Trophically Transmitted Parasites as “Cross-taxon Surrogates” of Biodiversity in Coastal Environments

by

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July, 2022

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Parasitism is a highly successful life history strategy, and estimates suggest that as much as 40% of life on earth is parasitic. Healthy ecosystems have high parasite diversity, which in turn reflects underlying patterns of host diversity. Cumulatively, these host-parasite interactions span multiple trophic levels and are sensitive to disturbance, making trophically transmitted parasites useful as conservation tools for addressing a multitude of applied and theoretical questions. The four chapters in this dissertation advance the theory and practice of using trophically transmitted parasites as cross-taxon surrogates of biodiversity. Parasite diversity in key host species was used to assess overall community diversity, habitat heterogeneity, and ecosystem restoration outcomes in the short-term (<1-year) and up to 25 years following the addition of structured habitat. Chapter 1 quantifies the population structure of the naked goby (*Gobiosoma bosc*), a species of fish that likely serves as a key host within host-parasite food webs in the Pamlico and Neuse rivers. There is extensive gene flow among populations of gobies, although there is also evidence of local adaptation as evidenced by the frequency of unique haplotypes. Interestingly, parasite diversity in these fish appeared to be greater in areas with intact natural habitat, in contrast to anthropogenically-modified areas, an observation that became the focus of Chapter 2. For this work, infra-communities of digenetic trematodes were sampled from the eastern mudsnail (*Ilyanassa obsoleta*), a key host for trematode parasites in

intertidal marine environments. Trematode community richness, evenness (Pielou's), and diversity (Shannon, Simpson) were greater in snails sampled from natural shorelines with complex habitat (e.g., oyster reefs, saltmarsh cordgrass) as opposed to shorelines artificially reinforced with bulkhead structures. There were also important differences at the community-level that likely reflect underlying patterns of host diversity at different shoreline types. For example, the trematodes *Lepocreadium seterifoides* (LS) and *Zoogonus lasius* (ZL) were the most common species sampled across all sites, although they dominated parasites communities in artificial shorelines. Polychaetes serve as intermediate hosts for LS and ZL, taxa that are ubiquitous but more common in polluted or habitat-poor environments. On the other hand, the trematode HQ, which requires mollusks and shorebirds as hosts, was only found at sites with natural shorelines, suggesting that these areas are less disturbed and have a greater diversity of taxa serving as intermediate hosts. Chapters 3 and 4 expand on the role of habitat by testing the short and long-term response of the host-parasite community to ecological restoration. Chapter 3 used parasite diversity in common intermediate hosts to assess how traditional forms of habitat restoration (e.g., shell bags) compared to novel methods (e.g., Oyster Catchers™) in the short-term (< 1-year) following restoration. Relative to areas with bare mud flats, most host-parasite taxa groups increased in response to the presence of structure, although habitat complexity (i.e., oyster density) mattered less than the overall volume of habitat added to the system. There were also clear shifts in the functional diversity of species present in the community from more generalist taxa (e.g., grass shrimp, naked gobies) to reef-resident organisms (e.g., striped blennies, snapping shrimp). However, in the short-term following restoration it can be difficult to determine whether organisms are responding to the presence of added structure or the disturbance posed by the restoration itself. Chapter 4 used parasite diversity as a tool to evaluate

long-term patterns of community succession in restored oyster reefs (5 to 22 years old). In general, the diversity of free-living taxa was highly variable and did not differ among New-Restored (<10 years), Old-Restored (>20 years), and Natural reefs. Conversely, parasite diversity increased with elapsed time post-restoration, and parasite communities in older restored reefs were more similar to those found in natural reefs. In addition, oyster toadfish (*Opsanus tau*) were identified as a key host species capable of facilitating parasite transmission and trophic ascent in oyster reefs food webs. According to the parasite data, trophic complexity in restored oyster reefs required at least 10 years to resemble that found in natural reefs. Altogether, this dissertation adds to a growing body of evidence demonstrating how parasites can serve as conservation tools. It also advances the rationale for using trophically transmitted parasites as biodiversity surrogates. Surrogate species in biodiversity monitoring studies must be capable of predicting the presence of other, more elusive taxa. Preference should be given to taxonomic groups from different trophic levels, especially cross-taxon surrogates that represent the functional links between organisms. Trophically transmitted parasites are ideal cross-taxon surrogates of biodiversity and trophic complexity, particularly when parasite diversity can be quantified in one or more key host species.

Trophically Transmitted Parasites as “Cross-taxon Surrogates” of Biodiversity in Coastal Environments

A Dissertation

Presented To the Faculty of the Department of Biology

East Carolina University

In Partial Fulfillment of the Requirements for the Degree

Doctor of Philosophy in Interdisciplinary Biological Sciences

by

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July, 2022

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magistris meis omnibus…alenda lux

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Chapter 1: Distribution and population structure in the naked goby *Gobiosoma bosc* (Perciformes: Gobiidae) along a salinity gradient in two western Atlantic estuaries

Abstract

Many species of fish produce larvae that undergo a prolonged dispersal phase. However, evidence from a number of recent studies on demersal fishes suggests that the dispersal of propagules may not be strongly correlated with gene flow. Instead, other factors like larval behavior and the availability of preferred settlement habitat may be more important to maintaining population structure. We used an ecologically-important benthic fish species, *Gobiosoma bosc* (naked goby), to investigate local and regional scale population structure and gene flow along a salinity gradient (~3 ppt to ~ 18 ppt) in two North Carolina estuaries. *G. bosc* is an abundant and geographically widespread species that requires complex but patchy microhabitat (e.g. oyster reefs, rubble, woody debris) for reproduction and refuge. We sequenced 155 fish from 10 sites, using a common barcoding gene (COI). We also included recent sequence data from Genbank to determine how North Carolina populations fit into the larger biogeographic understanding of this species. In North Carolina, we found a significant amount of gene flow within and between estuaries. Our analysis also showed high predicted genetic diversity based upon a large number of rare haplotypes found within many of our sampled populations. Moreover, we detected a number of new haplotypes in North Carolina that had not yet been observed in prior work. Sampling along a salinity gradient did not reveal any significant positive or negative correlations between salinity and genetic diversity, nor the proportion of singleton haplotypes, with the exception of a positive correlation between salinity standard deviation and genetic diversity. We also found evidence that an introduced European population

of naked gobies may have originated from an Atlantic source population. Altogether, this system offers a compelling way to evaluate whether factors other than dispersal per se mediate recruitment in an estuarine-dependent species of fish with a larval dispersal phase. It also demonstrates the importance of exploring both smaller and larger scale population structure in marine organisms to better understand local and regional patterns of population connectivity and gene flow.

Introduction

The Gobiidae is the largest family of marine fishes, with nearly 2000 species described worldwide, an estimated 320 of which are found in the Americas (Van Tassell 2011; pers. comm). Cryptic by nature, gobies are small (<70mm standard length) benthic fishes that are sedentary as adults. Most species inhabit tropical and sub-tropical regions (Thacker 2011); however, some taxa, including members of the genus *Gobiosoma*, are also found in temperate latitudes in the western Atlantic. Although common across a broad range of estuarine habitats and salinity gradients, the *Gobiosoma* remain relatively understudied (Carle & Hastings 1982; Van Tassell 2011).

One member of this genus, the naked goby *Gobiosoma bosc* (Lacépède, 1800), is a geographically widespread species – ranging from Connecticut to Campeche, Mexico – and the most commonly encountered gobioid fish in estuaries of the southeastern United States (Dawson 1969; Ross & Rhode 2004). This small goby (< 60 mm SL) is estuarine-dependent (Able & Fahay 1988, 2010) and prefers structured habitat (e.g. oyster reefs, woody debris). *G. bosc* is most abundant in waters of low-to-moderate salinity (Dahlberg & Conyers 1972), though it may also occupy sub-tidal mud flats or the shallow margins of marsh creeks (Miller & Guillory 1980; Peterson & Ross 1991; Hendon et al. 2000). Naked gobies function as an important trophic link

between benthic and pelagic communities (Markle & Grant 1970; Breitburg et al. 1995; Breitburg 1999).

Early studies characterizing the life history and distribution of *G. bosc* focused on its reproductive biology and the distribution and abundance of larvae and adults as a function of salinity (Massmann et al. 1963; Dawson 1966; Dahlberg & Conyers 1972; Crabtree & Middaugh 1982; Shenker et al. 1983; Conn 1989). Planktonic *G. bosc* larvae settle out and become part of the benthos at an approximate total length of 7-12 mm (Borges et al. 2011). Prior to settlement, larvae aggregate in low flow areas on the downcurrent side of oyster reefs (Breitburg et al. 1995), which serve as the preferred habitat for juveniles and adults and are also integral to goby refuge and reproduction (Nelson 1928; Massmann et al. 1963; Dahlberg & Conyers 1972; Shenker et al. 1983; Lederhouse 2009). In particular, adhesive egg masses are attached to the underside of oyster shells (e.g. Dahlberg & Conyers 1972; Crabtree & Middaugh 1982), and eggs hatch after approximately 1-2 weeks (Nero 1976). Early-stage larvae are subject to passive dispersal processes, while larvae in more advanced stages of development are capable of positive rheotaxis and have been observed to aggregate around oyster reefs, or other structured habitat like rubble or artificial structures, prior to settlement (Breitburg et al. 1989, 1991, 1995).

Due to its dependence on structured habitat for refuge and reproduction, spatially-mediated patterns of *G. bosc* larval settlement may determine local population densities throughout estuarine habitats (Breitburg et al. 1995). Oyster reefs in particular form a complex spatial mosaic in estuaries, along with mudflats, submerged aquatic vegetation (SAV), and emergent vegetation (e.g. Bell et al. 1991; Skilleter & Loneragan 2003; Gain et al. 2017), and this mosaic will in turn strongly influence goby abundance and distributional patterns. Further, larval *G. bosc* have been reported to migrate towards lower salinity regions in estuaries (e.g.

Shenker et al. 1983); however, the home range of adult naked gobies and their ability to disperse downstream remain unknown (Ross & Rhode 2004). In sedentary fishes like *G. bosc,* it is assumed that dispersal promotes connectivity between populations that are otherwise isolated within such habitat mosaics (Leis & McCormick 2002; Shanks 2009). In other words, long-distance dispersal of larvae over many kilometers would be expected to result in a relatively uniform population structure, especially at smaller spatial scales (Palumbi 1994; Shulman & Bermingham 1995; Mora and Sale 2002; Palumbi & Warner 2003). However, evidence from demersal tropical and Antarctic fishes suggests that self-recruitment and local adaptation can persist despite widespread gene flow (Ohman et al. 1998; Robertson 2001; Taylor & Hellberg 2003; Moody et al. 2015). This has led many to conclude that larval behavior and the availability of settlement habitat are more important predictors of population structure than dispersal alone (Shanks 2009; Riginos et al. 2011; Kohn & Clements 2011).

To date, few studies have attempted to quantify population connectivity in temperate fishes (e.g. Kohn & Clements 2011) like *G. bosc*. However, a recent study on the population genetics of naked gobies (Mila et al. 2017) has provided some understanding of the large-scale phylogeographic differences throughout much of the species' range. This investigation found that *G. bosc* is strongly structured based on geography, particularly between Gulf vs Atlantic populations, but within those major regions, gobies are further subdivided into subclades based upon geography (e.g., East and West of the Apalachicola River along the Florida panhandle), due to biogeographic breaks that inhibit gene flow. The Mila et al. (2007) study is therefore instrumental in understanding the broader phylogeography of *G. bosc* in North America. To date, however, there have been no studies investigating genetic diversity at smaller scales (i.e. at the estuary level). This is particularly relevant given the importance of suitable habitat in structuring

populations of naked gobies, which are otherwise isolated from one another as adults within a broader habitat matrix.

In our study, we collected *G. bosc* along a salinity gradient from two North Carolina estuaries in order to quantify population connectivity within and between estuaries, and also to better understand the role of habitat and salinity in mediating gene flow and local adaptation. While naked gobies have a broad salinity tolerance (<1.0 ppt – 32 ppt.), they have been noted as most abundant in salinities below 24 ppt (Massman et al. 1963; Shenker et al. 1983), and in North Carolina, we have observed adults to be most abundant in low-to-mid mesohaline habitats (5.0 ppt – 16.0 ppt) (personal observations). We therefore hypothesized that populations in oligohaline (< 5.0 ppt) and polyhaline (> 24.0 ppt) salinities may be more subject to processes that lower genetic diversity, like drift.

To quantify population structure in these estuaries, we used the common mitochondrial barcoding gene Cytochrome Oxidase I, which has been used as a tool for population genetics studies across multiple diverse taxa over the past 20+ years (COI 2014; Barcode 2018), and has proven effective at sequencing the genetic diversity of marine fishes (e.g. Ward et al. 2005). Furthermore, the use of this marker allowed us to explore our data in combination with other recent population genetics datasets for this species, like the Mila et al. (2017) study, and an additional goby phylogenetics study encompassing multiple goby species (e.g. Van Tassell et al. 2015). It is also noteworthy that although the comprehensive Mila et al. (2017) study sampled *G. bosc* throughout most of its range, it did not include samples from North Carolina, a state which features the second largest estuarine system in the USA (APNEP 2018). Our work provides a greater understanding of the importance of large estuaries in the life history *G. bosc* – particularly related to patterns of dispersal and connectivity among isolated populations of these

and similar fishes. This work could therefore have important implications for the management of marine protected areas (MPAs), or other conserved habitats that are spatially segregated but connected by gene flow.

