ABSTRACT: Temperature anomalies on coral reefs now routinely exceed coral stress thresholds, making temperature a critical variable to consider in coral host–pathogen systems. While temperature is widely hypothesized to drive coral disease outbreaks by decreasing coral resistance and increasing pathogen growth rates, tests of the temperature hypothesis are rare. Here we report evidence from the sea fan coral *Gorgonia ventalina*–*Aspergillus* host–pathogen system that temperature stress increases one component of sea fan resistance. Experimentally infecting sea fan fragments while increasing temperatures to reflect summertime highs in the Florida Keys, USA, caused a 176% increase in activity of host-derived antifungal compounds. Thus, temperature stress and infection induce higher levels of resistance. However, pathogen growth rate also increases over the same temperature range, providing an opportunity for pathogen establishment before host resistance is maximal. This dual effect of temperature emphasizes the need to test intact host–pathogen systems. Given predictions for future warming events, aspergillosis is predicted to continue causing sea fan mortality in the Caribbean Sea.

KEY WORDS: Temperature · Climate · Disease · Resistance · Sea fan · *Gorgonia ventalina* · *Aspergillus sydowii* · Infection
Disease prevalence and severity (the amount of tissue lost due to disease per colony) have been monitored since 1997 in the Florida Keys, USA (Kim & Harvell 2004). More than 50% of sea fan tissue in the Florida Keys has been lost due to complete or partial disease-induced mortality (Kim & Harvell 2004). Optimal pathogen virulence, quantified as maximum growth rate, is near 30°C (Alker et al. 2001). These temperatures regularly occur in the Florida Keys from May to October (Fig. 1) and may promote new infections or facilitate pathogen spread within an infected colony (i.e. increase severity).

Infection with Aspergillus sydowii induces a generalized host resistance response that includes a suite of physical and chemical defenses (Dube et al. 2002, Petes et al. 2003, Alker et al. 2004). One component of the resistance response is production of antifungal compounds near the site of infection (Kim et al. 2000). This response is likely affected by temperature as sea fans are thermoconformers (i.e. they do not actively regulate body temperature and therefore track ambient temperature) and previous work with thermoconforming insects indicates that the innate immune response is sensitive to temperature (reviewed in Thomas & Blanford 2003).

Because temperature could influence both pathogen virulence and host resistance, it is important to test the intact host–pathogen interaction over a range of temperatures. The Gorgonia–Aspergillus system is a tractable marine host–pathogen system with which to test the effects of temperature as the hosts are abundant, the pathogen is in culture, and hosts can be successfully infected in the laboratory. We predicted that temperature stress would decrease sea fan resistance, making sea fans more susceptible to fungal infection, while increasing pathogen growth rate. Here we report results of temperature-controlled experiments and discuss these results in the context of the current epizootic and climate change.

**MATERIALS AND METHODS**

Samples were collected in August 2001 from moderately sized (30 to 60 cm), healthy sea fans at Eastern Sambo Reef in the Florida Keys, USA (24°29.74' N, 81°38.92' W). We collected four 36 cm² fragments from the outer margin of each of 18 sea fan colonies. In all cases, 1 fragment was processed (see below) immediately to establish initial conditions. Of the remaining 3 fragments, one was placed into each temperature treatment (27, 30 or 31.5°C) at Mote Marine Tropical Research Laboratory on Summerland Key, Florida. Of the 18 fragments in each of the 3 temperatures, 9 were randomly blocked within infection groups and the remaining 9 in control groups. Temperature was maintained within ±0.5°C of target temperatures using a freshwater water bath circulating through a heater/chiller and around 38 l aquaria filled with seawater collected from the reef. Each tank was a closed system to prevent pathogen release to the environment.

Fragments were acclimated for at least 24 h before experimental infection. In each temperature treatment, 9 fragments were inoculated with Aspergillus sydowii using gauze strips covered with agar and fungus, pushed through 1 cm slits cut in the sea fan mesh. The remainder received control gauze/agar strips with no fungus. Control and treatment gauze strips were left on fragments for 7 d at which time each fragment was processed for zooxanthellae abundance and crude extract antifungal activity (a measure of host resistance; Kim et al. 2000).

**Zooxanthellae abundance.** A 1 cm diameter piece of tissue from each fragment was homogenized in 1 ml seawater using a Kontes 22 glass homogenizer and Teflon pestle attached to a variable speed power drill. Tissue homogenate was washed twice with seawater and resuspended in 1 ml of 5% formalin. Four 30 μl aliquots were removed from each sample, stained with 2.75 μl Lugol’s solution, and counted on a brightline hemacytometer at 40× magnification.

**Fungal inhibition.** Fungal inhibition of crude extracts was tested with methods modified from Alker et al. (2001) and Dube et al. (2002). Crude antifungal compounds were extracted from 16 cm² tissue samples twice overnight at –20°C in dichloromethane (DCM),
which was then evaporated under continuous N₂ flow. Extracts were weighed and resuspended in acetone to a final concentration of 20 mg ml⁻¹. All extracts were tested at an incubation temperature of 27°C, regardless of the temperature at which the sea fan fragments were held in the aquaria. This allowed comparison of activity of extracts across temperature treatments without the confounding effects of differences in fungal growth rates at different incubation temperatures.

