COMMUNITY ECOLOGY – ORIGINAL RESEARCH

Octocoral co-infection as a balance between host immunity and host environment

Allison M. Tracy[1] · Ernesto Weil[2] · C. Drew Harvell[1]

Abstract

Co-infection is the reality in natural populations, but few studies incorporate the players that matter in the wild. We integrate the environment, host demography, two parasites, and host immunity in a study of co-infection to determine the drivers of parasite interactions. Here, we use an ecologically important Caribbean sea fan octocoral, *Gorgonia ventalina*, that is co-infected by a copepod and a labyrinthulid protist. We first expanded upon laboratory studies by showing that immune suppression is associated with the labyrinthulid in a natural setting. Histological analyses revealed that immune cells (amoebocytes) were significantly suppressed in both labyrinthulid infections and co-infections relative to healthy sea fans, but remained unchanged in copepod infections. However, surveys of natural coral populations demonstrated a critical role for the environment and host demography in this co-infection: the prevalence of copepod infections increased with sea fan size while labyrinthulid prevalence increased with water depth. Although we predicted that immune suppression by the labyrinthulid would facilitate copepod infection, the two parasites did not co-occur in the sea fans more often than expected by chance. These results suggest that the distinct ecological drivers for each parasite overwhelm the role of host immune suppression in determining the distribution of parasites among hosts. This interplay of the environment and parasite-mediated immune suppression in sea fan co-infection provides insights into the factors underlying co-occurrence patterns in wild co-infections. Moving forward, simultaneous consideration of co-occurring parasites, host traits, and the environmental context will improve the understanding of host – parasite interactions and their consequences.

Keywords Disease ecology · Parasite interactions · *Gorgonia ventalina* · Labyrinthulid · Eco-immunology

Introduction

Hosts in wild populations are usually infected with more than one parasite at the same time (Rigaud et al. 2010; Tollenaere et al. 2016). These co-infecting parasites can influence each other through facilitative or antagonistic interactions (Graham 2008; Telfer et al. 2010; Hellard et al. 2015).

✉ Allison M. Tracy
amt279@cornell.edu

1 Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY 14853-2601, USA

2 Department of Marine Sciences, University of Puerto Rico, Mayagüez, PR 00680, USA

Depending on the nature of this interaction, co-infection can benefit or impair the success of each parasite. Co-infection can also influence the host population differently than in single infections with each parasite. For example, the presence of multiple parasites compromised host health when cassava plants were infected with different viral strains (Zinga et al. 2013) and led to elevated host mortality when African buffalo were infected with both worms and bovine tuberculosis (Jolles et al. 2008).

Given the importance of parasite interactions for the outcome of co-infection, laboratory studies have sought to determine what factors lead to facilitation and antagonism in co-infections. The results highlight central roles for resource competition and immune-mediated interactions between parasites (Hellard et al. 2015). However, field studies are necessary to understand how the environment and host characteristics influence co-infecting parasites in a natural setting. Although co-infection is the reality in natural populations, there are few studies on parasite interactions in the

field that incorporate the environment or host characteristics, such as immunity, host density, or demography. Moreover, the studies that take this approach are limited to just a few systems, including helminths and bovine tuberculosis in buffalo (Jolles et al. 2008; Henrichs et al. 2016), microparasites in voles (Telfer et al. 2010), helminths in rabbits (Hernandez et al. 2013), and helminths and anthrax in zebras (Cizauskas et al. 2014). There are fewer examples in invertebrates and plants compared to mammals (Honkavaara et al. 2009; Ezenwa 2016; Hajek and van Nouhuys 2016; Tollenaere et al. 2016), and no studies in the ocean. Given the paucity of field studies and the narrow taxonomic focus thus far, it is unclear how ecological factors work in concert with parasite resource competition and host immunity to determine the consequences of co-infection for hosts and parasites in wild populations.

Early research on wild co-infections has identified a suite of factors that affect parasite interactions in a natural setting. Consistent with laboratory studies, indirect parasite interactions via the host immune system and resource competition are paramount. Studying within-host mechanisms is critical for understanding wild co-infections because it provides insight beyond correlations. Correlations alone provide incomplete or even misleading information about parasite relationships and the consequences for host health (Fenton et al. 2014). For example, helminths and bovine tuberculosis are negatively correlated in African buffalo. However, this negative correlation results not from an antagonistic interaction, but rather from facilitation. The immune response of African buffalo to helminths constrains the host's defenses against bovine tuberculosis, facilitating the invasion of this microparasite (Jolles et al. 2008; Ezenwa et al. 2010). The parasites negatively co-occur in spite of immune-mediated parasite facilitation due to elevated mortality of the hosts during co-infection (Jolles et al. 2008). Likewise, Henrichs et al. (2016) highlight the importance of resource use through a study of haemoparasite co-infections of African buffalo. In this case, two parasites are negatively correlated. Because they are known to overlap in their use of blood cells, these parasites could be segregating due to competition or niche partitioning (Henrichs et al. 2016).

While immunity and resource competition matter in both the lab and the wild, field studies indicate that environment and demography play a role, and may even reverse expected outcomes for parasite co-occurrence. For example, the aforementioned trade-off in immune defenses against helminths and bovine tuberculosis in African buffalo occurs only during the dry season, suggesting that it is environmentally mediated. Demographic factors, specifically age, may also modify co-infection dynamics in this system because helminth infection is higher in younger animals while tuberculosis prevalence is higher in older animals (Jolles et al. 2008). For zebras in Etosha National Park,

immune-mediated parasite interactions and environmental factors together lead to unexpectedly high levels of anthrax infections in the wet season, a departure from the typical dry season outbreaks (Cizauskas et al. 2014). Cizauskas et al. (2014) hypothesize that increases in helminth infections in the wet season drive the unusual increases in anthrax through immune trade-offs between type 1 T-helper and type 2 T-helper cells during co-infections (Cizauskas et al. 2014). Further study in other natural ecosystems and host taxa are bound to uncover additional ways that environmental factors modify interactions between co-infecting parasites.