Materials and Methods

Study location

Gobiosoma bosc fish were sampled along a salient gradient spanning the Pamlico and Neuse River estuaries in the North Carolina coastal plain (Fig. 1; Table 1). Both the Pamlico and Neuse are shallow (average depth 1-3 m), microtidal (< 1 ft. tidal range), oligohaline-mesohaline (0.5 ‰ to 18 ‰) estuaries that combine to form Pamlico Sound: the largest lagoonal estuary in the United States (Bales & Nelson 1988). The Pamlico River estuary is a continuation of the freshwater Tar River, and it flows approximately 65 km from the town of Washington, NC to Pamlico Sound. The Neuse River estuary begins in New Bern, NC, and empties into Pamlico Sound. For comparison to samples collected from sites along the Pamlico and Neuse rivers, fish were also collected from Hoop Pole Creek Nature Preserve (HPC), which is located along Bogue Sound in Atlantic Beach, NC (Fig. 1) and is part of the Pamlico Sound.

Specimen collection

From February to July 2017, naked gobies were sampled (n = 155) from nine locations along the Pamlico and Neuse River estuaries, in addition to the site at Hoop Pole Creek (Fig 1; Table 1) (North Carolina Division of Marine Fisheries Scientific or Educational Permit Number 706671). Fish were collected using passive collecting devices: small plastic milk crates (19.05 x 22.10 x 15.75 cm) filled with approximately 1.7 kg of autoclaved oyster shell. This technique is modeled on the successful methodology employed by the Smithsonian Environmental Research Center (SERC) for the past twenty years, e.g. Roche & Torchin (2007), and within our lab for the

past 5 years. Although *G. bosc* and other organisms can freely move inside and outside the crates, they are attracted to the complex three-dimensional habitat that the shell provides. This is analogous to the collecting strategy used by D'Aguillo et al. (2014), who employed habitat traps (i.e. "shell-rubble trays": $0.8m^2$ plastic trays covered with 0.6 cm mesh netting and filled with oyster shell) to sample specifically for naked gobies.

Two replicate crates were deployed at each sample location, making for a total of ten collecting units on the Neuse River and eight on the Pamlico River. Crates were zip-tied to 0.75 m wooden stakes secured in the nearshore subtidal zone, or deployed from fixed or floating docks using rope. Crates on both rivers were checked every 6-8 weeks, and the contents sorted using a large sieve (55.9 x 55.9 x 12.7 cm) with 2 mm mesh. A maximum of ten naked gobies were collected from each sample site during each sampling event. In order to minimize selection bias, fish from both crates were pooled together, and ten individuals were randomly selected from a grid divided into four quadrants. Only sexually mature adults were used in this study. Therefore, all fish less than 20 mm SL (i.e. Dahlberg & Conyers 1973) were released at a minimum distance of 50 m from the collecting location. Fish were then live-transported to East Carolina University (ECU) and housed in aerated plastic aquaria (36.83 x 22.35 x 24.38 cm) at a salinity approximating that of the collecting location. All collection and housing protocols were approved by ECU IACUC: AUP #D346. Because our sample sites were located along a salinity gradient, salinity data were collected at each site using a handheld YSI (YSI Inc., Yellow Springs, OH). These point measurements were averaged across all sampling events (n=4) to provide an average salinity value for each site that could be regressed against genetic (haplotype) diversity at each sample location.

DNA Sequencing and Analysis

Naked gobies were dissected as part of an unrelated study assessing parasite diversity in these fish, and thus sampled fish were not released following capture. Once dissected, white muscle tissue was saved from each individual and immediately preserved at - 20 °C for later DNA extraction. Genomic DNA was isolated from tissue samples using proteinase K/SDS digestion, chloroform extraction, and ethanol precipitation (Kocher et al. 1989). Cytochrome Oxidase I (COI) PCR primers were designed based on sequence data from Van Tassell et al. (2015). These newly designed primers were: GOBY COI F: GCACCGCTTTAAGCCTTTTA and GOBY COI R: TGGTGTTGAGGTTTCGGTCT. The PCR profile is as follows: 95 °C for 2-min; 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s; and 72 °C for 5-min (Blakeslee et al. 2017). PCR amplicons were purified using ExoSAP-IT™ (ThermoFisher Scientific). Samples were then sent for Sanger sequencing to Macrogen USA (Rockville, MD).

Sequences were manually cleaned, inspected for ambiguities, and aligned without gaps to a reference sequence in Genbank (accession #: KM077829.1) using Geneious10.1.2 (Biomatters Ltd., Auckland, New Zealand). This resulted in a 530 base-pair fragment of the COI gene across all samples (n=155 individuals from North Carolina; accession numbers: MH680722-MH680751; TableS1). Sixty-six additional *G. bosc* sequences were located on Genbank following a BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with 100% coverage of the 530 base-pair COI fragment. These included 57 sequences (Popset: 1229627432; accession #s: MF168978-MF169034) from a recent and comprehensive population genetics investigation of *G. bosc* in several North American populations, including New York, Virginia, South Carolina, Atlantic Florida, Gulf Florida, Louisiana, Mississippi, and Texas (Mila et al. 2017). An additional 9 sequences came from a study by Van Tassell et al. (2015): 5 of these were from a

non-native population (Germany) and 4 were from the USA (Florida and the Gulf of Mexico) (accession #s: KT278516, KT278523, KT278535, KT278549, KT278552, KM077826, KM077829, KT278549, and KM077828).

Our new sequences were combined with the sequences from Genbank and aligned using Geneious 10.1.2. Sequences were then collapsed into haplotypes using TCS1.21 (Clement et al. 2002) (Table S1). The Mila et al. (2017) popset (#1229627432) from Genbank included an incidence-based understanding of the haplotypes found across the Atlantic and Gulf of Mexico. In other words, this popset contained no information on haplotype frequencies per population. On the other hand, our North Carolina dataset included frequency data that were explored between and among North Carolina populations and estuaries. As a result, two separate analyses were performed: (1) an investigation for North Carolina populations only, and (2) a geographic, incidence-based investigation of all haplotypes (including our new North Carolina ones) detected across the geographic range. This latter analysis determined how naked gobies in North Carolina fit into the larger biogeographic picture.

Genetic Analyses

For our first analysis focused on North Carolina, we estimated the hierarchical analysis of molecular variance (AMOVA) using ARLEQUIN311 (Excoffier et al. 2005). Resulting fixation indices helped pinpoint whether there was divergence among populations and between the two major estuaries in our study. Pairwise ϕSTs were calculated using ARLEQUIN (Table S2) and visualized in a non-metric multidimensional scaling analysis (using PRIMER 7.0.13 (Primer-e, Quest Research Limited, Auckland, New Zealand) to look for spatial patterns among populations. We used PopArt (http://popart.otago.ac.nz/index.shtml) to graphically create haplotype networks.

We also used ARLEQUIN to obtain genetic diversity values for each population to investigate whether there was any effect of salinity on haplotype diversity in the North Carolina populations. For this salinity analysis, we included sites that were sampled from the two rivers (n=9 sites: 4 in the Pamlico River and 5 in the Neuse River). We used JMP Pro 13 (SAS Institute Inc., Cary, NC) to regress salinity with a) genetic diversity and b) the proportion of singleton haplotypes (calculated from the haplotype analysis described above). This latter approach was used to determine whether there was any influence of salinity on the proportion of rare haplotypes in a population. Additionally, given the variability in salinity within these systems, we also explored whether there was any relationship between the standard deviation of salinity and genetic diversity.

In addition, Primer 7.0.13 was used to construct rarefaction and extrapolation curves of haplotype diversity in order to determine the accumulation of haplotypes with sample size, the expected haplotype richness in each estuary, and the number of samples that would produce an asymptote in haplotype richness (extrapolation). Nonparametric estimators have been found useful in a number of studies for predicting the eventual asymptote in richness of a particular population (Gotelli and Colwell 2001) and do so by including the effects of rare (or singleton) species/haplotypes (Witman et al. 2004, Chao 2005; Blakeslee et al. 2008, 2012). A clearly asymptoting accumulation curve indicates complete capture of the total richness in a population (Gotelli and Colwell 2001), thus estimator curves and accumulation curves that converge on the same asymptote can be very useful in determining whether there is adequate sampling in a population or region, or whether more sampling would reveal additional species/haplotypes (Walther and Morand 1998; Blakeslee et al. 2008).

For our second analysis focused on goby biogeography, we performed an AMOVA to explore differences at the subregional level and also at the larger regional level, specifically between the Atlantic and Gulf of Mexico regions. We again used PopArt to graphically create haplotype networks, using incidence-based data from Mila et al. (2017), Van Tassell et al. (2015), and our new North Carolina populations. In this analysis, we also included five sequences from a non-native population in Germany (Van Tassell et al. 2015) to identify if a source for the non-native population could be revealed within the overall dataset.

Results

North Carolina Estuaries

In our North Carolina dataset (Fig. 1; Table 1), we uncovered a total of 30 previously undescribed haplotypes. Among these haplotypes, 74% of our 155 individuals (from four sites in the Pamlico estuary and five sites in the Neuse estuary, and also a site from Bogue Sound) were found to share one dominant haplotype (HAP5). The dominance of this haplotype and the connections among it and the other haplotypes in the estuaries can be observed in the haplotype network of North Carolina populations (Fig. 2). For our two major estuaries (Pamlico, n=88, and Neuse, n=59), this haplotype (HAP5) was slightly more frequent in the Pamlico (78%) versus the Neuse (68%). At the haplotype level, most haplotypes were singleton occurrences: in the Pamlico, 60% of the haplotypes were singletons, and in the Neuse, 74% were singletons. When comparing between the Neuse and Pamlico sites, just 4 haplotypes (14%) were shared between the estuaries; thus at the haplotype level, there was much less overlap between them. However, at the individual level, 86% of the Neuse individuals shared haplotypes with the Pamlico, and 76% of the Pamlico individuals shared haplotypes with the Neuse. This was supported by non-significant differentiation in the AMOVA comparing these two estuaries (F_{CT}=-0.00149;

p=0.44282). It is important to note, however, that most of the sharing between estuaries and among populations occurred with the dominant haplotype, HAP5. In addition to the dominant haplotype (HAP5), the second most frequent haplotype (HAP27) was also comprised of individuals from both estuaries, as well as Bogue Sound.

At the population level (Fig. 3), the following sites were significantly (or nearly significantly) different from one another in pairwise ϕ_{ST} analyses after accounting for multiple pairwise comparisons (Bonferroni correction, p=0.005): MLC and CQC (p<0.001); MLC and HPC (p=0.009); and NCL and HPC (p=0.009). In addition, Table 1 lists the ratio of observed vs. expected haplotypes vis-à-vis the number of fish sampled at each site. GSC and CQC were found to have the greatest genetic diversity, with the number of expected haplotypes predicted to be roughly three times greater than what was actually observed. Both of these sites are classified as low in salinity (Table 1). Fish from all other sites (MLC, NCL, WRC, FSL, MTP, POC, CDI, HPC) occupied the full range of salinity (low-medium-high) and demonstrated between one and two times the expected number of haplotypes compared to the number sampled. At the estuary level (Fig. 4 A-B), 8 times more haplotypes were predicted for the Neuse (n=160) in the extrapolation curve than were actually detected (n=20), and for the Pamlico ~5 times more haplotypes were predicted (n=80) than were detected (n=15). Comparing between the estuaries, the Neuse was ~2.5 times greater in predicted diversity than the Pamlico. Such diversity differences between the estuaries were also supported by a Shannon diversity test, finding H'= 1.55 for the Neuse and H' = 1.06 for the Pamlico.

In explorations of salinity and genetic diversity/proportion of singleton haplotypes, we found no significant correlations for the following regressions: genetic diversity and salinity (R^2=0.004; p=0.873; Fig. 5A), the proportion of singleton haplotypes and average salinity

(R^2=0.070; p=0.490; Fig. 5B), and the proportion of singleton haplotypes and salinity standard deviation (R^2=0.187; p=0.245; Fig. 5D). At the population level, there was seemingly little influence of salinity and genetic relatedness. For example, two spatially similar sites (in terms of pairwise ϕSTs), CDI and NCL, had salinities that were separated on average by 6 ppt, and these sites were also found in different estuaries. However, there was a significant positive correlation between the standard deviation of salinity and genetic diversity (R^2=0.500; p=0.034; Fig. 5C). No correlations were found when estuaries were analyzed separately, nor did non-linear regressions improve fit and significance in any of these analyses, except in the case of the second polynomial for the proportion of singleton haplotypes and salinity standard deviation (R^2=0.792; p=0.009) (Table S3).

Biogeographic Comparison

In our second analysis (Fig. 6), we combined *G. bosc* sequences from the public sequence repository, Genbank, with our own North Carolina estuary samples. For this analysis, we explored regional differentiation across North America, including the mid-Atlantic (New York and Virginia), North Carolina (Neuse, Pamlico, Bogue), South Carolina, Atlantic Florida, Florida Gulf, Florida panhandle, and other Gulf states (Louisiana, Mississippi, and Texas). In addition, we included 5 sequences found in a non-native population in Germany (Weser estuary). Except for North Carolina, these samples came from two studies: Mila et al. 2017 and Van Tassell et al. 2015. Among all these samples, a total of 88 haplotypes were found. These haplotypes were significantly regionally differentiated (p<0.001), with North Carolina falling in with the southeast and mid-Atlantic samples. When comparing Atlantic versus Gulf of Mexico sites, there was significant differentiation between the two major regions (p<0.001) (Fig. 6).

