15)} = 11.2725$, $P < 0.0001$, Fig. 2). There were no significant differences in intracellular protease activity between the sea fan- and mangrove-isolated

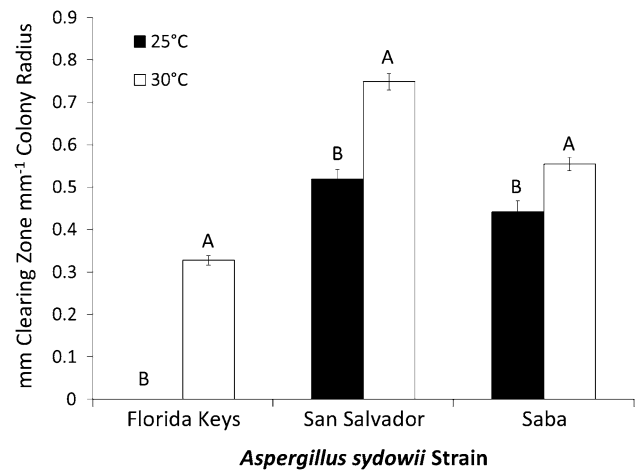


Fig. 1 Mean (\pm SE) extracellular protease activity of *Aspergillus sydowii* sea fan strains grown on casein-enriched agar ($n = 3$). Letters indicate statistical differences under 25 °C and 30 °C at $P < 0.05$ within strains

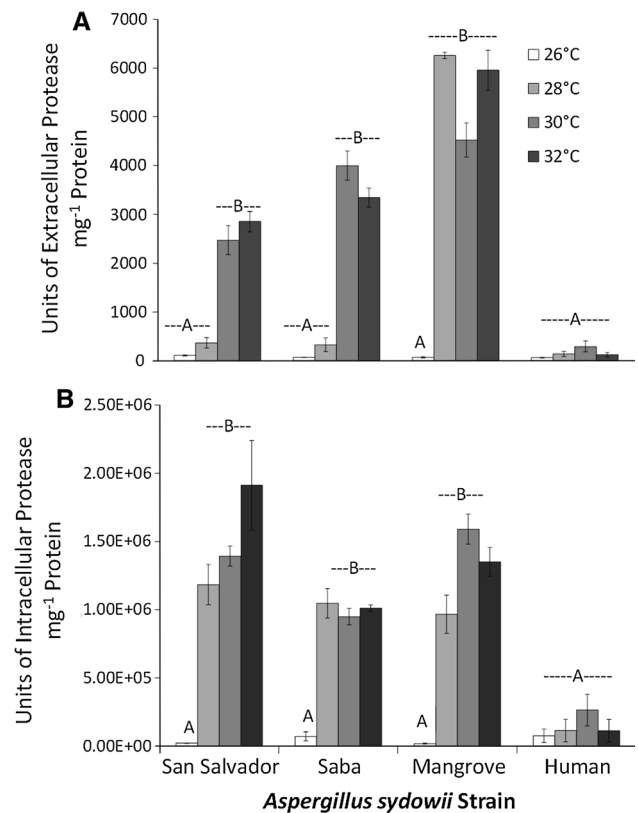


Fig. 2 Mean (\pm SE) **a** extracellular protease activity (per mg protein) and **b** intracellular protease activity (per mg protein) of *Aspergillus sydowii* strains from various host sources grown in PYG broth. Letters indicate significant differences under different temperatures at $P < 0.05$ within strains ($n = 3$)

strains, but all were different from the human-isolated strain, which had the lowest overall protease activity (Strain effect, ANOVA, $F_{(3,15)} = 53.80$, $P < 0.0001$). Temperature

effect was significant for intracellular protease activity in the fungal strains from the two sea fan-isolated strains and the mangrove-isolated strain, but not in the human-isolated strain. Intracellular protease activity increased at 28–32 °C and remained elevated at 30–32 °C in the fungal strains from sea fans and mangrove, respectively (Temperature effect, ANOVA, $F_{(3,15)} = 67.50$, $P < 0.0001$).