We added 75 μl of each extract suspension to the surface of 3 replicate 35 mm diameter Petri plates containing peptone yeast glucose (PYG) agar (1 l deionized water, 1.25 g peptone, 1.25 g yeast extract, 3.0 g glucose, 30 g Instant Ocean®, 20 g bactoagar, 0.05 g tetracycline) and spread with a glass rod. Unamended agar plates, acetone-amended plates, and plates with hygromycin (an antifungal agent) served as controls. We placed 2 μl of stock spore solution (2250 spores μl⁻¹) in the center of each plate. Plates were closed with the self-sealing film, Parafilm, and incubated at 27°C for 6 d.

Digital images of circular fungal colonies were taken 4, 5, and 6 d post-inoculation to avoid a short lag in colony growth that occurs after inoculation (Alker et al. 2001). Fungal colony area was calculated from 2 perpendicular measurements of colony diameter with digital imaging software (NIH Image v 1.62, National Institutes of Health). Antifungal activity was calculated as inhibition of fungal growth on extract-amended plates relative to unamended and acetone-amended control plates (Dube et al. 2002) as follows:

\[ I = 1 - \frac{\text{Area}_{\text{extracts}}}{\text{Area}_{\text{controls}}} \]

where \( \text{Area}_{\text{extracts}} \) = area of fungal colonies on extract-amended plates, and \( \text{Area}_{\text{controls}} \) = mean area of fungal colonies on unamended and acetone-amended control plates. Both sets of control plates were used since there was no difference in fungal growth on the unamended and acetone-amended plates (data not shown). The change in inhibition from initial fragments was used in analyses (\( \Delta I = I_{\text{Final}} - I_{\text{Initial}} \)).

**Results**

Zooxanthellae abundance

Both temperature and infection affected the abundance of zooxanthellae in sea fan tissue (Fig. 2; temperature \( F = 139.85, p < 0.0001 \); infection \( F = 21.05, p = 0.0003 \); temperature \( \times \) infection \( F = 4.30, p = 0.0148 \)). The combination of thermal stress (31.5°C) and infection resulted in a 31.4% loss of zooxanthellae (Tukey-Kramer post-hoc tests \( p < 0.0001 \)). In all other treatments, zooxanthellae increased with the largest increase occurring in 27°C control fragments (105.5%) and the smallest in 31.5°C control fragments (19.7%; Tukey-Kramer post-hoc tests \( p < 0.0001 \)). All infected fragments contained fewer zooxanthellae than respective controls (Fig. 2).

Field surveys indicated that similar losses in zooxanthellae occurred in naturally infected sea fans (Fig. 3). Healthy sea fans contained more zooxanthellae than healthy areas of diseased sea fans. Healthy areas of
diseased sea fans in turn contained more zooxanthellae than diseased areas of diseased sea fans (ANOVA, $F = 98.91$, $p < 0.0001$, Tukey-Kramer post-hoc tests $p < 0.0001$).

**Fungal inhibition**

Temperature and infection affected inhibitory activity of host-derived extracts with a significant temperature $\times$ infection interaction (Fig. 4; temperature $F = 4.91$, $p = 0.009$; infection $F = 5.85$, $p = 0.03$; temperature $\times$ infection $F = 9.57$, $p = 0.0001$). Little to no change in inhibition occurred in controls while inhibition increased in all infected fragments. The greatest increase in inhibition occurred in infected fragments at the warmest ($31.5^\circ$C) temperature (Tukey-Kramer post-hoc tests $p < 0.05$).

**DISCUSSION**

Understanding the mechanisms underlying host–pathogen interactions, including the synergistic roles of abiotic stressors and disease, is essential to predict and mitigate disease outbreaks (Lafferty & Holt 2003). In corals, which live near their upper thermal thresholds on many reefs (Porter & Tougas 2001), host and pathogen performance curves may peak at different temperatures, with pathogen optima occurring at higher temperatures than host optima (i.e. host resistance is lower than pathogen growth or virulence; Fig. 5A). Pathogens may also adapt to temperature increases more rapidly than their hosts due to more rapid growth (Chubb 1980) and shorter generation times (Pojmanska et al. 1980).

Counter to the prediction, we found that sea fan host resistance (measured as inhibitory activity of crude extracts) was almost 2-fold greater at warmer ($31.5^\circ$C) relative to cooler ($27$ and $30^\circ$C) temperatures 7 d post-

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**Fig. 3. Gorgonia ventalina.** Zooxanthellae abundance (±SE) in healthy and naturally infected sea fans sampled on Pickles Reef. Samples 1 cm in diameter were collected from completely healthy sea fans (open bar), healthy portions of diseased sea fans (HD, light gray bar), and diseased portions of diseased sea fans (DD, dark gray bar), $N = 20$. Different letters above bars indicate significant differences.