Introduced German Population

At this point in time, any comparison with the non-native population (Weser estuary, Germany) must be taken with caution, as there are just a few (n=5) representative sequences from this region (Van Tassell et al. 2015). However, in the haplotype analysis (see red coloration in Fig. 6) all five of those sequences aligned within the Atlantic network, with two individuals sharing haplotypes with Atlantic Florida and the mid-Atlantic. There was no evidence for a connection to the Gulf of Mexico. Additional data from the non-native region could help pinpoint a more specific origin for this non-native population.

Discussion

G. bosc is an important member of estuarine communities, but much remains unknown about the dispersal potential of this species, especially at smaller scales, and how it may influence gene flow among populations within a habitat mosaic, or among estuaries. Recent genetic work has focused on broader questions of phylogenetic relationships among the genus *Gobiosoma* (Van Tassell et al. 2015) and large-scale differentiation in widespread populations of *G. bosc* (Mila et al. 2017). Our study is the first to focus on smaller-scale patterns of connectivity and gene flow in naked gobies using a major estuarine system as our focal region. Our study also contributes previously undocumented sequence data from North Carolina populations of *G. bosc*, which will help in further resolving questions related to gene flow in this species at both the local and biogeographic scales. In addition, we incorporated a salinity gradient into our study design, as previous research has suggested that salinity is an important abiotic factor in the life history of this fish. To the contrary, we found that salinity alone was not a major predictor of genetic diversity in this species (with the exception of salinity variability), even though reports in the

literature find naked gobies to be most abundant in moderate salinity habitats. In the sections that follow, we expand upon these findings and discuss their implications.

Genetic Diversity, Gene Flow, and Connectivity

Spatially-structured populations are linked by the dispersal of individual organisms (Cote et al. 2010). Dispersal itself is a fundamental life-history trait (Schludermann et al. 2012), and connectivity between distant groups is a major driver of population dynamics (Bignami et al. 2013). Moreover, the availability of stable epibenthic substrate is crucial for maintaining populations of estuarine fishes like *G. bosc* as well as other organisms (Allen & Barker 1990; Shima & Swearer 2009; Gain et al. 2017). In North Carolina, gene flow was broadly distributed across *G. bosc* populations between the Pamlico and Neuse estuaries (Fig. 2), and genetic diversity was quite high. The latter is especially reflected in the proportion of singleton haplotypes within populations (Table 1), which reached as high as 75% in one Neuse population and was close to 50% when averaged across all 9 populations. Such a preponderance of singleton haplotypes suggests a significant amount of genetic diversity remains unaccounted for in this system. In fact, rarefaction and extrapolation curves (Fig. 4 A-B) suggest upwards of 1000s of individuals would need to be sampled to produce an asymptote; in turn, greater sampling would produce significantly more haplotypes in each estuary than were initially detected.

In addition to demonstrating high diversity and a large number of singleton haplotypes (just a couple mutation steps away from the dominant haplotype), the star-like pattern of the haplotype network is also a well-known signature of populations that have undergone recent expansion (e.g., Mila et al. 2000; Frattini & Vannini 2002). For example, star-like patterns (together with other molecular analyses; e.g., Fu's F_s and Tajima's D) detected in the haplotype network of the protist *Plasmodium falcipram* (which infects African mosquitos) demonstrated

clear population expansions for the parasite (Joy et al. 2003). In a marine example, star-like patterns were observed in haplotype networks of the spiny lobster (*Palinurus gilchristi*) in South Africa, suggesting a recent bottleneck and/or population expansion (Tolley et al. 2005). Specific to *G. bosc*, there is little understanding of this fish's ecological and demographic history in this region, and as a result, any explanations of the mechanisms responsible for population expansion are inherently speculative. However, as winds are the main driver of currents and water level in the Pamlico and Neuse estuaries (Luettich et al. 2002; Whipple et al. 2006), larval *G. bosc* would be highly susceptible to wind-driven dispersal while remaining planktonic in the water column. Given the severe storms that frequent the region (and also coincide with seasonal spawning activity of *G. bosc*), our evidence for a possible population expansion could be a response to one or more of the 35 tropical cyclones to affect coastal North Carolina over the past two decades (Paerl et al. 2018), or perhaps could be due to recent temperature and climactic changes in the region (Harley et al. 2006). A greater understanding of the interaction between the biology of this species and the multiple abiotic factors shaping its distribution, reproduction, and gene flow in the past and present is therefore needed.

Naked gobies also demonstrate extensive gene flow between and among populations in both estuaries, but especially within the Pamlico River. For example, in the MDS plot of pairwise ϕSTs (Fig. 3), Pamlico sites are closer together spatially than Neuse sites. The location of our sample sites along each river, as well as the topography of the two rivers themselves, could be influential in differentially affecting gene flow in these rives. Most of our sites were located on creeks that were tributary to the main stem of either river. Although these sites were all positioned within 1 km of the river, some creeks would be subject to more flushing than others, while more hydrodynamically-isolated areas would favor greater larval retention. In

addition, the orientation of the Pamlico itself is straighter (Fig. 1) and thus may be more favorable to wind-driven dispersal relative to the Neuse, the latter demonstrating an orthogonal bend between the upper and lower parts of the estuary. In some areas of the Neuse estuary, this may possibly lend itself to greater larval retention, greater isolation, and potentially more locally adapted populations. Retention zones have been detected in numerous marine systems and are highly important to the genetic structure and diversity exhibited among populations of organisms connected by larval dispersal (Palumbi 1994; Pringle & Wares 2007; Pringle et al. 2011). Larval dispersal is especially important in organisms that are much less mobile (or sessile) as adults (Sotka et al. 2004). While fish tend to be highly mobile during both larval and adult stages, fish species like *G. bosc* that are more associated with the benthos and have strict habitat requirements may be more influenced by retention zones that will influence gene flow and genetic structure within a region. In future studies, it would be important to map the availability of preferred settlement habitat in each river to determine how well it corresponds to the observed distribution of haplotype frequencies among our sample locations.

The Role of Salinity and Habitat Preference

Fish were collected along a salinity gradient averaging 3 to 14 ppt (averaged across four time periods in 2017) (Table 1). Although *G. bosc* is a euryhaline species, salinity alone did not seem to affect the distribution of haplotype frequencies across our sample sites, even though the greatest abundance of larval naked gobies in plankton tows has been reported from mesohaline habitats (e.g. Dawson 1966; Shenker et al. 1983). In our study, adult naked gobies were most abundant in sites ranging from 4 -12 ppt. We therefore expected greater genetic diversity in fish collected from mesohaline sites because of their relative abundances. However, our results (Fig. 5) revealed few significant linear or non-linear trends with salinity (albeit our sampling only

incorporated a single polyhaline site) with two exceptions: 1) we found a significant positive relationship between genetic diversity and salinity standard deviation (Fig. 5C), whereby sites with greater salinity variability possessed greater genetic variability; and 2) we found a significant positive relationship between the proportion of singleton haplotypes and salinity standard deviation (Table S3). Such an outcome could signify the influence of neutral processes like the effects of waves and currents as one moves upriver, or potentially more fine-scale genetic structure as a result of varying salinity (Beheregaray & Sunnucks 2001). Alternatively, it may signal some kind of salinity adaptation occurring along salinity gradients in both estuaries. Though COI is generally treated as a neutral marker (i.e., it is widely used as a "barcoding" gene), this may not always be the case, particularly when linked to genes that are under greater selective pressures (Moritz & Cicero 2004). Thus, differences in genetic variation along a salinity gradient may suggest an adaptive response (i.e., more variable salinity represents less stable conditions in a population); however, a genome-wide approach would be necessary to elucidate this potential influence on genetic variation in this system.

Further, previous studies have recognized the importance of habitat in structuring populations of *G. bosc*, but have also advocated for the major synergistic role that salinity plays for this fish species. Larval *G. bosc* approaching competence-to-settle are known to congregate near the downstream edges of oyster reefs, rubble, or artificial structures (Breitburg 1989, 1991, 1995). Adults are mostly sedentary and require hard substrate (e.g. oyster shell) for the attachment of egg masses, and it has been proposed that spawning is confined to the downriver portions of estuaries where this habitat is more common (Massamann et al. 1963; Shenker et al.1983). Shenker et al. (1983) reported that the abundance and size of larval *G. bosc* increased with time in the upriver portions of the Patuxent River estuary in Maryland, and they speculated

that larvae were selectively using flood tides to move upriver. Upriver displacement of larvae offers a low salinity refuge from predation and is common in other estuarine-dependent species like weakfish *Cynoscio regalis* (Lankford & Targett 1994) and red drum *Sciaenops ocellatus* (Stewart & Scharf 2008). However, the synchronous spawning movement of adult *G. bosc* seems highly unlikely given that the species is cryptic and adapted to a benthic lifestyle. Adult *G. bosc* are opportunistic in their choice of spawning substrate, and in lieu of oyster shell will use rubble, woody debris, or other material like discarded cans (Nero 1976; Lehnert & Allen 2002). Much like oyster shell, this habitat is often distributed haphazardly, and so population connectivity would depend on the quality, scale, and proximity of available habitat patches. *G. bosc* is adept at using such material, as evidenced by Miller et al. (2014), who extensively sampled a 155 km portion of the middle St. Johns River in Florida averaging 0.2 to 1.2 ppt. Based on the size and abundance of nearly 50,000 larval *G. bosc*, they unequivocally concluded that adults were spawning at all of their sample sites and that larvae were not being transported upriver. They also confirmed the presence of spawning adults and larvae in freshwater lakes and tributary streams of the St. Johns, which accords with our sampling of adult *G. bosc* in sites at the freshwater interface of the Pamlico River (< 0.1 ppt). Altogether, the evidence from prior studies and our own suggests that gene flow in this species is mediated by multiple biotic and abiotic factors – principally among them the type of epibenthic substrate available as habitat, how the distribution of this habitat changes with salinity, and the location of suitable habitat relative to wind-forced circulation patterns.

Biogeography of U.S. Populations

Biogeographic breaks leading to genetic differentiation have been detected in a number of marine organisms, and North Carolina in particular is positioned at one of the sharpest marine

thermal boundaries in the world (Pietrafesa et al. 1985). As such, it functions as an important biogeographic break between temperate and sub-tropical regions. However, while we did not see a clear biogeographic break in Atlantic populations around North Carolina (as there were for the Gulf of Mexico; see discussion below), no shared haplotypes were detected between North Carolina and any other regions/subregions in the Atlantic—demonstrating that while there is a large amount of local gene flow within estuaries, there is much less gene flow at the regional level. Occasional stochastic weather events like tropical storms (AOML, NOAA 2017) may move individuals (particularly larvae) beyond these local boundaries, but these movements appear rare. For example, Ross and Rohde (2004) report just a single record of an adult *G. bosc* collected from an offshore scallop bed, and there are occasional reports of juveniles collected off Beaufort, N.C., located close to the Atlantic Ocean (Hildebrand & Cable 1938).

In fact, Atlantic populations of *G. bosc* demonstrated less noticeable differentiation among geographically spaced locations than within the Gulf of Mexico (i.e., Atlantic locations were separated by fewer sequence changes than were Gulf locations). For example, in the Gulf, geographic differentiation was much more apparent with a considerable break at Apalachicola Bay, sub-dividing the Florida panhandle in two (Mila et al. 2017). In the Atlantic, it may be that the Gulf Stream is playing a role in the greater genetic connectivity (albeit still subregionally differentiated) that was detected among Atlantic populations compared to the Gulf of Mexico populations. The Gulf Stream is known to shape dispersal patterns in many marine fauna. For example, it is thought to be responsible for the presence of Caribbean mesopelagics in the northern Sargasso Sea (Jahn 1976), and some western Atlantic groups in the eastern Atlantic Azores archipelago (Avila et al. 2009). In addition, it also promotes gene flow among such widely distributed organisms as sea turtles (Blumenthal et al. 2009) and American and European

eels (Kleckner & McCleave 1982). These organisms all represent pelagic-spawning species whose larvae would be subject to long-distance transport, in contrast to *G. bosc*, which is an estuarine-resident organism. If the Gulf Stream does play a substantive role in gene flow among Atlantic populations of *G. bosc*, this might also help explain the importance of the tip of Florida representing a major biogeographic break. Off the southern tip of Florida in the Florida Straits, the Gulf Stream current flows east and is at its narrowest and strongest (Gula et al. 2015), which would likely reinforce divergence between Atlantic and Gulf populations at the regional scale.

Status of a Non-native German Population

Very little is known about the non-native population of naked gobies in the Weser estuary, Germany. Thiel et al. (2012) reported that multiple individuals were collected in a stow net in 2009 by a commercial fishery vessel at a depth of between 11.0 and 14.3 m – unusually deep for this species. Without more information, it is unclear whether the population in Germany is more widespread or isolated to this particular estuary. Moreover, it is not possible at this point to determine a likely source location for this introduction, except that it appears likely to have come from an Atlantic source (i.e., in our network analysis, two Germany individuals were found to share haplotypes with an Atlantic Florida and a New York individual; Fig. 6). Given the distance between these two US Atlantic populations, this may suggest multiple introduction events from different source populations—a common occurrence among non-native species introduced via ballast water from shipping (e.g., Blakeslee et al. 2017). The Weser estuary is located near the border with the Netherlands and serves as an important commercial shipping hub, which led Thiel et al. (2012) to speculate that *G. bosc* was introduced via ballast water. An additional introduction has previously been reported from the Orinico Delta in Venezuela (Lasso-Alcala et al. 2005), which is also a major international shipping destination. In all

probability, introduced populations of *G. bosc* are underreported owing to the species' small size and cryptic nature.