Similar to the intracellular protease assay, the strain identity and temperature had significant effect on extracellular protease activity (Two-way ANOVA, $F_{(9,15)} = 9.37$, $P < 0.0001$, Fig. 2). There was different protease activity among strains: the two sea fan-isolated strains had significantly higher protease activity than the human-isolated strain but not the mangrove-isolated strain (Strain effect, ANOVA, $F_{(3,15)} = 56.34$, $P < 0.0001$). The effect of temperature was significant for extracellular protease activity in the *A. sydowii* strains isolated from sea fans and mangrove but not the human-isolated strain (Temperature effect, ANOVA, $F_{(3,15)} = 37.56$, $P < 0.0001$). Specifically, extracellular protease activity was higher at 28–32 °C for the mangrove-isolated strain and 30–32 °C for the sea fan-isolated strains (San Salvador and Saba) compared to activity at 26 °C.

Sea fan protease inhibitor activity

Sea fan extracts were able to inhibit the activity of all three proteases, fungal-derived, trypsin, and α -chymotrypsin. There was an overall effect of health condition on fungal-derived protease inhibition (ANOVA, $F_{(2)} = 4.0880$, $P = 0.0303$), trypsin protease inhibition (ANOVA, $F_{(2)} = 5.1521$, $P = 0.0130$), and α -chymotrypsin protease inhibition (ANOVA, $F_{(2)} = 18.3048$, $P < 0.001$). Fungal protease inhibition was lower in lesion tissue of the diseased colony than both in healthy tissues of diseased and healthy colonies (Fig. 3a). Trypsin inhibition was systemically lower in diseased colonies (both lesion and healthy tissue) than the healthy control colonies (Fig. 3b). In contrast, α -chymotrypsin inhibition was highest in the infected tissue and lower in both healthy tissues of the diseased and healthy colonies (Fig. 3c).

Sea fans exposed to elevated temperatures for 14 days demonstrated no significant changes in protease inhibition activity against fungal-derived proteases (ANOVA, $F_{(11)} = 0.00$, $P = 0.9977$), trypsin (ANOVA, $F_{(11)} = 0.777$, $P = 0.3903$), or α -chymotrypsin (ANOVA, $F_{(11)} = 0.0052$, $P = 0.9434$).

Discussion

The interplay between host and pathogen can manifest itself through the specific virulence factors of a pathogen and countermeasures employed by the host (Rolff and

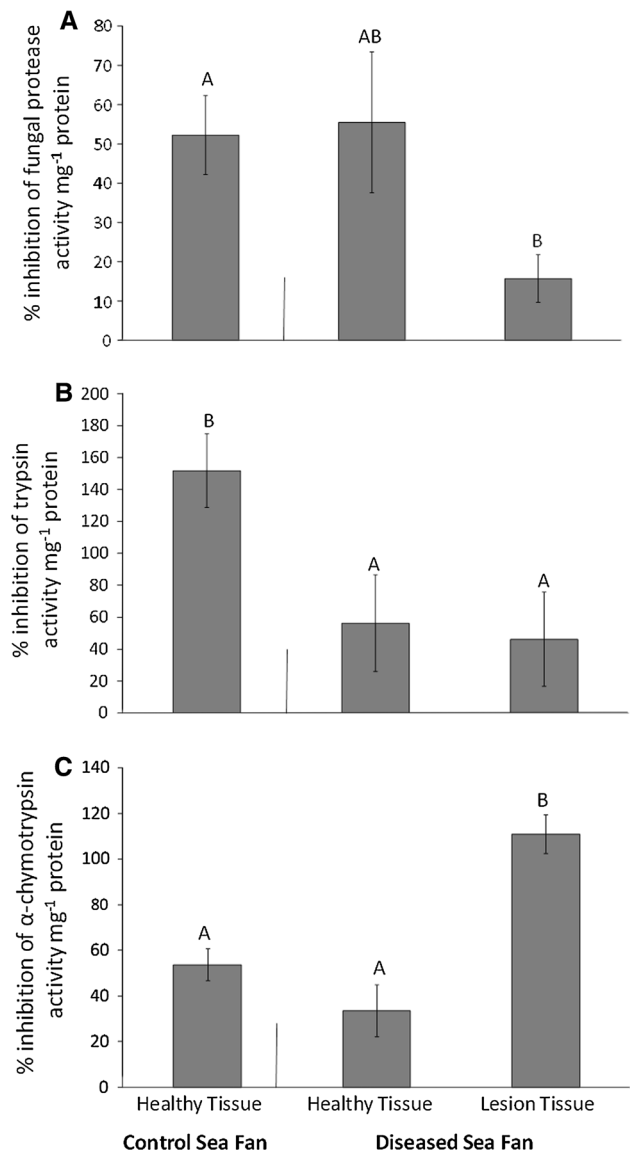


Fig. 3 Mean (\pm SE) protease inhibitor activity of **a** fungal-derived, **b** trypsin, and **c** α -chymotrypsin proteases ($n = 8$). Letters indicate statistical differences at $P < 0.05$