The clear importance of temperature, host density, and host immunity for coral disease suggests that multiple factors are likely to influence wild co-infections in these marine invertebrates (Harvell et al. 2007; Altizer et al. 2013; Lafferty and Harvell 2014). In this study, we untangle the drivers of parasite interactions in a wild population of the Caribbean sea fan octocoral, *Gorgonia ventalina*. To our knowledge, this is the first study of host immunity in co-infected hosts of a wild marine system. Sea fans are a tractable system for studying the importance of multiple factors in co-infection because there are more than 20 years of research on their wild pathogens and immune capabilities (Kim and Harvell 2004; Burge et al. 2012, 2013a, b; Ivanenko et al. 2015; Weil et al. 2017). Studies have shown that sea fans can respond to infection using amoebocytes (a type of immune cell), melanization, and other immune responses (Mydlarz et al. 2008; Couch et al. 2013). Amoebocyte density is one of the best immune metrics in sea fans because it is significantly higher in sea fans with fungal infections, attenuates with increasing distance from a lesion (Mydlarz et al. 2008), and peaks in response to infection (Couch et al. 2013). The melanization associated with amoebocytes is thought to function as a physical–chemical barrier to infection (Mydlarz et al. 2008). In addition to the advances in disease ecology in sea fans, they are also important organisms on an ecosystem level. Sea fans and other octocorals may be especially critical for the future of reefs because they have greater resilience than scleractinian corals in the face of environmental stressors (Aronson and Precht 2001; Raymundo et al. 2008; Miller et al. 2009; Weil and Rogers 2011; Tsounis and Edmunds 2017).

The sea fans in La Parguera, Puerto Rico, are infected with a labyrinthulid protist (Fig. 1a) and a copepod (Fig. 1b). The copepod is linked to an emerging disease, multifocal purple spots (MFPS), which was first observed in 2005 in Mexico and later appeared in many Caribbean localities (Fig. 1c; Ivanenko et al. 2015; Weil and Rogers 2011; Weil et al. 2017). The copepod is found encysting in the host's mesoglea, or connective tissue, with evidence of infection by multiple life stages at once (Fig. 1b; Ivanenko et al. 2015). On the outside of the sea fan, the characteristic signs of MFPS are small purple spots of 1–3 mm in size that can
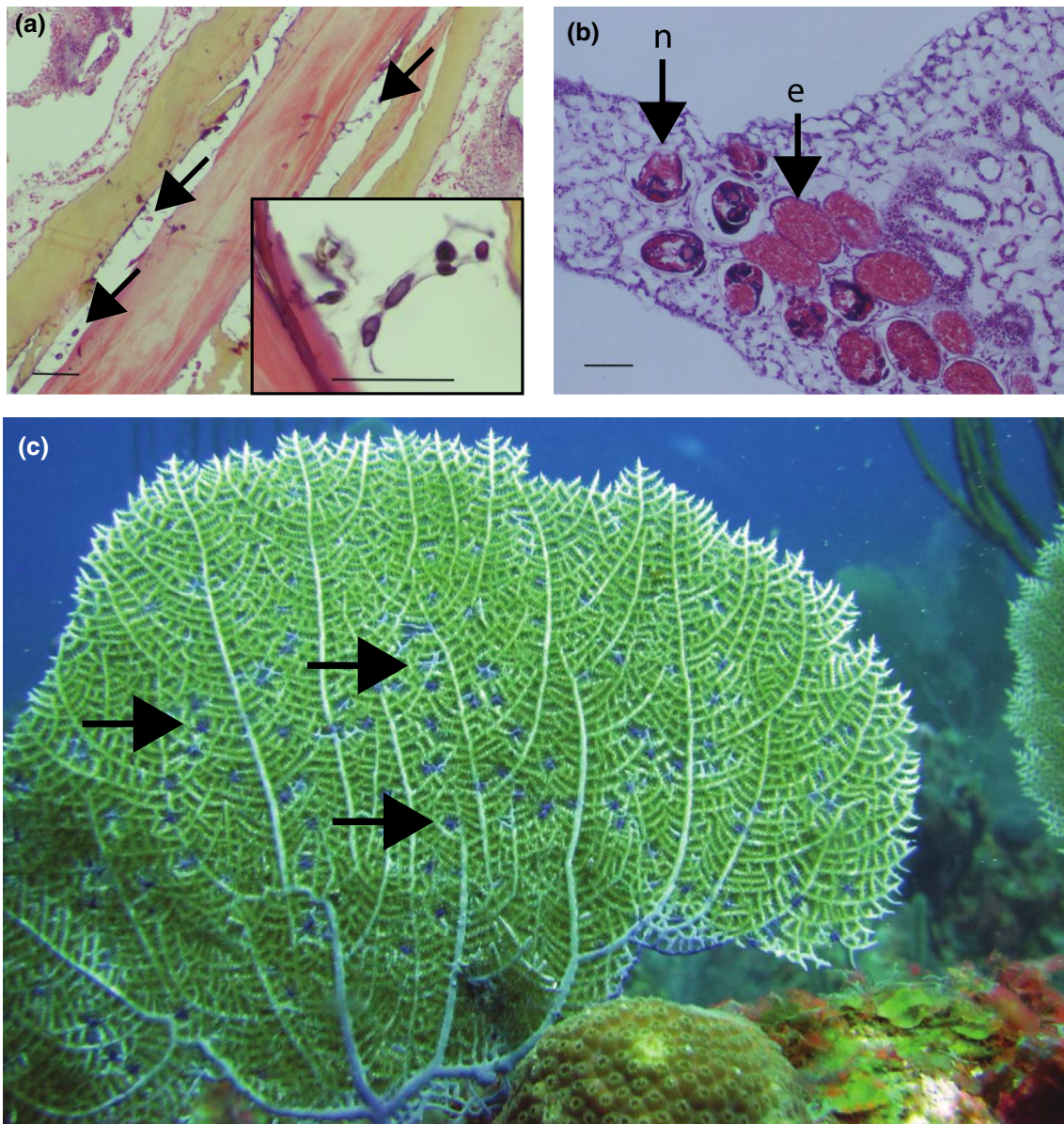
Fig. 1 *Gorgonia ventalina*, the Caribbean sea fan octocoral, can be infected with multiple parasites. **a** The labyrinthulid parasite is a protist that infects the gorgonin skeleton. The higher magnification inset shows the mucus net around the ovoid parasites. **b** The copepod parasite has characteristics clusters of what appear to be different stages of eggs ("e") and nauplius larvae ("n") (hematoxylin and eosin stain, scale bars = 50 um). Arrows point to the respective parasites. **c** Multifocal purple spots (MFPS), examples of which are identified by arrows, is an emerging disease linked to copepod infection (Color version available online)