Conclusions

We stand to learn a great deal from studying common fish like *G. bosc* that, notwithstanding their abundance, remain relatively understudied in the literature. Only recently, for example, was a comprehensive analysis published on the feeding ecology of this species (e.g. D'Aguillo et al. 2014), which addressed basic questions like diet and daily patterns in foraging activity. In the future, we intend to continue to address the limited understanding of the basic biology of this species by resolving questions of adult dispersal—in particular by studying the movement patterns of adult *G. bosc* that reside in specific habitat patches. The distribution of adults also appears to be related to shoreline exposure, as areas with significant fetch tend to have fewer naked gobies and a higher relative abundance of other species like skilletfish *Gobiesox strumosus* and striped blennies *Chasmodes bosquianus* (pers. obs.). While adult *G. bosc* are relatively cryptic and do not appear to stray far from complex habitat, this remains an untested assumption that could potentially inform our understanding of population connectivity in this species at the estuary level. Further resolution of the genetic connectivity among populations should also include genome-level markers (i.e. RAD-Seq), as it can be difficult to develop a detailed understanding of the magnitude of gene flow at smaller scales using only frequency-based approaches (Waples 1998; Hellberg 2009). Even so, our study provides an initial understanding of the importance of investigating genetic diversity and population structure at local and regional scales.

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Figures

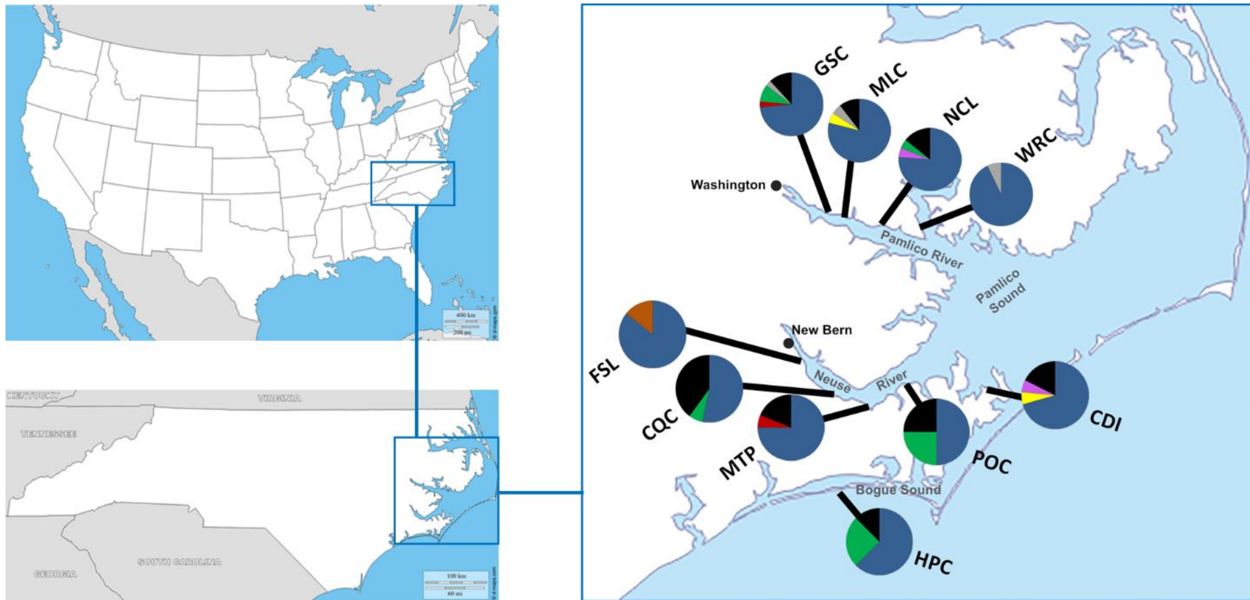

Fig 1. Map of study system. Within the eastern part of North Carolina (USA), *Gobiosoma bosc* were collected from 5 sites on the Pamlico River (3-10 ppt), and 5 sites along the Neuse river (3-15 ppt), as well as a single site from Bogue Sound (30 ppt), which feeds into the Pamlico Sound. Haplotype frequencies are shown for sites along the Pamlico (GSC = Goose Creek; MLC = Mallard Creek; NCL = North Creek Landing; WRC = Wright's Creek), the Neuse (FSL = Fisher's Landing; CQC = Cahooque Creek; MTP = Matthew's Point; POC = Pin Oak Court; CDI = Cedar Island), and Bogue Sound (HPC = Hoop Pole Creek). Colored pie pieces represent shared haplotypes within North Carolina, and black pie pieces represent the collective proportion of singleton haplotypes per population (see Table 1 and Table S1 for more information).

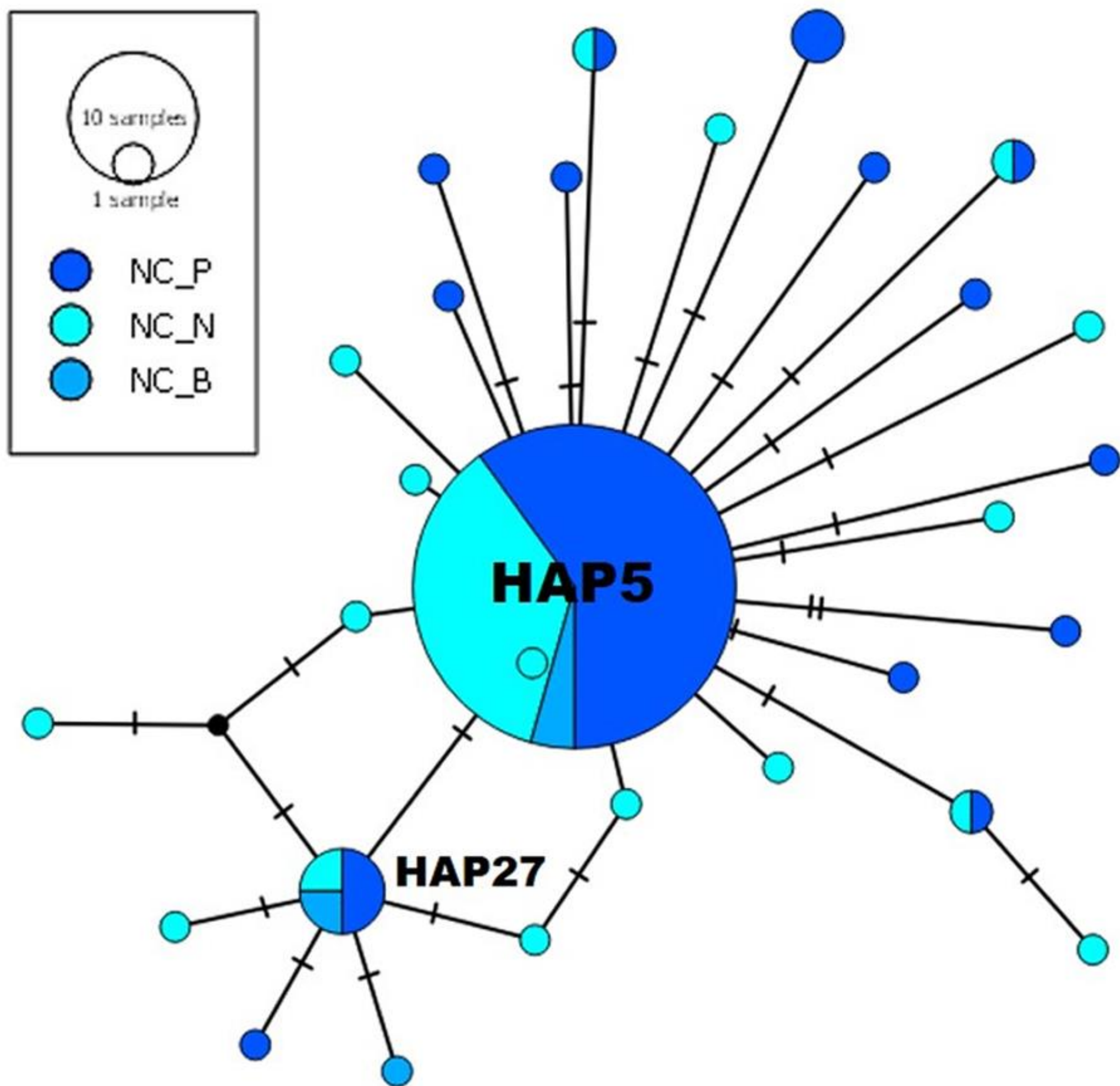

Fig. 2. Haplotype network – North Carolina. Subregions include: NC-P (Pamlico), NC_N (Neuse), NC_B (Bogue Sound). The size of the circle is representative of the number of occurrences for each haplotype (see key in upper left).

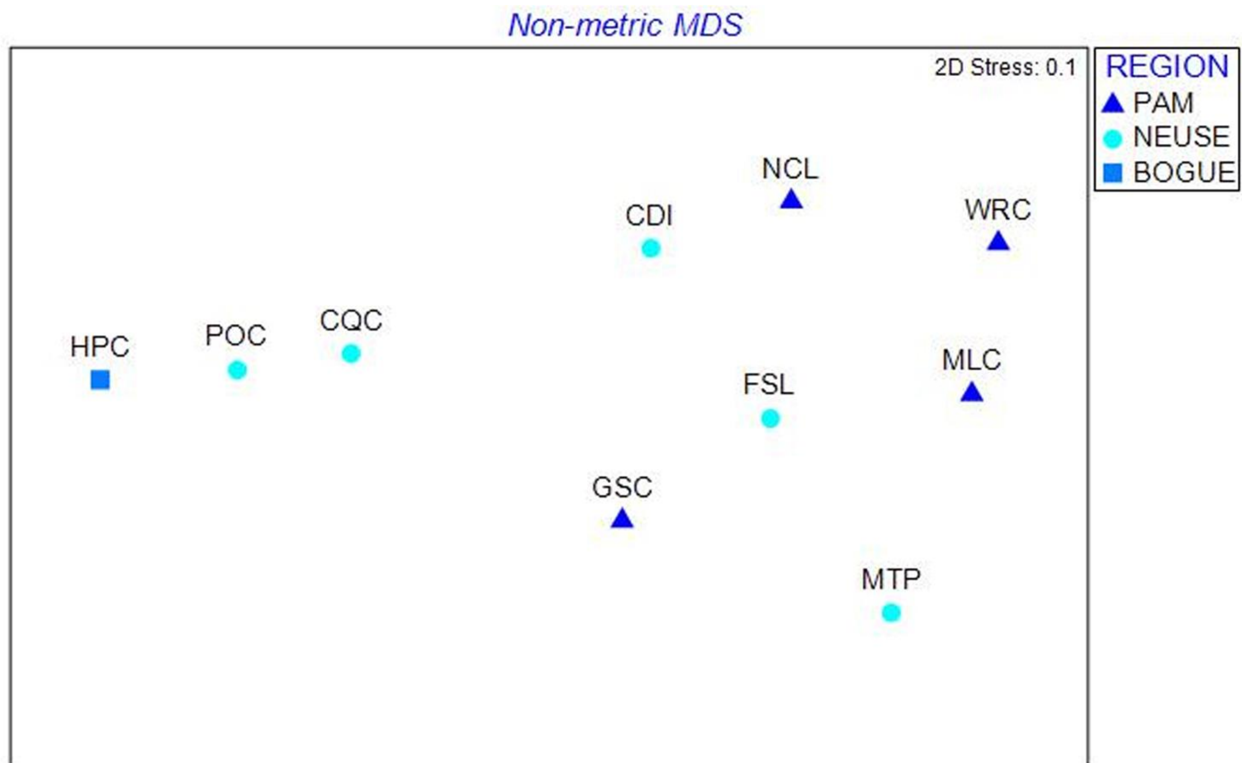

Fig. 3. MDS Plot of North Carolina Populations. Pairwise FST data were analyzed using a resemblance matrix and plotted using nMDS. Points closer together are more similar in their haplotype frequencies than those more distant. Fish sampled from sites along the Pamlico River appear as triangles (GSC, MLC, NCL, WRC), and fish sampled from the Neuse appear as circles (FSL, CQC, MTP, POC). Fish from Bogue Sound appear as a square (HPC).