Siva-Jothy 2003). Furthermore, host and pathogen dynamics can be shaped by changes to environmental conditions favoring a particular side of the relationship (i.e., pathogen virulence or host immunocompetence) (Altizer et al. 2013). Through the observation of pathogen protease and host protease inhibitor activity in the sea fan—*A. sydowii* pathosystem, we highlight antagonistic mechanisms shaping host–pathogen interactions and contribute to the understanding of how climate change may influence this interaction.

Intracellular and extracellular proteases play varying roles in cellular metabolism but can also serve as potential virulence factors (Armstrong 2006). Intracellular proteases are commonly involved with cellular metabolic processes such

as protein maturation and cascade initiation (Bond and Butler 1987; McGillivray et al. 2012), while extracellular proteases are more involved in acquiring nutrients and/or evasion of host immune defenses (Hoge et al. 2010). Protease secretion (particularly of serine proteases) is common in many species of *Aspergillus* and can serve as a virulence factor in pathogenic *Aspergilli* (Hanzi et al. 1993; Wang et al. 2005). The present study confirms the presence and activity of both intracellular and extracellular serine proteases in *A. sydowii* strains isolated from sea fans, mangroves, and humans.

Increases of both extracellular and intracellular proteases can occur when there is metabolic demand for nutrients (Cohen 1973) during either stasis or stress. In the sea fan and mangrove strains of *A. sydowii* used in this study, intracellular protease activity significantly increased at 28 °C, while extracellular activity increased at 30 °C, suggesting there may be an increase in metabolic activity and need for larger amounts of nutrients at these temperatures. The temperature optima of the protein may also contribute to higher activity seen at elevated temperatures. Isolated proteases from other fungi show optimal temperatures which can exceed 35 °C (Schomburg et al. 2013). Therefore, increasing metabolic demands and/or suitable functioning temperatures for the enzymes themselves could translate into greater potential virulence and pathogenesis in the sea fan—*Aspergillus* system.

In the human *A. sydowii* strain, a clear out-group in this study, intracellular and extracellular proteases did not increase at elevated temperatures (28–32 °C). In other *Aspergillus* species that cause human diseases, optimum temperatures for growth and virulence are closer to 37 °C (Hedayati et al. 2007); therefore, the expected optimum temperature for protease activity in the human *A. sydowii* strain is beyond the ecologically relevant range examined here.

To maintain resistance to disease, the host must be able to conserve basic cellular function and produce a counteractive immune response to prevent pathogenesis (Little et al. 2005). As we shown in the first part of this study, serine proteases are present in many fungal pathogens as virulence factors to help colonize host tissue (Dunaevskii et al. 2006). Although many serine protease inhibitors (aprotinin or leupeptin) have endogenous or intracellular functions, proteins, peptides, or small molecules can also be secreted for anti-pathogenic purpose as seen in plants and invertebrates (Faisal et al. 1998; Kim et al. 2009). Our study shows that the sea fan has protease inhibitors that effectively work against trypsin, α -chymotrypsin, and fungus-derived proteases and suggests a potential for resistance against *A. sydowii* infection and proliferation.

Lower levels of trypsin and fungal protease inhibition were observed in infected sea fan tissue compared to healthy tissue from both diseased and control colonies. Conversely, α -chymotrypsin inhibitory activity was higher or induced

in the infected tissue compared to the healthy tissue of both diseased and control colonies. The varying patterns of protease inhibitor activity in diseased and healthy sea fans suggest differing functions of each inhibitor. Various types of protease inhibitors can have a distinct endogenous or exogenous function: either as intracellular regulatory factors for cellular processes (Van de Ven et al. 1993), or extracellular anti-pathogenic compounds (Xue et al. 2006).