be present in small numbers or peppering the entire colony (Weil and Hooten 2008). The purpling is evidence of a generalized defensive response in sea fans, which they use against threats from macroalgae to fungal infection (Alker et al. 2004). The labyrinthulid parasite is related to the thraustochytrid parasite of hard clams, quahog parasite unknown (QPX) (Burge et al. 2012), as well as a seagrass labyrinthulid (Sullivan et al. 2017). In sea fans, labyrinthulid infections occur first in the gorgonin, or skeletal matrix, and can progress to severe lesions that destroy sea fan polyps

(Burge et al. 2013a). Exposure to the labyrinthulid is known to alter immune gene expression in *G. ventalina* (Burge et al. 2013b). In a functional study of labyrinthulid infection and sea fan immunity, Mann (2014) found that the labyrinthulid parasite suppressed antibacterial activity, antifungal activity, and other defenses of the sea fan host (Mann 2014). In contrast to fungal infections, where amoebocytes are induced and melanization serves as a physical–chemical barrier, the labyrinthulid suppressed key steps in the melanization cascade (Mann 2014).

Given this evidence for immune suppression by the labyrinthulid, labyrinthulid infections could be partly responsible for the emergence of MFPS in sea fans through parasite–parasite facilitation. We measured amoebocytes as a metric of host immunity to assess labyrinthulid-mediated immune suppression in a natural field setting and to evaluate the consequences for co-infection in *G. ventalina*. We hypothesized that labyrinthulid infections would be associated with suppressed immunity, and a higher incidence of copepod infections. Recognizing that environment and demography can lead to unexpected patterns in co-infection, we also investigated the role for depth, temperature, coral cover, host density and host size in driving prevalence and severity for both parasites. To address immune-mediated parasite interactions, we asked whether amoebocyte density varied with infection status, the environment, and host demography. This approach integrates important information about the context of co-infection to understand patterns of parasite co-occurrence and the outcome of co-infection in a wild host.

Materials and methods

Study sites and *Gorgonia ventalina* surveys

G. ventalina colonies ($N = 1056$) were surveyed at 10 different reef sites in La Parguera Natural Reserve off the southwest coast of Puerto Rico from 19 June 2013 to 5 July 2013 to test the association of the environment, host demography, and infection status with parasite prevalence, severity, and host immunity (Table 1 and Fig. 5, Online Resource 1). Site selection was haphazard with respect to sea fan health, but contingent on the presence of *G. ventalina* colonies. At each site, we marked three permanent 10×2 m belt transects beginning with a haphazard endpoint. Depth was measured at the center of each transect using calibrated depth gauges. 1 m long PVC sticks were used on each side of the transect tape to survey a standard width of 2 m along the 10 m long tape (a 20 m² band transect). Three sea fans with external signs of MPFS and three apparently healthy sea fans were tagged on each transect using non-invasive numbered tags with thin cable ties ($N = 60$).

We first estimated the area of every sea fan on each transect ($N = 1056$) as the product of the height and the perpendicular width. This metric was used for sea fan size. Live coral cover was assessed as the proportion of live coral and octocoral cover using the line-intercept method (Loya 1972). In short, coral cover is the proportion of the midline of the transect that consisted of octocorals and scleractinians. Sea fan density was calculated as the total number of sea fans in the 20 m² band transect. The health status of each sea fan on each transect was checked using the guidelines in the Coral Disease Handbook (Raymundo et al. 2008) and the Underwater Cards for Assessing Coral Health on Caribbean Reefs (Weil and Hooten 2008). We distinguished between diseases and other compromised health signs involving tissue loss, injuries, and discolorations using a key and a decision tree. MFPS was identified using the characteristic signs of the disease, conspicuous oblong purple lesions of 1–3 mm on the outside of a sea fan (Fig. 1; Weil and Hooten 2008; Burge et al. 2012). In each affected sea fan, the number of spots was counted to assess MFPS severity. We counted spots individually up to 50 spots per sea fan, above which we categorized MFPS severity as "between 50 and 100 spots" due to time constraints underwater.

Temperature loggers recorded data at each site every 30 min from June 19 to July 31 2013. We used two kinds of temperature loggers, both of which are commonly used in ecological studies and have been shown to be comparable (Angilletta and Krochmal 2003). We fastened Thermochron iButton loggers (Embedded Data Systems, Lawrenceburg, KY, USA; DS1921G-F5#; Table 1, Online Resource 1) 0.5–1 m above the substrate at six of the sites. HOBO® temperature loggers (Bourne, MA, USA) have been recording temperatures every 2 h for several years at the other four sites. We averaged the data for each site for statistical analyses.