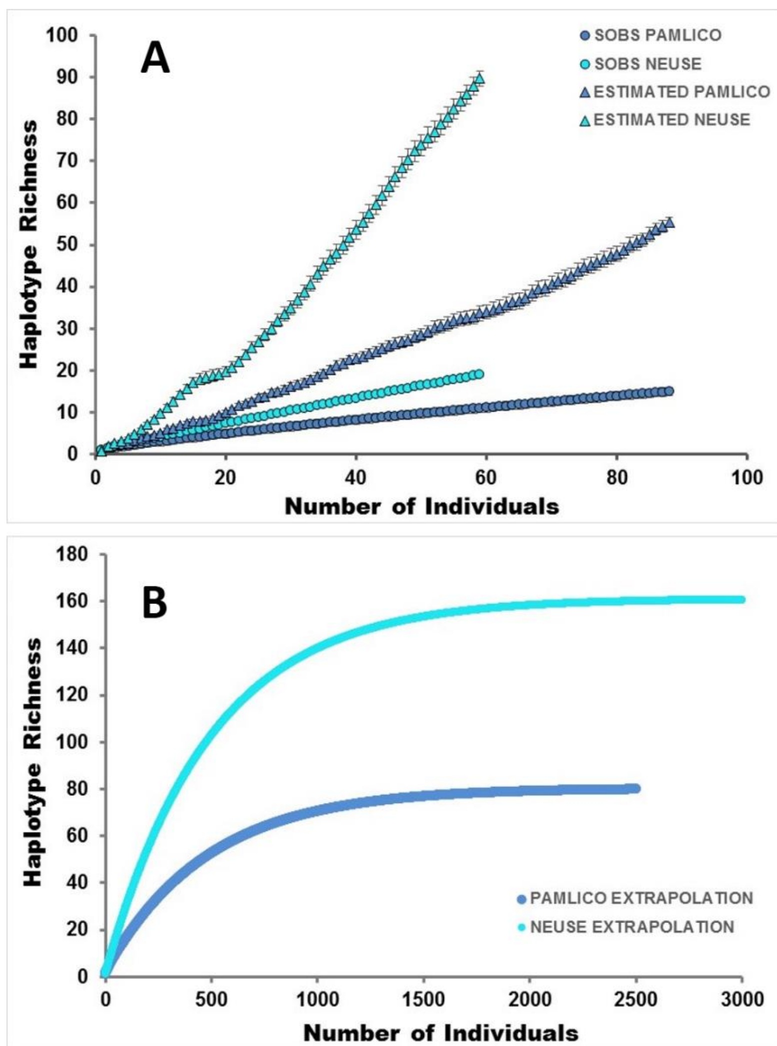

Fig. 4. Rarefaction and Extrapolation Curves. The upper graph represents accumulation (SOBS) and estimator curves of haplotypes in the two North Carolina estuaries, scaled by the number of individuals. The lower graph extrapolates an asymptote based on the number of unique haplotypes sampled from fish within each estuary.

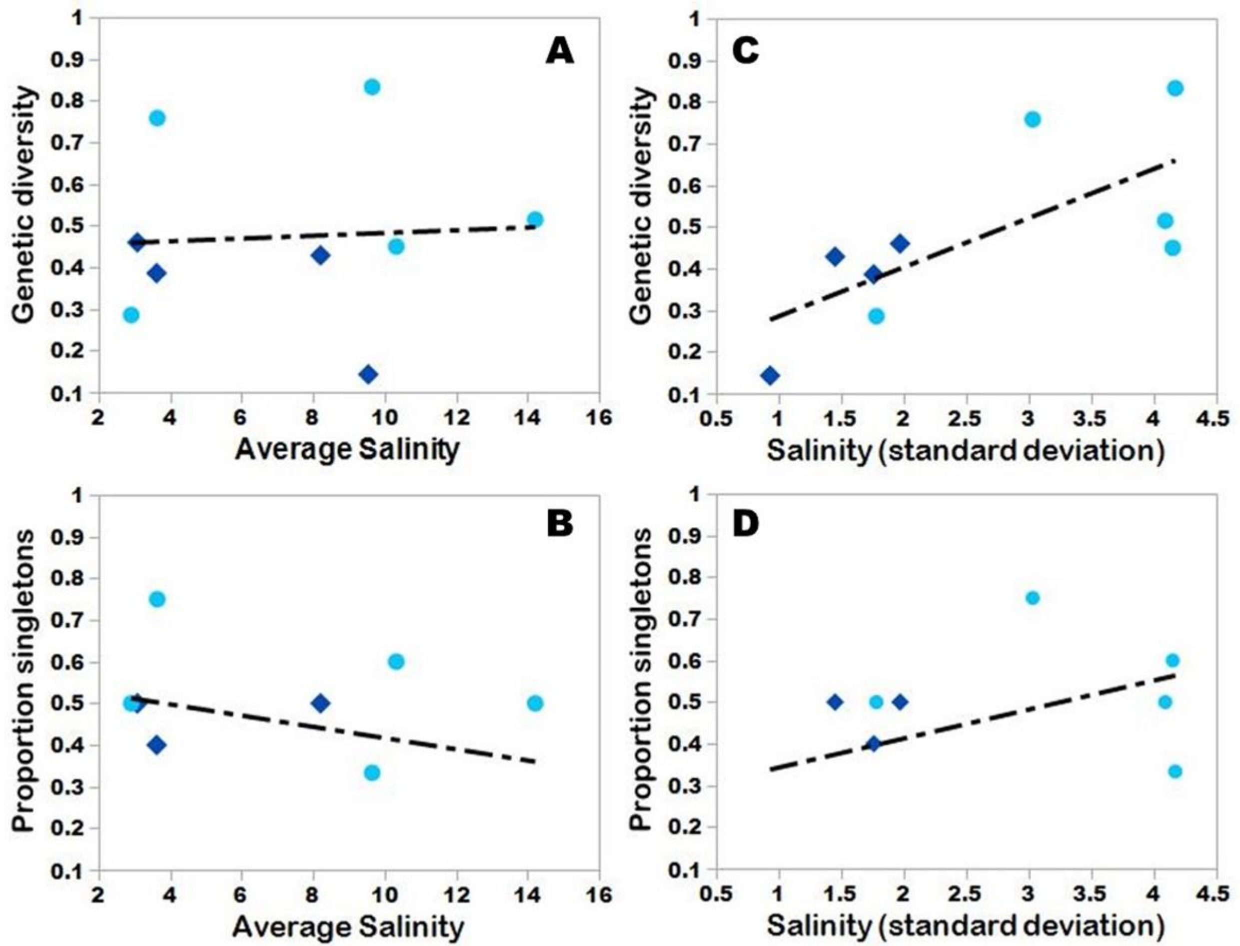

Fig. 5. Salinity with Genetic Diversity and Salinity with Proportion of Singleton Haplotypes. Dark blue diamonds = Pamlico; light blue circles = Neuse. Salinity was averaged across 4 time intervals and regressed with (A) genetic diversity and (B) proportion of singleton haplotypes. Salinity standard deviations are also plotted for genetic diversity (C) and proportion of singleton haplotypes (D).

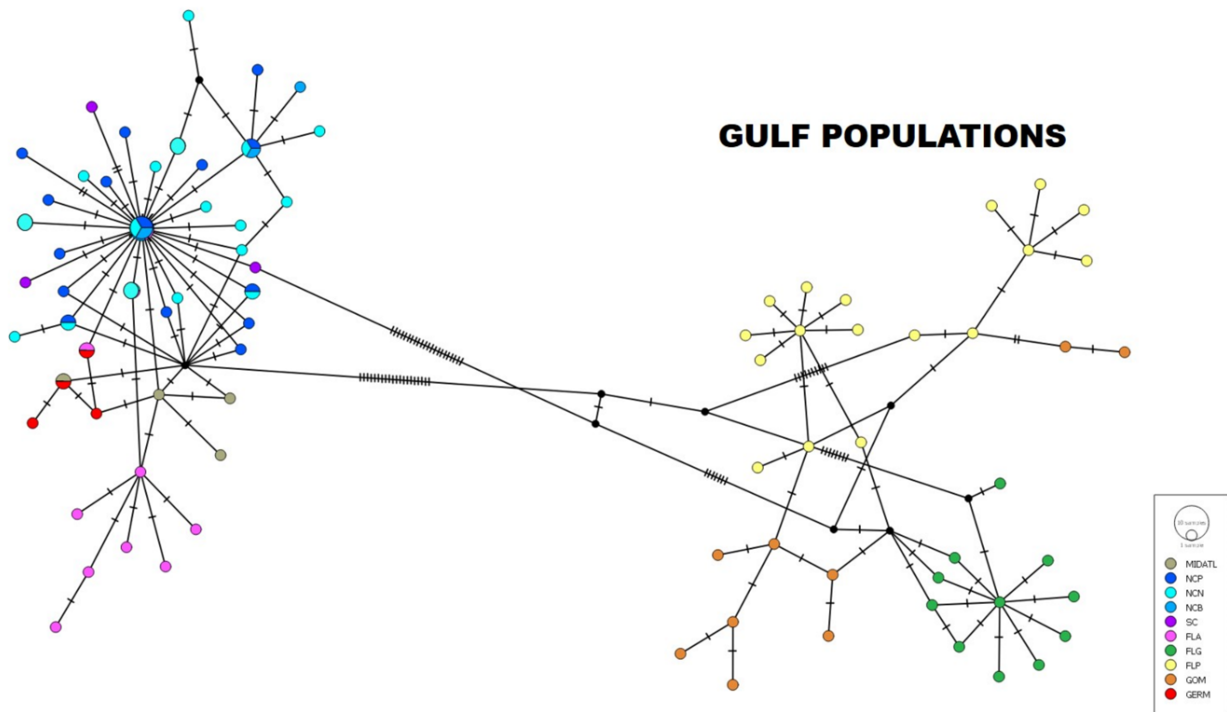

Fig. 6. Haplotype Network of Atlantic and Gulf of Mexico Populations. Subregions included the following: MIDATL (Mid Atlantic, including New York and Virginia), NC_P (NC Pamlico), NC_N (NC Neuse), NC_B (NC Bogue), SC (South Carolina), FLA (Atlantic Florida, including Jacksonville (JAFL) and Indian River (IRFL), FLG (Gulf Florida, including Cedar Key (CKFL) and Tampa Bay (TBFL)), FLP (Florida panhandle, including Apalachicola (APFL) and Destin (DEFL)), GOM (Gulf of Mexico, including Empire, Louisiana (EMLA), Ocean Springs, Mississippi (OSMS), and Galveston, Texas (GATX)), and GERM (Weser Estuary, Germany). This figure represents an incidence-based TCS network analysis of all haplotypes and their connections throughout the sampled region in the Atlantic and Gulf of Mexico. Much of the data comes from Mila et al. (2017) (acquired from Genbank) and also our recent data from North Carolina estuaries.

Tables

Table 1. List of site names and abbreviations, locations, average salinities, salinity classification, number of haplotypes per population, population-level genetic diversity, and the proportion of singleton haplotypes (out of all detected haplotypes) per population. Population-level genetic diversity was not calculated for HPC.

Site	Site Name	Location	Mean Salinity (ppt)	Standard Deviation Salinity	Salinity Classification	Number of Samples	Total Number of Haplotypes	Expected # haplotypes	# samples to reach asymptote	Genetic Diversity	Proportion of Singleton Haplotypes
GSC	Goose Creek, Washington, NC	Pamlico	3.08	1.97	low	34	8	22.53 (±2.36)	550	0.46	0.50
MLC	Mallard Creek, Washington, NC	Pamlico	3.62	1.76	low	19	5	9.65 (±1.33)	65	0.39	0.40
NCL	North Creek Landing, Belhaven, NC	Pamlico	8.21	1.45	medium	21	6	13.40 (±1.23)	80	0.43	0.50
WRC	Wright's Creek, Belhaven, NC	Pamlico	9.55	0.93	medium	14	2	2.44 (±0.14)	15	0.14	0.00
FSL	Fisher's Landing, New Bern, NC	Neuse	2.91	1.78	low	7	2	2.57 (±0.14)	8	0.29	0.50
CQC	Cahooque Creek, Havelock, NC	Neuse	3.64	3.03	low	15	8	29.94 (±2.91)	365	0.76	0.75
MTP	Matthew's Point Marina, Havelock, NC	Neuse	10.33	4.15	medium	16	5	9.62 (±1.27)	55	0.45	0.60
POC	Pin Oak Court, Merrimon, NC	Neuse	9.65	4.17	medium	4	3	4.36 (±0.21)	12	0.83	0.33
CDI	Cedar Island, NC	Neuse	14.21	4.09	medium	17	6	13.61 (±1.70)	70	0.51	0.50
HPC	Hoop Pole Creek, Atlantic Beach, NC	Bogue Sound	30	n/a	high	8	3	3.57 (±0.12)	30	n/a	0.33

Supplementary Tables

Table S1. Haplotype data and DNA sequences

In the table below, the first column represents the haplotype number (the actual sequence information for each haplotype follows this table). Columns 2-10 represent newly collected raw data from populations in North Carolina. These include the abundance of a particular haplotype per site. Columns 11-12 represent data extracted from Genbank that were part of Van Tassell et al. (2015). Columns 13-23 represent incidence data extracted from Genbank that were part of Mila et al. (2017). Below the table are the individual sequences for each of the 88 haplotypes represented in the table.