**Fig. 4. Gorgonia ventalina.** Change in extract inhibition activity (±SE). □ $= 27^\circ$C; ○ $= 30^\circ$C; Δ $= 31.5^\circ$C
The pathogen is also temperature sensitive, growing >25% more rapidly at 30 to 31°C than at 27°C (Fig. 5B). This may provide an opportunity for the pathogen to infect a host at summer temperatures (near 30°C, Fig. 1) that promote pathogen growth but not host resistance (Fig. 5B). Once established, the pathogen is concentrated in the proteinaceous gorgonin axis (Petes et al. 2003) where it may avoid host antifungal compounds circulating in the coenenchyme.

It is also possible that host resistance is not constant through time. In some instances, after induction of a response to herbivores or pathogens, a relaxation in the response occurs. Evidence from other studies suggests that sea fan host resistance does not decline over time as the activity induced in warm (31.5°C) infected fragments is comparable to that found near disease lesions on naturally infected sea fans (Kim et al. 2000, Dube et al. 2002). The naturally infected sea fans were likely infected for weeks to months before sampling, given lesion formation and amount of tissue purpling apparent. This indicates that induced levels of antifungal activity are sustainable over time.

An essential component of coral health is the association of the coral animal with endosymbiotic zooxanthellae. Zooxanthellae increased in all control and in the cooler (27 and 30°C) infected fragments (Fig. 2), The increase may have occurred as autotrophy increased to compensate for reduced heterotrophy, (i.e. acquisition of energy from phyto- and zooplankton; Ribes et al. 1998) since closed-system tanks were not amended with plankton during the experiment. The increase in zooxanthellae may also have been in response to wounding as zooxanthellae densities have been shown to peak at wound sites on a related gorgonian coral 7 d post-injury (Meszaros & Bigger 1999). If so, this suggests an important role of temperature in regulating wound healing in gorgonian corals.

Both temperature and infection affected the magnitude of changes in zooxanthellae abundance, with a significant temperature × infection interaction. This was particularly evident in the loss of zooxanthellae with infection at the warmest (31.5°C) temperature (Fig. 2). Naturally infected sea fans sampled on the reef also have fewer zooxanthellae, in both healthy and diseased areas of the infected sea fan, relative to those in completely healthy sea fans (Fig. 3), suggesting that infection causes at least partial bleaching. Reduction of zooxanthellae abundance in infected tissue is likely due to a reduction in number of polyps in infected areas (Kim & Harvell 2002). It is not possible to distinguish from these data whether lower zooxanthellae abundance in healthy tissue of infected colonies occurred before, or was a consequence of, infection.

The fragments infected at 31.5°C and naturally infected sea fans had lower zooxanthellae abundances than control fragments or healthy sea fans, respectively (Figs. 2 & 4). However, high levels of antifungal activity were associated with 31.5°C infected fragments (Fig. 4) and lesions on naturally infected sea fans (Kim et al. 2000). These results suggest that zooxanthellae do not play a critical role in production of antifungal compounds. However, the identity of zooxanthellae in coral tissue can be as important as their abundance. Symbiont shuffling, or acquisition of different, more stress-tolerant clades, has been suggested as a process of host acclimation to temperature stress (Baker et al. 2004).

The effects of temperature and infection on the zooxanthellae clade were investigated in a separate study (Kirk et al. 2005). Symbiont associations were stable and identical in healthy, experimentally infected, and naturally infected sea fans (Fig. 3), suggesting that symbiont shuffling does not occur in sea fans regardless of temperature or disease stress (Kirk et al. 2005).
The factors that control pathogen transmission and spatial distribution of infections in marine systems are often unknown. Sea fan aspergillosis is patchily distributed with high prevalence and severity at some sites, but not others, along the same reef tract (Jolles 2002, Kim & Harvell 2004). One hypothesis to explain patchiness of aspergillosis is that environmental stressors such as temperature and nutrient enrichment (Bruno et al. 2003) facilitate infections at some sites. Experimentally testing the role of these abiotic factors on host–pathogen interactions may reveal approaches for disease control.

Temperature can have complex effects on disease dynamics (Lafferty & Holt 2003). In some cases, hosts may become more susceptible to infection while in others they are released from infection (Harvell et al. 2002, Lafferty & Holt 2003). We found that short-term (i.e. 7 d) thermal stress increases host resistance to infection (quantified as fungal inhibition by crude extracts) in sea fan corals. However, pathogen growth rate increases more rapidly than host resistance over the same temperature range (Alker et al. 2002, Lafferty & Holt 2003). We found that pathogen growth rate increases more rapidly than host resistance over the same temperature range (Alker et al. 2001). In addition, constitutive levels of host resistance (Kim et al. 2000) and induction of resistance with temperature stress are not uniform within the sea fan colony (J. Ward pers. obs.), providing an opportunity for rapid pathogen infiltration in less-resistant areas of the colony during periods of warm (30°C) summer temperatures. Prevalence of aspergillosis is currently low in the Florida Keys (Kim & Harvell 2004). However, given the thermal sensitivity of this host–pathogen system and predictions of more frequent, longer duration thermal anomalies (Hoegh-Guldberg 1999), we predict that this disease will continue to affect sea fans in the Caribbean Sea.

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