Sample collection and histology

We collected cross-sectional data on the population by taking one sample of each of the 60, tagged sea fans to diagnose disease and quantify amoebocyte density. The following three samples were collected from each of the three transects at a given site: one 4×4 cm sample from an apparently healthy sea fan and two 4×4 cm samples (one with disease signs of MFPS, one asymptomatic) from a sea fan with MFPS (General Collection Permit of the Department of Marine Sciences, University of Puerto Rico). In total, we collected 9 samples across 6 sea fan colonies at each of the 10 sites ($N = 90$ samples, $N = 60$ sea fan colonies).

We collected samples for disease diagnosis because we can only verify the presence of the labyrinthulid and the copepod in sea fan tissue using histology. While external signs of MFPS are indicative of copepod infection (Fig. 1c), histology provides a more robust diagnosis. For the labyrinthulid, histological evidence is the only reliable method for diagnosis as this parasite lacks clear external signs of disease.

Samples were cut from the sea fans using titanium scissors to minimize damage and transported them to the laboratory in Ziplocs with seawater to stabilize temperature until processing at the University of Puerto Rico at Mayagüez's marine station on Isla Magueyes. We photographed and preserved 4 cm² of each sample in 10% seawater-buffered

formalin for histology. After 24 h, the samples were switched to 70% ethanol and then shipped to Cornell University in ethanol-dampened paper towels. Samples were decalcified in a 50/50 mix of 10% citric acid and 30% formic acid followed by a 6-h water rinse. The Cornell University Veterinary Histology Laboratory embedded the samples in paraffin wax, sectioned them into 5 μm sections, mounted them on slides, and stained them with hematoxylin and eosin. Colonies checked a year after the collections all showed signs of regrowth in the cut areas.

Prior to analysis of the histology slides, the identity of each sample was confirmed using the photographs and the location of the purple spots on the tissue sample was noted. Diagnosis of labyrinthulid infection relied on previous histological classification linked to molecular identification (Burge et al. 2012; Fig. 1a). We identified the copepod using characteristic structures described in Ivenanko et al. (2015; Fig. 1b).

Amoebocyte density was analyzed in healthy samples by photographing 12, random coordinates at 40 × with an Olympus BH-2 compound light microscope system. For diseased samples, we photographed coordinates in four directions within 1 mm of 1–3 lesions because the response attenuates after this distance (Mydlarz et al. 2008). Amoebocyte density was then quantified by calculating the amoebocyte area as a percentage of the total image surface area in ImageJ, as described in Couch et al. (2013).

Data analyses

All analyses were conducted in R (version 3.1.2, R Development Core Team 2014). We analyzed several predictor variables for disease prevalence, disease severity, and amoebocyte density. Five variables are shared among these models: the environmental variables temperature, depth, and coral cover; and the demographic variables sea fan density (hereafter "density") and sea fan area (hereafter "size"). For the sixth variable, we analyzed labyrinthulid prevalence as a predictor of copepod prevalence, copepod prevalence as a predictor of labyrinthulid prevalence, and infection status (zero, one, or two parasites) as a predictor of amoebocyte density. We used the survey data for MFPS prevalence ($N = 1056$ sea fans) given the external signs, but a subset of 60 sea fans with histology data for labyrinthulid prevalence, copepod prevalence, and amoebocyte density ($N = 978$ photos). In a separate analysis excluding sea fans with labyrinthulid infections, we tested whether MFPS tissue status predicted amoebocyte density better than the null model using the likelihood ratio test (LRT) to evaluate the relevance of the amoebocyte response for MFPS.

In each analysis, we confirmed a lack of co-variation of the predictor variables, re-scaled numeric variables (Gelman 2008), and used step-wise model selection. We included only additive terms for labyrinthulid prevalence and amoebocyte density due to the low number of labyrinthulid infections. For all other linear models, we included additive, interactive, and quadratic terms. Models of MFPS, copepod, and labyrinthulid prevalence were fit using binomial generalized linear mixed-effect models (GLMM) with maximum likelihood estimation and site as a random effect (Bates et al. 2014). Transect was further nested within site for MFPS prevalence. For MFPS severity, we log-transformed the response variable to address overdispersion and then used the censReg package for censored data, with site as a random effect, to address the bias introduced by counting a maximum of 50 spots (Henningsen 2013). For analyses of the immune cells, amoebocyte density was square-root transformed to meet linear mixed model (LMM) assumptions of normality and homogeneity of variance. Sea fan colony nested within transect nested within site was used as a random effect (Bates et al. 2014). In each case, the best model was selected with Akaike's information criterion (ΔAIC) and parsimony (Bolker 2008). We used a significance level of alpha = 0.05 in GLMMs, FDR-adjusted p values in multiple comparisons (R package multcomp), and 95% confidence intervals for LMMs and censReg (Hothorn et al. 2008). PiecewiseSEM allowed us to calculate the marginal R^2, the variance explained by the fixed effects in the best model (Lefcheck 2015). We calculated confidence intervals and odds ratios for logistic regressions (GLMM) in lme4. For LMM and censReg, we used the confint function to calculate confidence intervals for the best model and pairwise comparisons. Effect sizes are reported as raw estimates due to non-normality.

We conducted Chi-square analyses to determine whether co-infection with the copepod and the labyrinthulid was more or less frequent than expected by random chance. We considered MFPS and the copepod to be linked due to previous studies, and because we confirm that the presence of MFPS significantly predicts the presence of the copepod in sea fans (estimate = 4.39, CI 2.62, 7.37; $P = 6.2$ E-05; OR 81.0; Table 11, Online Resource 1). In histology samples, the copepod was detected at the same spot on the sea fan tissue as the 1–3 mm purple lesions characteristic of MFPS in 80% of the samples with MFPS (Fig. 1). These observations support a causal relationship between MFPS and the copepod, as suggested in Ivanenko et al. (2015).