HAP	GSC	MLC	NCL	WRC	FSL	CQC	MTP	POC	CDI	HPC	FL	GERM	MIDATL	SC	JAFL	IRFL	CKFL	TBFL	APFL	DEFL	EMLA	OSMS	GATX
1												2											
2												1	1										
3												1											
4											4	1											
5	25	15	16	13	6	8	12	2	12	5													
6			1																				
7						1																	
8							1																
9	1																						
10	1																						
11					1																		
12							1																
13	1																						
14			1																				
15							1																
16									1														
17						1																	
18		1																					
19	1	1		1																			
20						1																	
21	1						1																
22		1							1														
23			1																				
24		1																					
25									1														
26			1						1														
27	3		1			1		1		2													
28									1														
29	1																						
30										1													
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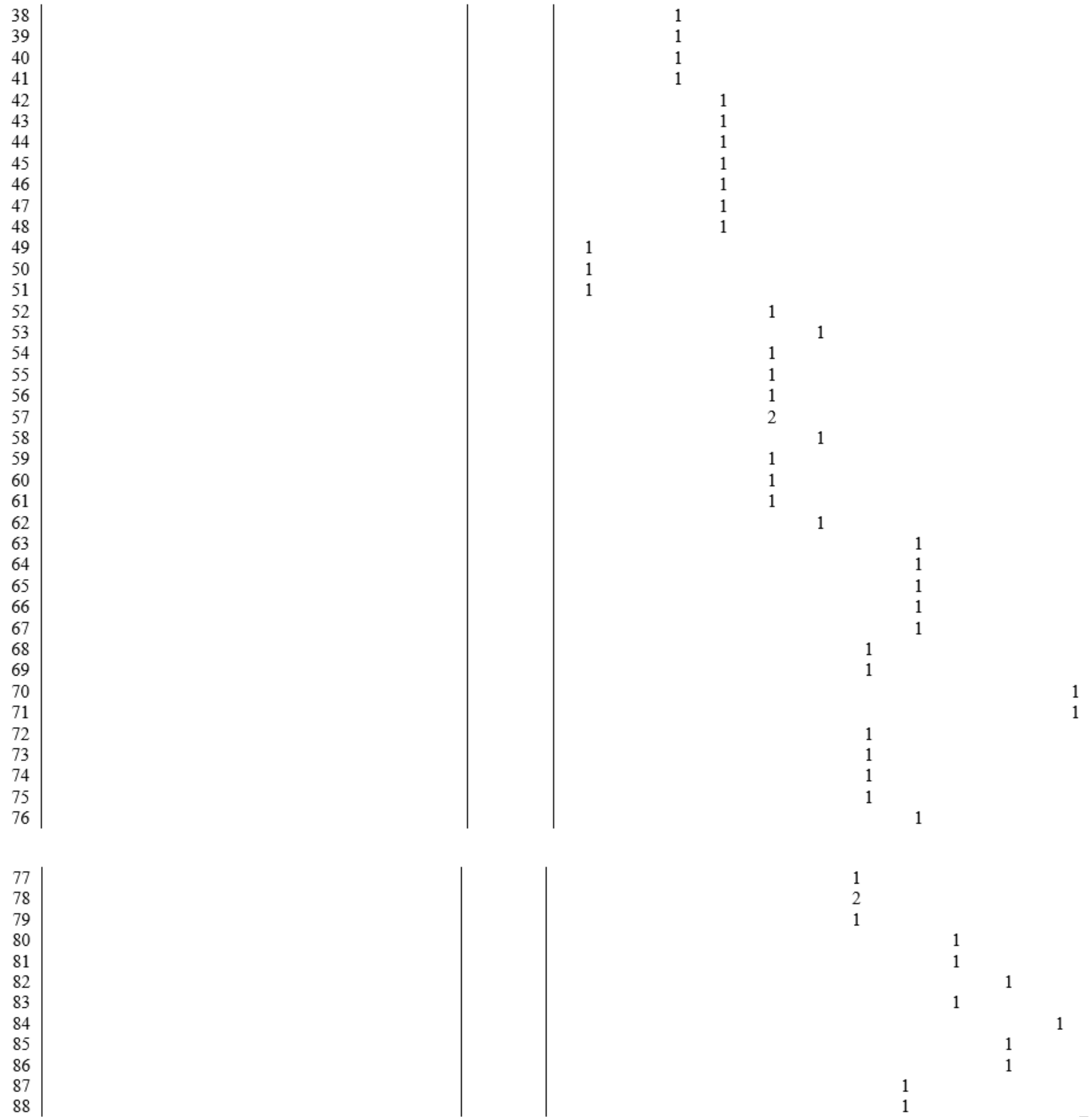

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HAP4 ATCCGCGCGGAGGCTCAGCCAGCCCGGCG-
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Table S2. Pairwise FST values.

	GSC	MLC	NCL	WRC	FSL	CQC	MTP	POC	CDI	HPC	GULF	GERM
GSC	0											
MLC	0.00927	0										
NCL	-0.00334	-0.00032	0									
WRC	-0.00797	-0.03473	-0.012	0								
FSL	-0.0161	-0.0167	-0.02228	0.02778	0							
CQC	0.02474	0.07242	0.04755	0.067	0.01743	0						
MTP	0.00506	-0.00059	-0.00179	-0.00403	-0.01087	0.06667	0					
POC	-0.00875	0.1062	0.05469	0.21127	0.07508	-0.1131	0.11432	0				
CDI	0.01005	0.00396	-0.01223	0.00587	-0.02402	0.03491	0.01079	-0.00338	0			
HPC	0.05418	0.18767	0.13095	0.25396	0.15569	-0.035	0.19251	-0.08367	0.07985	0		
GULF	0.62101	0.72604	0.69638	0.89472	0.83768	0.47105	0.75498	0.66667	0.57618	0.65692	0	
GERM	0.87808	0.85445	0.85855	0.84423	0.74778	0.78472	0.84228	0.64052	0.81896	0.74536	0.63704	0

Table S3. Regressions with salinity.

Analysis	R^2	p
Genetic diversity & Average salinity (linear, ALL DATA)	0.004	0.873
Genetic diversity & Average salinity (linear, NEUSE)	0.010	0.870
Genetic diversity & Average salinity (linear, PAMLICO)	0.477	0.309
Genetic diversity & Average salinity (polynomial (2), ALL DATA)	0.004	0.987
Genetic diversity & Average salinity (polynomial (2), NEUSE)	0.203	0.796
Genetic diversity & Average salinity (polynomial (2), PAMLICO)	0.817	0.428
Genetic diversity & Average salinity (polynomial (3), ALL DATA)	0.049	0.965
Genetic diversity & Average salinity (polynomial (3), NEUSE)	0.897	0.401
Genetic diversity & Average salinity (polynomial (3), PAMLICO)	n/a	n/a
Genetic diversity & Salinity standard deviation (linear, ALL DATA)	**0.499**	**0.034**
Genetic diversity & Salinity standard deviation (linear, NEUSE)	0.244	0.398
Genetic diversity & Salinity standard deviation (linear, PAMLICO)	0.798	0.107
Genetic diversity & Salinity standard deviation (polynomial (2), ALL DATA)	**0.639**	**0.047**
Genetic diversity & Salinity standard deviation (polynomial (2), NEUSE)	0.548	0.453
Genetic diversity & Salinity standard deviation (polynomial (2), PAMLICO)	0.918	0.286
Genetic diversity & Salinity standard deviation (polynomial (3), ALL DATA)	0.662	0.118
Genetic diversity & Salinity standard deviation (polynomial (3), NEUSE)	0.723	0.631
Genetic diversity & Salinity standard deviation (polynomial (3), PAMLICO)	n/a	n/a
Proportion singletons & Average salinity (linear, ALL DATA)	0.07	0.49
Proportion singletons & Average salinity (linear, NEUSE)	0.157	0.509
Proportion singletons & Average salinity (linear, PAMLICO)	0.401	0.367
Proportion singletons & Average salinity (polynomial (2), ALL DATA)	0.185	0.542
Proportion singletons & Average salinity (polynomial (2), NEUSE)	0.199	0.801
Proportion singletons & Average salinity (polynomial (2), PAMLICO)	0.824	0.420
Proportion singletons & Average salinity (polynomial (3), ALL DATA)	0.210	0.732
Proportion singletons & Average salinity (polynomial (3), NEUSE)	0.352	0.899
Proportion singletons & Average salinity (polynomial (3), PAMLICO)	n/a	n/a
Proportion singletons & Salinity standard deviation (linear, ALL DATA)	0.187	0.248
Proportion singletons & Salinity standard deviation (linear, NEUSE)	0.058	0.697
Proportion singletons & Salinity standard deviation (linear, PAMLICO)	0.724	0.147
Proportion singletons & Salinity standard deviation (polynomial (2), ALL DATA)	**0.792**	**0.009**
Proportion singletons & Salinity standard deviation (polynomial (2), NEUSE)	0.647	0.354
Proportion singletons & Salinity standard deviation (polynomial (2), PAMLICO)	0.909	0.302
Proportion singletons & Salinity standard deviation (polynomial (3), ALL DATA)	**0.793**	**0.037**
Proportion singletons & Salinity standard deviation (polynomial (3), NEUSE)	0.672	0.688
Proportion singletons & Salinity standard deviation (polynomial (3), PAMLICO)	n/a	n/a

Chapter 2: Trophically transmitted parasites as cross-taxon surrogates of biodiversity in coastal shoreline environments

Abstract

Coastal shorelines are vulnerable to erosion and storm surge, natural processes that are exacerbated by anthropogenically-induced climate change and sea level rise. In response, managers are increasingly incorporating nature-based solutions like "living" shorelines that offer natural habitat protection as part of a broader strategy of coastal defense. In addition to their protective benefits, living shorelines also support and enhance biodiversity relative to shorelines that use artificial structures like bulkheads or seawalls. Routine monitoring is crucial for evaluating the long-term performance of all forms of coastal infrastructure, although monitoring can often prove extensive and time-consuming. We conducted a survey of biodiversity in both natural and artificial shoreline environments using digenetic trematode parasites as proxies for the presence and abundance of their host taxa. Past workers have indicated that trematode surveys can address many of the shortcomings of traditional monitoring programs, and trematodes themselves are readily collected and identified in their first-intermediate snail hosts. We sampled infra-communities of digenetic trematodes from the eastern mudsnail (*Ilyanassa obsoleta*), a key host for trematode parasites in intertidal marine environments. Trematode community richness, evenness (Pielou's), and diversity (Shannon, Simpson) were greater in snails sampled from natural shorelines with complex habitat (e.g., oyster reefs, saltmarsh cordgrass) rather than shorelines artificially reinforced with bulkhead structures. There were also important differences at the community-level that likely reflect underlying patterns of host diversity at different shoreline types. For example, the trematodes *Lepocreadium seterifoides* (LS) and *Zoogonus lasius* (ZL) were the most common species sampled across all sites, although

they dominated parasites communities in artificial shorelines. Polychaetes serve as intermediate hosts for LS and ZL, taxa that are ubiquitous but more common in polluted or habitat-poor environments. On the other hand, the trematode HQ, which requires mollusks and shorebirds as hosts, was only found at sites with natural shorelines, suggesting that these areas are less disturbed and have a greater diversity of taxa serving as intermediate hosts. Overall, there were stark differences in the trematode-host community between natural and artificial shorelines, which is surprising given the small-scale of our study. Wave energy is likely an important variable responsible for structuring the underlying host community, and future studies should quantify differences in the energy and sediment profiles at different shoreline environments.

Introduction

Hardened or "grey" shorelines (e.g., bulkheads/seawalls, rock sills) provide protective features that mitigate erosion and storm surge (Dugan et al. 2011). While "shoreline hardening" has become increasingly prevalent over the last century (Gittman et al. 2015), hardened infrastructure usually fails in the absence of significant investment and upkeep (Thieler & Young 1991; Gittman et al. 2014). In addition, shoreline hardening can also negatively affect biodiversity and other ecosystem services (Sutton-Grier et al. 2015). In contrast, natural or "green" shorelines (e.g., saltmarsh cordgrass, oysters) are capable of mitigating erosion while also conserving biodiversity (Sutton-Grier et al. 2015) (Fig. 1).

Increasingly, practitioners have called for an "ecosystem-based" strategy for coastal defense, one focused on creating "living shorelines" to improve the resilience of coastal ecosystems (Temmerman et al. 2013). Resilience is the ability of an ecosystem or biological community to quickly recover from a disturbance, or adapt to changing environmental conditions (Timmerman 1981). In the aftermath of Hurricane Matthew (2016), living shorelines with natural

habitat were found to be more resistant to landward erosion than hardened shorelines in the same comparable environment (Smith et al. 2018). In addition to wave attenuation and erosion mitigation, natural shoreline habitat is also capable of adapting to sea-level rise and enhancing the resilience of surrounding biological communities (Gittman et al. 2018). Collectively, biodiversity and related ecosystem services like flood protection are often referred to as "natural" or "ecological" infrastructure (Sandifer & Sutton-Grier 2014; Rosati et al. 2015). As a metric of resilience, biodiversity represents the trait-based diversity of organisms (Cadotte et al. 2011) and the ecological services associated with these traits (Palmer et al. 2014). Degraded coastal landscapes, or shorelines lacking complex natural habitat, are thus missing more than a variety of different species; they are also deficient in the ecological complexity characteristic of a resilient ecosystem.