The inhibitor activity against the fungal-derived proteases by the extracts from the infected tissue of the diseased sea fan colonies was lower in the lesion than in the healthy tissues from both the diseased and control healthy colonies. As an anti-pathogenic defense, it is unexpected that the inhibition of fungal-derived proteases would be lower in the lesion than in the rest of the colony. However, due to the variation of protease inhibitor activity in healthy tissue from diseased colonies, it is possible that this defense is acute in nature and occurs only when and where the fungus is actively invading the coral. Similar patterns of lower activity in the lesion than in the healthy tissue have also been found in protein content and peroxidase activity of diseased colonies (Mydlarz and Harvell 2007). The lesion represents the end point of an infection, and so accordingly, melanin and other cellular defenses may be the remaining active defenses. Inducible anti-pathogenic responses could be occurring earlier in the infection process, which may not be observed in the later infection as seen in the lesion. In sea fans experimentally exposed to pathogens (demonstrating this early stage of infection), induced protein and transcriptomic changes of putative anti-pathogenic processes have been observed, such as antifungal and peroxidase activity (Ward et al. 2007; Burge et al. 2013). The antifungal protease inhibitors examined here possibly serve as direct pathogenic defenses that occur early in the infection process and the resources for these processes are spent by the time the lesion is visible or detectable for collection.

The microbial flora is also essential in overall immune function of any organism. Native bacterial species are known to produce compounds that can inhibit other non-native microbes such as pathogenic fungi (Gil-Turnes et al. 1989). Since our coral extracts include all the symbiotic organisms, such as algae endosymbiont and bacteria, there may be additional contributions to the protease inhibitor activity. The natural microbial flora of the sea fan is significantly different between the lesion tissue and healthy tissue (Gil-Agudelo et al. 2006). Therefore, if the microbial community does play a role in protection against *A. sydowii*, then perhaps this protection is diminished in the lesion tissue.

Trypsin-like proteases have shown to be involved in the melanin-synthesis and prophenoloxidase cascades of many invertebrates, including corals (Cerenius and Soderhall 2004; Palmer et al. 2011). Melanin synthesis is a key mechanism in the encapsulation of pathogens and sea fans

effectively use these pathways to fight *A. sydowii* (Petes et al. 2003; Mydlarz et al. 2008). In fact, early immune responses to immunogens show strong upregulation of genes encoding a serine protease in hard corals (Weiss et al. 2013). Since the melanin-synthesis cascade including prophenoloxidase requires proteolytic cleavage to be activated, any inhibitors may prevent this. It is possible that the trypsin inhibitors in the sea fan are playing a role in the melanin-synthesis cascade rather than having direct anti-pathogenic effects. The strong systemic decrease in trypsin inhibition in the entire diseased sea fan (both lesion and healthy tissue) may actually help prevent spread of infection throughout the colony by allowing activation of the melanin-synthesis cascades.

The pattern of α -chymotrypsin inhibition was opposite to that of trypsin and fungal-derived protease inhibition and showed a strong induction in infected tissue. Although chymotrypsin-like proteases have been suggested to be involved in the melanin-synthesis cascade in insects (Sugumaran et al. 1985), α -chymotrypsin inhibitors also have other functions (McManus et al. 1994). Chymotrypsin proteases are involved in digestion and growth, which need to be regulated by protease inhibitors (Novillo et al. 1997; Sunde et al. 2001). In heavily infected tissue of the sea fan, growth and digestion are not needed and may be suppressed by the α -chymotrypsin inhibitors.

Changes in environmental conditions can alter the dynamics and relationship between hosts and associated pathogens, compromising host immunity and/or exacerbating pathogen virulence (Harvell et al. 2002; Mydlarz et al. 2010). However, under experimental conditions, elevated temperatures did not affect the protease inhibitor activity in the sea fan tissue. It is possible the duration of the experiment was not long enough to elucidate any protease inhibitor response on its own, or exposure to a pathogen is needed to act in concert with elevated temperatures.

Using *in vitro* analysis, this study demonstrates a strong theoretical link for how environmental change, such as elevated temperatures, can affect the dynamics of a host–pathogen relationship. Under current climate change conditions, increased temperatures promote *A. sydowii* protease activity while having no immediate effect on sea fan inhibitor activity. The fact that sea fans still succumb to aspergillosis and many of the largest outbreaks have followed unseasonably warm air and sea temperatures (Flynn and Weil 2009) indicates that the sea fan is still losing the disease “arms race” against *A. sydowii*. In this case, perhaps the effects of temperature on pathogen growth (Ward et al. 2007) and virulence (i.e., extracellular protease activity) shift the power toward the fungus. In the future, it is necessary to address these questions further using *in vivo* experimentation to elucidate other specific mechanisms that are changing within the host and/or pathogen under various

climate change scenarios. Nonetheless, the contribution of this study to the understanding of host–pathogen relationships among corals is important toward determining causes behind epizootics and emergence of new diseases.

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