Results

Environment and demography predict parasite prevalence

All the metrics of disease and immunity depended on environmental factors, demographic factors, or both. Overall,

copepod prevalence was predicted by the size of the sea fan (Fig. 2b) while labyrinthulid prevalence was predicted by the depth of the transect (Fig. 2c). However, the correlates segregated for the two parasites as copepod prevalence was not predicted by depth (Fig. 2d) and labyrinthulid prevalence was not predicted by sea fan size (Fig. 2a). The best model of labyrinthulid prevalence includes depth alone, explains 68.6% of the variance, and shows that prevalence increases significantly with increasing depth ($N = 60$, Fig. 2c; GLMM; $P = 0.0027$; estimate = 5.37, CI 2.35, 10.1; OR = 214; Table 2 and 3, Online Resource 1). The best model of copepod prevalence includes size, explains 25.1% of the variance, and shows that prevalence increases with sea fan size ($N = 60$, GLMM; $P = 0.0091$; estimate = 2.84, CI 0.827, 5.13; OR = 17.1; Table 4 and 5, Online Resource 1). Larger sea fans are more likely to be infected. The best model also includes the quadratic term, $size^2$, but while there is indeed a significant decrease in copepod prevalence in the very largest sea fans (GLMM; $P = 0.044$; estimate = − 2.25, CI − 4.89, − 0.539; OR = 0.105; Table 5, Online Resource 1), this decrease disappears when the two largest sea fans are omitted with a best model that explains 50.0% of the variance ($N = 58$, Table 6, Online Resource 1). Thus, the most robust result is that copepod prevalence increases with size (Fig. 2b; GLMM; $P = 0.0026$, estimate = 3.11, CI 1.31–5.42; OR = 22.4; Table 6, Online Resource 1). As further confirmation, the best model of MFPS includes size and depth and explains 15.3% of the variance ($N = 1056$, Table 7, Online Resource 1). Just as in the models of copepod prevalence, MFPS prevalence increases with increasing

sea fan size (GLMM; $P < 2.0$ E-16; estimate = 2.10, CI 1.65, 2.56; OR = 8.14), except for the largest sea fans in approximately the top 20% (GLMM; $P = 1.3$ E-06; estimate = − 0.826, CI − 1.18, − 0.519; OR = 0.438; Table 8, Online Resource 1). In addition to changing with size, MFPS prevalence decreases in sea fans at greater depths (GLMM; $P = 0.00099$, OR = 0.424). In analyses of MFPS severity, the best model also includes both size and depth, but only size is significant with an increase in MFPS severity in larger sea fans (censReg; $P = 3.3$ E-04; estimate = 0.527, CI 0.239, 0.815; Table 9 and 10, Online Resource 1). Temperature was not a significant predictor for disease or immunity. The temperature values for all sites spanned a range of 1.02 °C and did not separate by logger type.

Infection status and the environment predict immune cell density

The best model of amoebocyte density includes coral cover, depth, and infection status (labyrinthulid, copepod, neither, or co-infected; $N = 60$, Table 12, Online Resource 1). This model explains 10.6% of the variance in amoebocyte density. Amoebocyte density increases significantly with depth (estimate = 0.414, CI 0.0389, 0.789) and decreases significantly with coral cover (estimate = − 0.370, CI − 0.667, − 0.0720; Table 13, Online Resource 1).

In all cases, amoebocyte density is suppressed in infected tissue, but the magnitude depends on the parasite. In pairwise comparisons, amoebocyte density is 18.7% lower in sea fans infected with the labyrinthulid, which have a mean