Schultz et al. (2012) called for more indirect (i.e., proxy) indicators for calculating resilience and recovery following a disturbance and for evaluating the ability of coastal systems to withstand multiple stressors. An increasing number of biodiversity studies prioritize the use of indicator or "surrogate" species in biological monitoring – organisms that can serve as proxies for a broad array of taxa (Lindenmeyer et al. 2015). Multiple studies have shown that trophically transmitted parasites can serve as proxies, or surrogates, of overall biodiversity (Huspeni & Lafferty 2004; Hechinger & Lafferty 2005; Hechinger et al. 2007; 2008; Moore et al. 2020; Moore et al. in review). Trophically transmitted parasites (e.g., digenetic trematodes) typically require multiple invertebrate and vertebrate hosts to complete their life cycles (Blakeslee et al. 2012; Phelan et al. 2016). Given the strong positive correlation between host and parasite abundance (Kamiya et al. 2014; Wood & Johnson 2016), high parasite diversity in easily collected host organisms can provide evidence that the other hosts required by the parasite are

also recruiting to the area. In fact, variation in the parasite community can be informative for understanding how a system is "recovering" and "adapting" (i.e. CERB 2014) in response to disturbance or long-term environmental change (Huspeni & Lafferty 2004; Huspeni et al. 2005; Anderson & Sukhdeo 2013).

We sampled digenetic trematode parasites from the eastern mudsnail *Ilyanassa obsoleta* at shorelines containing both artificial (bulkheads) and natural (oyster reef/*Spartina*) habitat. Mudsnails are commonly found in tidal creeks and mudflats along the Atlantic coast of North America (Abbot 1974), where they may aggregate in dense clusters exceeding 1000 m^{-2} (Scheltema 1964; Curtis 2002), particularly in areas with low wave energy (Levinton et al. 1995). These snails are an integral part of estuarine food webs (Kelaher 2003). They also serve as first-intermediate hosts for nine species of digenetic trematodes, each of which requires one or more additional hosts (e.g., polychaetes, mollusks, fishes, shorebirds) to complete its lifecycle (Miller & Northrup 1926; Curtis 2007; Blakeslee et al. 2012; Phelan et al. 2016). We tested whether parasite diversity in mudsnails could serve as a proxy for overall community diversity in areas with artificial and natural shoreline habitat. We predicted that areas with more complex, natural habitat would have greater parasite diversity. As cross-taxon surrogates for multiple invertebrate and vertebrate taxa (Rodrigues & Brooks 2007; Caro 2010), snail-trematode parasites offer a potential "shortcut" for inventorying biodiversity. In fact, an analysis of sampling methodologies by Huspeni et al. (2005) suggests that larval trematode surveys have a distinct advantage over more traditional survey methods in terms of effort and assessment value. Parasites thus offer a promising method for monitoring biodiversity and ecosystem function over the long-term, as changes in the broader host community will be reflected in the relative abundance of parasite species present.

Materials and Methods

Field Sampling

Mudsnails were collected along the north-facing shoreline of Taylor's Creek, part of the Intracoastal Waterway in Beaufort, North Carolina (Fig. S1). Four pairs (n=8 sites total) of artificial/natural shorelines were sampled beginning in January, 2018 through October, 2019. Each paired site (n=4) was approximately 100-m in length. Sites were classified as artificial or natural based on the presence of a bulkhead and the overall condition of the marsh. Sites with artificial shorelines had a bulkhead and generally < 5-m of intact marsh between this structure and the water's edge. Sites with natural shorelines did not have a bulkhead and featured > 5 m of intact marsh between the water's edge and the upper marsh.

In January, 2018, two replicate plastic sampling units were staked in the shallow-subtidal at all sites (n=16 units total). These samplers were filled with oyster shell and passively recruited resident and transient fauna like mud crabs and small benthic fishes. From March, 2018 through April, 2019 taxonomic diversity in these collectors was quantified and resident species of xanthid crustaceans and benthic fishes were removed and later dissected for parasites (n=10 sampling events). In addition, a minimum of n=100 snails (*Ilyanassa obsoleta*) were haphazardly collected from each site using a dip net at low-tide. Snails were collected from all sites from January, 2018 through October, 2019 (n=12 sampling events). Abiotic measurements of salinity (ppt), water temperature (°C), and dissolved oxygen (mg/L) were collected at each site during each sampling event with a handheld YSI (ProODO).

Estimates of habitat complexity were collected from all sites in September, 2018. The density of *Spartina* shoots and live oysters was quantified along a 50-m transect oriented perpendicular to the shoreline. The number of shoots and live oysters were counted within a

0.25m^2 quadrat at 1-m intervals until reaching the upper marsh edge. Mudsnail densities were also collected from each site in June, 2019. At low tide, a 50-m transect tape was oriented parallel to the shoreline at the water's edge. A 0.5 m^2 quadrat was used to count the number of mudsnails present at 5-m intervals along the tape until reaching the 25-m mark (n=5 quadrat measurements total). Following sampling, the average number of snails across all 5 quadrats was tabulated, which was then used to compute an estimate of snail density for each site. Field collections were authorized by the North Carolina Division of Marine Fisheries (Scientific or Educational Permit #706671), and housing and dissection protocols were approved by East Carolina University's IACUC (AUP #D346).

Host Dissection and Parasite Identification

Snails were dissected for parasites using a stereomicroscope (Zeiss Stemi 508). Prior to dissection, snails were measured (mm) from the base of the siphon to the upper spire. Snails were then dissected by cracking the shell with a hammer and extracting the soft tissue, which was teased apart in a watch glass using forceps. The gonadal region of the snail was separated and scanned for larval trematodes, which are readily identifiable to species using ordinary light microscopy (Blakeslee et al. 2012). Both mature and immature-stage infections were noted. Snail-trematode parasites were identified using standard protocols and keys (Blakeslee et al. 2012; 2015; Phelan et al. 2016).

Resident species of crabs and fish were also dissected for parasites. Crabs were measured (carapace width, mm), sexed, and dissected by separating the upper and lower carapace. Tissue squashes of hepatopancreas and gonad tissue were then scanned for parasites at low power (4-10x) using a compound microscope (Zeiss AxioScope A1). Fish were measured (TL, mm), sexed, and dissected by removing the entire gastrointestinal tract (stomach, liver, gallbladder,

spleen, intestine) and scanning for parasites at low power. The gut cavity of each fish was then rinsed and the wash examined for parasites that had been dislodged during dissection. Lastly, the head, body, and fins were checked for subcutaneous trematode cysts by viewing each fish at low power under a stereomicroscope (Zeiss Stemi 508). Only xanthid crabs ≥5 mm, and benthic fishes ≥20 mm were dissected for parasites because the macroparasites in our system primarily infect adult individuals (Moore et al. 2020). All parasites were identified using standard protocols and keys (e.g., Yamaguti 1971). Although smaller mud crabs and fish were not dissected for parasites, they were still included as part of our free-living diversity analyses. We also counted and identified other mobile taxa (e.g., blue crabs, shrimps, transient fishes) found within the crates and minnow traps at each site.

Statistical Analyses and Data Visualization

Statistical analyses were performed on data for identifiable species of larval trematodes in snails; early-stage immature infections were excluded from all analyses. Parasite count data was zero-inflated and did not meet the assumptions of a normal distribution. As such, non-parametric statistical tests were used for assessing differences in parasite diversity in snail, crustacean, and fish hosts from artificial and natural shoreline sites. Univariate analyses were conducted in R (version 4.0.3) using R core functionality and the package *rstatix* (Alboukadel 2020) for basic statistical tests. Wilcoxon rank sum tests with continuity correction were used to test for pairwise differences in richness, evenness, diversity, and snail density between artificial and natural shorelines. Wilcoxon effect size tests were used to calculate effect sizes and extract confidence intervals. Kruskal-Wallis and Wilcoxon rank sum post-hoc tests were used to test for differences in habitat metrics across multiple pairwise comparisons (i.e., n=8 sites). Shannon-Weiner and Simpson diversity values were calculated using the *vegan* package (Oksanen et al. 2020).

Pielous' evenness was derived by dividing Shannon-Weiner (H) index values by log (#species) (Pielou 1966). Rank-abundance curves were constructed for the snail-trematode data to understand patterns of relative abundance in artificial and natural shoreline communities. This log-series model predicts the number of species at different levels of abundance (Fisher et al. 1943). Snail-trematode data from both shoreline communities were arranged in decreasing order of abundance, and log-abundance (y-axis) was plotted against community rank (x-axis). In total, eight of nine possible trematode species were sampled from *I. obsoleta* snails in Taylor's Creek (n=6 from Artificial; n=8 from Natural). Curves from both shorelines were fitted along the same domain (1-8) to make it easier to compare community data. Data were fitted using loess smoothing (non-parametric), a method that is appropriate for smaller sample sizes (Gardener 2014).

For visualizing site-level patterns in the data, a cluster dendrogram was constructed for the snail-trematode data. Snail trematode data were presence/absence transformed and pooled by site. The resulting matrix was used to construct the dendrogram based on Jaccard dissimilarity. We also conducted chi squared tests on the abundance data to determine if species of trematodes were positively or negatively associated with specific shoreline types (Gardener 2014). Trematode abundance at all shorelines was formatted as a contingency table and analyzed using chi-squared approximation. The resulting Pearson residuals for species at each shoreline were then plotted using basic R functionality. A threshold value of [2] is considered to be a significant test of an "indicator," or association (Gardner 2014).

We used coverage-based rarefaction methods in addition to conventional sample-based rarefaction to determine whether we had fully accounted for trematode diversity in artificial and natural shoreline communities. Estimators of richness cannot distinguish between rare and

common species in a community (see discussion in Chao & Jost 2012). In communities with multiple rare species, sample richness may never asymptote with increasing sample size (Coddington et al. 2009). While rare species of trematodes were present in both shoreline communities, they were overly-abundant in the artificial shoreline community. As a result, sampling-based rarefaction/extrapolation estimators may not be appropriate and we opted to standardize diversity in these different communities using additional coverage-based estimators (Chao & Jost 2012; Chao et al. 2014; Hsieh et al. 2020). The R package *iNEXT* plots Hill Numbers (species richness, Shannon diversity, Simpson diversity) and their associated 95% confidence intervals using a conventional sample size estimator (Type 1), sample completeness (Type 2), and coverage-based (Type 3) rarefaction/extrapolation estimators (Hsieh et al. 2020). Of note, the Type 2 curve provides a link between sample size and coverage-based estimation methods (Chao & Jost 2012). Snail-trematode community data were analyzed in *iNEXT* using all three methods.

Lastly, we constructed a table of invertebrate and vertebrate taxa found in coastal North Carolina that could serve as downstream hosts for snail-trematode parasites in our system. The range and distribution for all hosts was assessed using either the World Register of Marine Species (WoRMS), FishBase, or the Cornell Lab of Ornithology. This effort builds upon similar work done for *Ilyanassa obsoleta* and its trematode parasites conducted in coastal Delaware (Curtis 2007) and in native vs. introduced ranges of the snail (Blakeslee et al. 2012).

Results

Trematode Diversity and the Host Community

Shannon-Simpson diversity of trematode communities was greater in sites with natural shorelines (W=229.5, p=0.003; W=198, p=0.0006) (Fig. 2). There were also compelling differences in the richness and evenness of trematode communities, which may have implications for the types of downstream host taxa frequenting these environments. Overall, richness was greater in sites with natural shorelines (W=273, n=64, p=0.008) (Fig. S2). However, this difference disappeared when trematode richness was partitioned into fish-using trematode species (n=4) vs. bird-using trematode species (n=4), neither of which differed as a function of shoreline type (Fig. S3). Similarly, there was a much more even abundance of trematode species in sites with natural shorelines (W=229.5, p=0.003) (Fig. S4). Collectively, these results are illustrated in Figure 3, which depicts how the diversity of snail-trematode parasites can be used to extrapolate the presence of additional host taxa required for life cycle completion. Based on work done by Blakeslee et al. (2012), there are a multitude of potential hosts for these parasites, all of which are abundant in coastal North Carolina (Table S1).

Parasite Community Metrics

To better visualize the trematode community data, rank-abundance curves were created for the data from artificial and natural shorelines (Fig. S5). When comparing data from both communities, the slope of the curve is greater in the figure depicting snail-trematode data from artificial shorelines, indicating lower overall diversity. While the trematode LS was the most abundance species at both types of shorelines (rank=1), the rate-of-change to the next abundant species, ZL (rank=2, Artificial) vs. HQ (rank=2, Natural), is more abrupt at sites with artificial shorelines. Note that DN and GA are paired together at artificial shorelines (rank=5) because there was only a single collection record for each species. The trematode LS was overly-abundant in artificial shoreline sites (Fig. S5; Table S2). While there were no differences in the

overall prevalence of infected snails between artificial and natural shorelines (Table S3), removing LS from both sites resulted in greater infection prevalence at sites with natural shorelines (W=242, p=0.004) (Fig. S6). Interestingly, the trematode HQ was detected in high abundance (n=45 infections) at natural shorelines but was absent from artificial shorelines (Table S2). LS and HQ may serve as indicators of artificial or natural shoreline sites (Fig. S7). Artificial shorelines were positively associated with the trematode LS, a species requiring polychaetes and fish as second-intermediate and final hosts, respectively. Of note, the trematode LS is still highly abundant in natural shorelines (n=165 infections) (Table S2). There is, however, an uneven abundance of LS in artificial shorelines, which may indicate the preference of this species, or its host taxa, for this type of environment (i.e., Gardener 2014). On the other hand, artificial shorelines were negatively associated with HQ, which requires mollusks and shorebirds as hosts. This species of trematode was positively associated with natural shorelines.