Fig. 2 **a** Size is not a significant predictor of labyrinthulid prevalence; **b** however, size is a significant predictor of copepod prevalence (GLMM; $P = 0.0091$; estimate = 2.84, CI 0.827, 5.13; OR = 17.1; Table 6, Online Resource 1). Depth is a significant predictor of (**c**) labyrinthulid prevalence (GLMM; $P = 0.0027$; estimate = 5.37, CI 2.35, 10.1; OR = 214; Table 2 and 3, Online Resource 1); **d** but not copepod prevalence. (Color version available online)
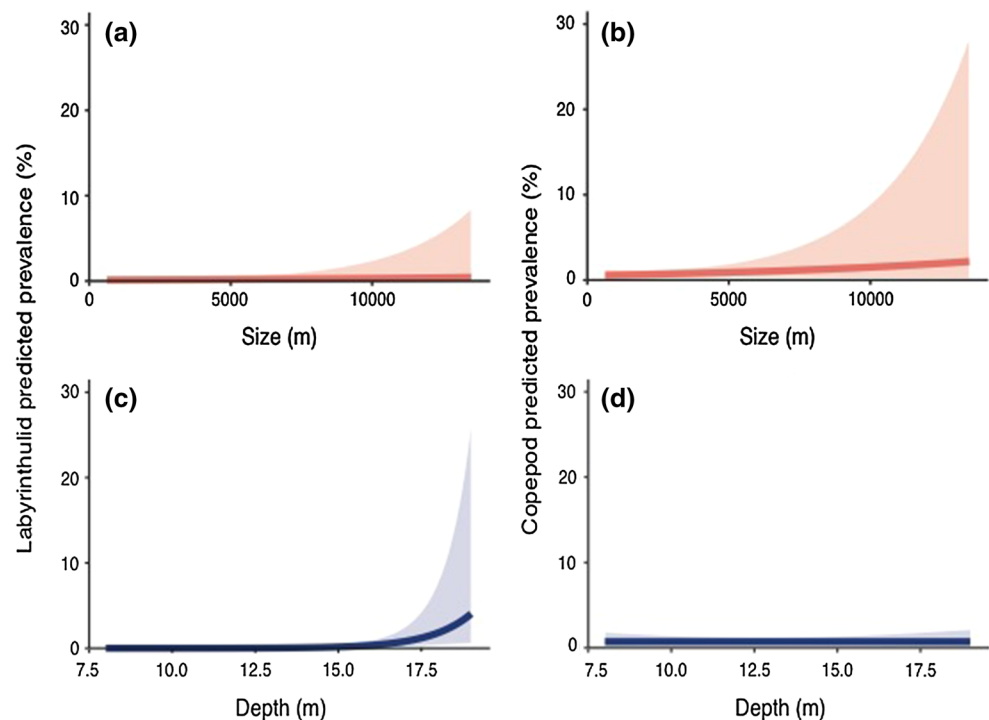
density of 14.8%, relative to uninfected sea fans with a mean density of 18.2% (General linear hypothesis test; $P = 0.026$; estimate $= -0.662$, CI -1.34, 0.0126; Fig. 3a; Table 14, Online Resource 1). Amoebocyte density is 6.6% lower in copepod-infected sea fans than in uninfected sea fans, with a mean density of 17.0% compared to 18.2%, respectively, but this is not significant. Amoebocyte density is the lowest in co-infected sea fans with a mean density of 11.8%, which is 35.2% lower than in uninfected sea fans ($P = 0.00058$, estimate $= -0.994$, CI -1.64, -0.348) and in sea fans with single copepod infections ($P = 0.0074$, estimate $= 0.797$, CI -1.46, -0.130; Table 14, Online Resource 1). In a separate analysis of amoebocyte density excluding all labyrinthulid infections, MFPS status was a better predictor than the null model (LRT, $P = 0.023$) but explained only 2.6% of the variance (Table 15, Online Resource 1). Amoebocyte density was higher in healthy tissue than in asymptomatic tissue ($P = 0.020$, CI -0.582, -0.0460), while diseased tissue with the characteristic spots of MFPS was intermediate between the two (Fig. 3b; Table 16, Online Resource 1).

The copepod and the labyrinthulid infect different tissues

In addition to identifying a role for ecological, demographic, and immune variation in coral co-infection, we also observed differential use of coral tissues by the two parasites (Fig. 1). The labyrinthulid and the copepod both damage sea fan polyps, but the labyrinthulid infects the gorgonin skeleton while the copepod invades the mesoglea and polyps.

Parasite co-occurrence

Histological analysis for the 60 tagged sea fans revealed 25 (41.7%) that had only the copepod, 8 (13.3%) with only the labyrinthulid, and 31 (50.2%) with neither. Four sea fans (6.67%) were co-infected with both parasites (Fig. 4). These infection and co-infection levels are comparable to other coral and wildlife surveys (Hersh et al. 2014; Lamb et al. 2015). The Chi-square analysis indicates that the copepod and labyrinthulid do not co-occur more, or less, often than expected by chance (Pearson's Chi-squared test, Yates' continuity correction; X-squared $= 0.0165$; $P = 0.90$).

Discussion

This study highlights the roles of the environment, host demography, and immune suppression in wild co-infections of the Caribbean sea fan, *Gorgonia ventalina*. The environment and host demography influenced the prevalence of the sea fan parasites. Specifically, copepod prevalence increased with the size of the sea fan colony while
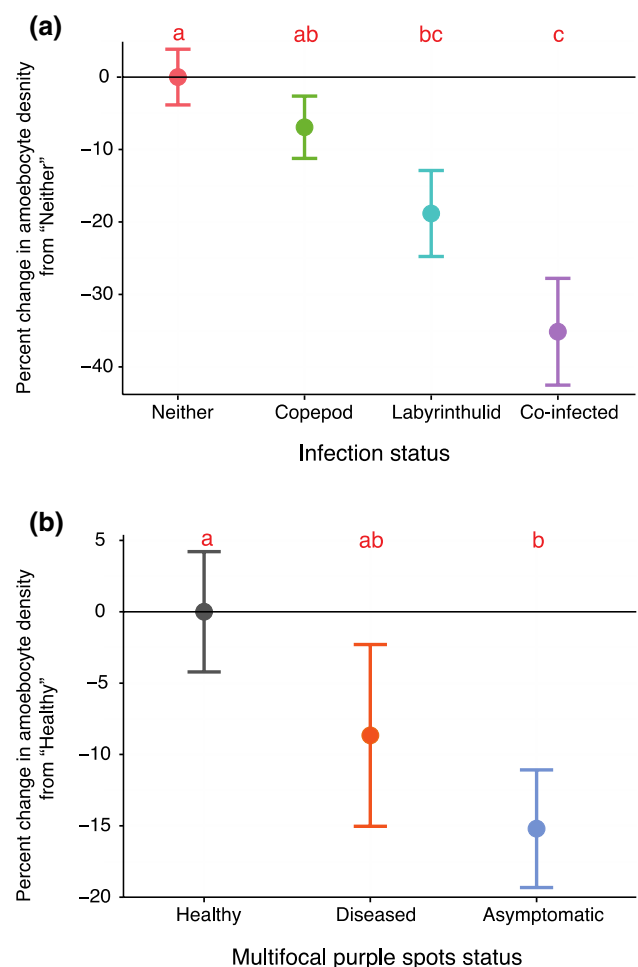

Fig. 3 a Percent change in amoebocyte density (% area) in infected and co-infected sea fans relative to the baseline, sea fans with neither parasite. Mean amoebocyte density is 18.2% in uninfected sea fans ($N = 31$), 17.0% in copepod infections ($N = 21$), 14.8% for labyrinthulid infections ($N = 4$), and 11.8% in co-infected sea fans ($N = 4$). Letters denote significance: "Labyrinthulid" vs. "Neither" (General linear hypothesis test; $P = 0.026$; estimate $= -0.662$, CI -1.34, 0.0126; Table 14, Online Resource 1); "Co-infected" vs. "Neither" ($P = 0.00058$, estimate $= -0.994$, CI -1.64, -0.348; Table 14, Online Resource 1); and "Co-infected" vs. "Copepod" ($P = 0.0074$, estimate $= 0.797$, CI -1.46, -0.130; Table 14, Online Resource 1). Error bars are 2SE. Each sea fan has multiple photos for replication. (Color version available online). **b** Percent change in amoebocyte density (% area) in healthy, diseased, and asymptomatic sea fan tissue relative to the baseline, sea fans without MFPS ("healthy"). "Diseased" tissue is diseased tissue from a MFPS-affected colony and "asymptomatic" tissue is healthy tissue from a MFPS-affected colony. The mean amoebocyte densities are 19.1% in healthy sea fans, 17.4% in diseased tissue, and 16.2% in asymptomatic tissue. MFPS status was a better predictor than the null model (LRT, $P = 0.023$). Letters denote significance: "Healthy" vs. "Asymptomatic" ($P = 0.020$, CI -0.582, -0.0460; Table 16, Online Resource 1). Error bars are 2SE. Each sea fan has multiple photos for replication. (Color version available online)
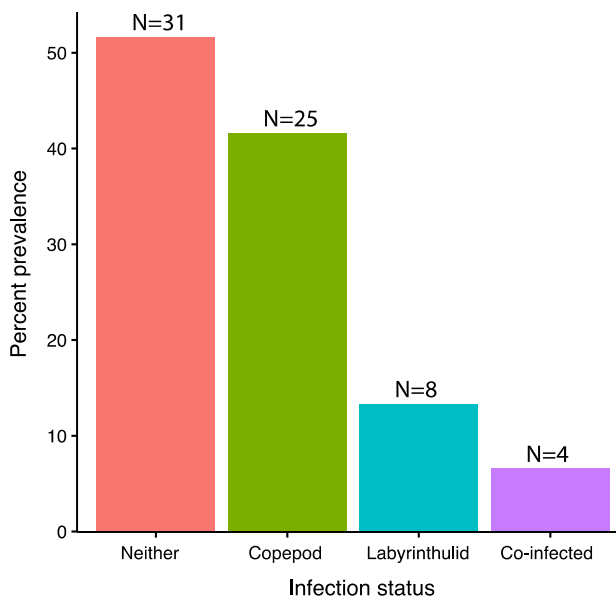
Fig. 4 Histology diagnoses for the 60 tagged sea fans revealed 25 (41.7%) sea fans infected with the copepod, 8 (13.3%) infected with the labyrinthulid, and 31 (50.2%) with neither. Four sea fans (6.67%) were co-infected with both parasites. The two parasites occur neither more nor less often than expected by chance. (Color version available online)

labyrinthulid prevalence increased with water depth. Additionally, host immunity varied with parasite infection status: amoebocyte density was significantly suppressed in both single labyrinthulid infections and co-infections relative to sea fans infected with neither parasite. This supports our hypothesis that suppressed immunity is associated with the labyrinthulid. However, the results do not support our hypothesis that increases in copepod infections are linked with the labyrinthulid, via suppressed immunity. Despite the immune suppression in labyrinthulid infections, which could facilitate the invasion or persistence of the copepod, the two parasites did not co-occur more often than expected by chance in the host population. These findings provide new insights for wild co-infections because it appears in this case that environmental and demographic drivers overwhelm immune-mediated parasite interactions to determine parasite co-occurrence patterns.

As in many other instances of co-infection in wild hosts, we find that infection alters immunity in sea fans. Overall, there is a trend towards immune suppression in all infections relative to healthy sea fans (Fig. 3). The consistent reduction in amoebocyte density during labyrinthulid infections supports our hypothesis. While this finding alone does not establish that the labyrinthulid causes immune suppression, as the data are from a single time point for each sea fan, the evidence of labyrinthulid-mediated immune suppression in Mann (2014) strengthens this interpretation. Mann (2014)

found that the labyrinthulid suppressed antibacterial activity and antifungal activity at both 18 h and 18 days after exposure. There was also evidence of labyrinthulid-mediated suppression of the sea fan prophenoloxidase cascade, which controls barriers to infection through melanization; protease inhibitors, which impede parasite invasion; and superoxide dismutase, which is involved in scavenging free radicals to minimize host damage during infection (Mann 2014). We have now shown that immune suppression is associated with the labyrinthulid not only in the lab, but also in a natural field setting, thus highlighting its ecological relevance. The best model of amoebocyte density predicted only 10.6% of the variation, but this is a notable finding in sea fans because the amoebocyte response can vary greatly among colonies, likely accounting for this low R^2 value (Couch et al. 2013).

Given the evidence for immune suppression by the labyrinthulid, we hypothesized that labyrinthulid infection would benefit the copepod. We detected a random pattern of parasite co-occurrence, showing that copepod infections are not more frequent during labyrinthulid infections. However, it is also clear from research in other systems that the pattern of parasite co-occurrence may be decoupled from immune interactions due to the combined importance of several factors (Jolles et al. 2008; Honkavaara et al. 2009; Fenton et al. 2014). For example, trematode parasites of amphibians co-occur frequently in hosts due to similar resource requirements in spite of their antagonistic interaction through the host immune system (Johnson and Buller 2011). In the sea fans here, the non-overlapping resource use by the copepod and the labyrinthulid in both single and co-infections suggests that they do not have an antagonistic relationship mediated by resource competition (Fig. 1). Thus, they might still be expected to co-occur frequently due to immune facilitation. Having considered immunity and resource use, we then assessed the nature of the parasite interaction in the context of the natural environment.

The importance of both environment and host demography for parasite interactions and host immunity in *G. ventalina* confirms past studies while introducing a novel perspective. As in other studies in a variety of organisms, disease prevalence increases with host size (Rohde et al. 1995; Dube et al. 2002; Lafferty and Harvell 2014; Groner et al. 2014; Yoshioka et al. 2016). Larger sea fans are more likely to have copepod infections (Fig. 2b), signs of MFPS, and more severe MFPS (Tables 5–10, Online Resource 1). Given the correlational nature of the data, causality is not clear. Size may be important because larger hosts present a larger target for parasites, accumulate infection for longer, or mount a weaker immune response (Ellner et al. 2007; Lafferty and Harvell 2014). In this case, the best model of amoebocyte density did not include size, allowing us to reject the hypothesis of size-based immune compromise. Whatever the mechanism for the copepod's size-dependent

prevalence, size did not significantly affect labyrinthulid prevalence (Fig. 2a). Labyrinthulid prevalence increased with water depth (Fig. 2c), yet copepod prevalence did not change and MFPS prevalence (based on a larger sample size) decreased only slightly with depth (Table 8, Online Resource 1). Just as size is important for the copepod but not the labyrinthulid, the two parasites show different relationships with depth. This is illuminating because studies have not identified a role for depth in wild co-infections.

The role for depth is interesting whether or not depth itself is the causal factor. Aside from the change in pressure, depth could influence disease through changes in abiotic conditions, such as temperature or light. Temperature did not predict copepod or labyrinthulid prevalence, but light could be important. Interestingly, increased light supersedes temperature in driving black band disease of corals (Sato et al. 2011). Sea fans at different depths may also experience differential exposure to the labyrinthulid or the copepod due to changes in the biotic community.

The R^2 values for the model of labyrinthulid prevalence (68.6%) and copepod prevalence (50.0%) indicate that depth and size, respectively, explain much of the variation for these parasites. However, the low R^2 value for MFPS prevalence (15.3%) suggests that there are other factors structuring disease prevalence that we did not measure. For example, sites with higher water motion may have lower disease prevalence due to decreased residence times of the parasite (Couch et al. 2014). Nutrient concentrations are also known to influence disease in corals, including *G. ventalina* (Baker et al. 2007, Vega Thurber et al. 2014). Although these are avenues for further research, the high explanatory power in the models of labyrinthulid and copepod prevalence establishes a clear separation for the parasites across the sea fan population.

The distinct role of host size and water depth for the two sea fan parasites expands the present understanding of wild co-infections. There are other studies of co-infection where the two parasites are separated in the population, as in African buffalo where one parasite increases with age while the other decreases. Yet in that case host immunity still drove the parasite co-occurrence patterns (Jolles et al. 2008). In the sea fans, distinct ecological requirements for each parasite seem to prevent them from interacting directly within the host. The different environmental and demographic correlates of the parasites provide a sensible explanation for random parasite co-occurrence at the population level in light of the minimal resource overlap and the potential for immune-mediated facilitation (Fig. 2).

Immune suppression may not result in positive co-occurrence when one parasite experiences minimal benefits from immune suppression. For example, the immunosuppressive myxoma virus in rabbits does not affect the occurrence of *Graphidium strigosum*, a co-infecting parasite, because *G. strigosum* is not regulated by host immunity and therefore does not benefit from immune suppression (Cattadori et al. 2008). Accordingly, it is possible that the suppression of the sea fan amoebocyte response during labyrinthulid infections is not relevant for the copepod or is too spatially localized. We lack information to evaluate the spatial scale of immune influence, but it is likely that amoebocytes play a role in the response to the copepod, since copepod infections are usually surrounded by purpling that marks one of the sea fan's defensive responses (Alker et al. 2004). We also found that immunity varies between healthy tissue and asymptomatic tissue from sea fans with MFPS, though with low explanatory power (Fig. 3).

The insights from studying this sea fan co-infection in the wild provide a more nuanced view of host immune suppression and the ecological factors that can determine the outcome of co-infection. Immune suppression in the sea fan host was marked in both labyrinthulid and co-infections. However, it was not linked to changes in parasite co-occurrence, likely due to the distinct ecological drivers of each parasite in the wild. The natural setting of the host-parasite interaction is of clear importance for sea fan disease. Parasites that could facilitate each other, at great expense to the host, appear to be driven instead by the environment and host demography. It is possible that these ecological drivers reversed the expected outcome of immune-mediated facilitation, i.e., positive parasite co-occurrence. Finally, the results suggest that labyrinthulid-mediated immune suppression is not responsible for the emergence of MFPS in this system.

Our results highlight how the environment and host demography are critical in infections of wild populations and may determine the outcome of co-infection. Without considering these realistic drivers of wild co-infections, studies may make incorrect conclusions about parasite interactions and co-infection in important host populations like octocorals. Careful consideration of the environment, host demography, parasite resource use, and host immunity in wild co-infections will help future studies uncover the effects of co-infection on parasites and hosts.

Author contribution statement AMT, EW and DH conceived and designed the study. AMT and EW conducted field surveys. AMT

performed data analyses and wrote the manuscript with editorial input from EW and DH. All authors gave final approval.

Compliance with ethical standards

Data accessibility All datasets and code are available on Figshare: https://figshare.com/s/41bb927b86d802e90698 (https://doi.org/10.6084/m9.figshare.4996754).

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable institutional and/or national guidelines for the care and use of animals were followed.

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