Overall, the snail-trematode data tended to cluster by shoreline type (Artificial vs. Natural), although data from the Boathouse-Artificial site was more similar to data from other natural shoreline sites (Fig. S8). Interestingly, two out of three rare trematode species sampled from artificial shorelines (AV=2; DN=1; GA=1) came from this site. This included both observations of the trematode AV, which requires shorebirds as hosts, and the single record of GA, which requires crustaceans and shorebirds as second-intermediate and final hosts, respectively. The lone record for DN at artificial shoreline sites, which also requires shorebirds, was sampled at the Duke Marine Lab. The Boathouse-Artificial site is unusual among its cohort in that it has a relatively intact saltmarsh between the bulkhead and the water's edge. The habitat data were noteworthy at this site. While there were no differences overall in *Spartina* shoot or

live oyster density across all sites, densities of both types of habitat were greater at the Boathouse-Artificial site compared to its natural shoreline counterpart (W=12, p=0.05) (Fig. S9).

Sample Coverage

Mudsnails tend to aggregate in large groups (Scheltema 1964; Curtis 2002), and densities were greater at sites with natural shorelines (W=0, n=4, p=0.03) (Table S4; Fig. S10). However, estimates of coverage-based rarefaction and extrapolation showed that our sampling effort was adequate in both shoreline types. If standardizing by sample size, as is traditional for most estimators, it would appear as if richness in artificial shorelines has been undersampled. (Fig. S11a). However, one major problem (i.e., Chao & Jost 2012) is that the estimator in this case is equally sensitive to rare species in the community (e.g., DN (n=1), GA (n=1), AV (n=2)) as it is to common species like LS (n=103) (TableS2). It is therefore difficult to differentiate a sampling effect from a true community effect, the latter an indication that artificial shoreline communities are host-poor compared to natural shoreline communities. Chao & Jost (2012) argue instead for standardizing by sample completeness and coverage rather than size. When formatted in this manner, both estimators show near-complete saturation for artificial shorelines (~97%) and 100% saturation for natural shoreline communities (Fig. S11b-c).

Additional Analyses

Summary statistics for abiotic data can be found in Table S5a. Mean estimates of temperature, salinity, and dissolved oxygen (± 1SD) stratified by site and shoreline type can be found in Table S5b. Site-resident and transient species of crustaceans and fish were also collected to quantify the overall taxonomic diversity of nekton associated with different shoreline types. Mean abundances (± 1SD) of these taxa are depicted in Tables S6a-b. Site-resident species

(e.g., mud crabs, small benthic fishes) were dissected for parasites. There were no differences in the parasite richness of crustacean and fish taxa as a function of shoreline type (Fig. S12).

Discussion

Measurements of biodiversity are notoriously scale-dependent (Magurran 2003). Effect sizes can radically differ in both magnitude and direction, making it impractical to rely on a single metric for quantifying biodiversity over time (*see* Chase et al. 2018). To address this issue, Chase et al. (2018) recommended using several biodiversity metrics (e.g., functional, phylogenetic, and taxonomic) that reflect the multidimensional scale of biodiversity. As cross-taxon surrogates for their host taxa, trophically transmitted parasites rely on an array of functional groups at different trophic levels for life cycle completion, making them ideal indicators of overall community diversity at multiple scales. Nevertheless, our results differ from recent analyses concluding that scale is not an important driver of the post-parasite diversity relationship. Kamiya et al. (2014) analyzed 38 case studies of host and parasite species richness, literature used by Wood & Johnson (2016) to conduct a meta-regression on the effects of scale. According to their results, for each 1-species increase in host diversity, parasite diversity increased by 0.44 species. Of note, spatial scale was not an important predictor of the host-parasite relationship. It should be emphasized that most studies used by Wood & Johnson (2016) dealt with terrestrial systems at extensive scales (100-1000 kilometers). While host diversity and parasite diversity are tightly correlated (Kamiya et al. 2014; Wood & Johnson 2016), over vast distances the relationship between surrogates and their target taxa can weaken (Wiens et al. 2008). At smaller scales, however, cross-taxon surrogates like trophically transmitted parasites are useful for predicting underlying patterns of host diversity (Wiens et al. 2008; Rossiter & Sukhdeo 2014).

The null hypothesis guiding our study was that the data from each paired site (A vs. N) should be similar since they are adjacent to one another. Instead, snail-trematode communities at artificial and natural shoreline sites were remarkably different and tended to cluster by shoreline type (Fig. S8). Trematode communities from natural shorelines exhibited greater richness (Fig. S2), evenness (Fig. S4-S5), and diversity (Fig. 2) compared to their artificial counterparts. This was surprising given the small scale of our work – paired sites were located adjacent to one another and were generally less than 100-m in length. However, despite the scale involved, it is unlikely that mud snails were moving between shorelines and mixing trematode communities. Movement patterns of *I. obsoleta* have been studied extensively, both in the field (Curtis 1987; 1990; 2005; 2007a) and in the lab (Borowsky 1979; Dimock 1985). On average, parasitized snails move less than unparasitized individuals (Curtis 2002; 2003). In fact, parasitized individuals are infected for life, decades in some cases (Curtis 2003), as the parasite castrates the snail and slows its growth (Curtis & Hurd 1983). Other than infection status, environmental conditions are mostly responsible for mediating patterns of snail distribution. Rossiter & Sukhdeo (2012) GPS-tracked mudsnails over a three-year period, finding that snails released in intertidal pannes buffered by *Spartina* did not migrate into nearby habitats, despite their proximity, while those released into exposed coastal flats (with greater wave action) moved more on average and sought out less disturbed areas. Our study area has low fetch and is subjected to wave energy from boat wake, which can be destructive to shallow sub-tidal habitats (Parnell et al. 2007). Shorelines reinforced with artificial structures tend to have more reflective wave energy, which contributes to scouring and sediment resuspension (Currin 2019), whereas natural shorelines typically have large sections of intact marsh capable of dissipating this energy (Manis et al. 2015; Currin 2019). This may explain why mudsnails were more scattered in areas with

artificial shorelines (Table S4), although it does not mean that snails in natural shorelines were more likely to be infected. The prevalence of infected snails was similar between artificial (8.4%) and natural shorelines (9.0%) (Table S3). In a study of *I. obsoleta* and its trematode parasites in Delaware Bay, Curtis (2009) identified six species (HQ, LS, ZL, AV, GA, ST) in a population with low infection prevalence (14%), which he argued resulted from wave energy and current velocity inhibiting the environmental transmission phase from final hosts to snail first-intermediate hosts. The population that Curtis (2009) studied was fringed with narrow channels, leading him to theorize that trematode eggs were being advected from the area. In some cases, snail populations within only a few meters of each other exhibited markedly different infection prevalence, which he attributed to local hydrodynamics (Curtis 2007b). There are a multitude of factors that affect cercarial transmission and infection at the free-living stage (Combes et al. 1994; Thieltges et al. 2008; Koprivnikar & Poulin 2009). In our system, potential differences in wave energy did not affect the overall infection prevalence of snails in artificial and natural shorelines (Table S3). However, the energy profile of each shoreline environment could mediate the types of host taxa present, which in turn would structure the parasite communities found in these areas.

The trematode community data imply that there are underlying differences between the host communities in each shoreline type. The top three most abundant trematodes at artificial shorelines (LS=103; ZL=15; ST=8) all use fish as final hosts, while the most abundant species at natural shorelines use both fish (LS=165; ZL=68) and shorebirds (HQ=45) (Fig. S5). Of note, the trematode HQ – a species requiring mollusks and shorebirds as second-intermediate and final hosts – was abundant at natural shorelines (n=45 observations) but completely absent from artificial shoreline sites (Table S2). For HQ to complete its lifecycle, larval cercariae shed from

snails must infect a molluscan second-intermediate host, which must then be consumed by a shorebird final host. Based on field studies, confirmed second-intermediate hosts for HQ include the polychaete *Scoloplos fragilis* (Stunkard 1986) and the bivalve *Mya arenaria* (Uzmann 1951). In addition, experimental studies have shown that ribbed mussels *Geukensia demissa,* jackknife clams *Ensis directus*, quahog clams *Mercenaria* spp., horsemussels *Modiolus modiolus*, and scallops *Argopectans irradians* can also serve as competent second-intermediate hosts for HQ, although apparently not the eastern oyster *Crassostrea virginica* (Cheng et al. 1966). Bivalve communities are sensitive to wave-energy and sediment deposition (Ysebaert et al. 2019). Studies of sub-tidal benthic communities adjacent to bulkheads have reported lower diversity compared to those near natural marshes (Seitz et al. 2006). Similar studies have shown that installing living shorelines with natural habitat can result in profound changes in benthic community composition such as higher densities and biomass of bivalves (Davenport et al. 2018). In our system, a high abundance of HQ at natural shorelines implies that bivalves and other second-intermediate hosts are also present and are being consumed by herring gulls *Larus argenatus*, common terns *Sterna hirundo*, and other final hosts (Stunkard 1938) (Table S1).

Multiple studies have concluded that the long-term maintenance and stability of parasite populations is best explained by the abundance of final hosts (Hoff 1941; Robson & Williams 1970; Hughes & Answer 1982; Matthews et al. 1985; Smith 2001; Kube 2002; Skirnisson et al. 2004; Byers et al. 2008; Levakin et al. 2013; Byers et al. 2016). In any system, parasite component communities from different hosts are expected to reach a state of dynamic equilibrium determined by the relative abundance of autogenic vs. allogenic host taxa (Esch & Fernandez 1994; Fellis & Esch 2005; Feis et al. 2015). Fish-driven systems are said to be autogenic and have less colonization potential compared to allogenic systems that use shorebirds

as final hosts (Esch et al. 1988). Trematode parasite communities in artificial shorelines lacked a strong allogenic signature, suggesting that these hosts are not frequenting artificial shorelines even though their colonization potential is higher overall. In addition, HQ may be absent from artificial shorelines because second-intermediate hosts like mussels and clams are not present in large numbers. The absence of HQ could thus be thought of as an indicator of a disturbed shoreline environment with low host diversity (Table S7). Similarly, a high relative abundance of the trematode LS may also serve as an indicator of artificial shorelines or disturbed environments (Table S7). Multiple species of polychaetes within the family Spionidae have been confirmed as second-intermediate hosts for LS including *Pygospio elegans* (McCurdy et al. 2000; Curtis 2007), *P. ciliata*, *P. ligni*, and *Spio* spp. (Peoples 2013). It is highly likely that LS can further its lifecycle using a range of polychaetes, flatworms, and other invertebrates at this stage (Table S1). This species has many potential hosts, which may explain why it was the most abundant parasite sampled from our system, although it was more common in sites with artificial shorelines (Table S2). The third most abundant trematode, ZL, also uses several species of polychaetes as second-intermediate hosts (Peoples 2013) (Table S1). Polychaetes in general are a highly diverse group of invertebrates that occupy a wide range of environments (Glasby & Timm 2008). They are especially common in soft-bottom habitats, where they function as deposit feeders in sediment-rich environments (Tian et al. 2019). Polychaetes are opportunistic taxa tolerant of disturbance and pollution (Dauvin 2018). For this reason, they have been used as indicators of human impacts alongside more sensitive groups like amphipods (Dauvin 2018). Habitats with higher polychaete diversity relative to other invertebrates are often found in anthropogenically modified estuaries subject to a variety of chemical and physical stressors (Dafforn et al. 2013). The polychaete-using trematodes LS and ZL were some of the most

common trematode parasites in our system. However, they were overly abundant at sites with artificial shorelines (Fig. S5). Altogether, the parasite data indicate that polychaetes dominate the host community at artificial shoreline sites, while the data from less disturbed natural shorelines suggests that mollusks are abundant in addition to polychaetes and other invertebrates (Table S1, S2, S5).

Routine monitoring is crucial for evaluating the long-term performance of coastal infrastructure (La Peyre et al. 2022). Although there are well-recognized financial and logistic challenges associated with monitoring programs (Yoccoz et al. 2001), trematode-based studies have several advantages over more conventional methods of quantifying biodiversity (Huspeni et al. 2005). With a minimum amount of training and effort (Huspeni et al. 2005), trematodes can be readily surveyed and identified in common snail-hosts, making them ideal indicators of community diversity and ecological resilience. Where *I. obsoleta* is locally abundant, parasite surveys in these snails could serve as a "rapid assessment" method analogous to the EPA's protocol for monitoring biodiversity in freshwater streams (EPA 1999). Moreover, there are multiple snail-trematode systems that could be substituted for *I. obsoleta* in other locations. For example, trematode-host communities have been characterized for the Littorine snails *Littorina saxatilis* and *Littorina obtusata* in the northeastern United States and western Europe (Blakeslee et al. 2012; Granovitch & Maximovich 2013; Levakin et al. 2013) as well as the California Horn Snail *Cerithidea californica* along the Pacific Coast of the United States (Huspeni & Lafferty 2004). Horn snails have a world-wide distribution (Houbrick 1984) as do hydrobiids, which have diversified across freshwater, estuarine, and marine environments (Miller et al. 2018). There are multiple records of trematode diversity in both horn snails (Harada & Suguri 1989; Mani & Rao 1993; Al-Kandari et al. 2000) and hydrobiids (Heard 1970; 1976; DeBlock 1980; Heard &

Overstreet 1983; Font et al. 1984; Heard et al. 2002; Skirnisson et al. 2004; Pung et al. 2002; 2006; 2008). However, in many cases we lack detailed knowledge of the relevant host communities needed by these parasites (although see Heard & Overstreet 1983; Pung et al. 2006; 2008). Future efforts should therefore focus on identifying these host taxa, particularly the final hosts needed for lifecycle completion (autogenic vs. allogenic).

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Figures

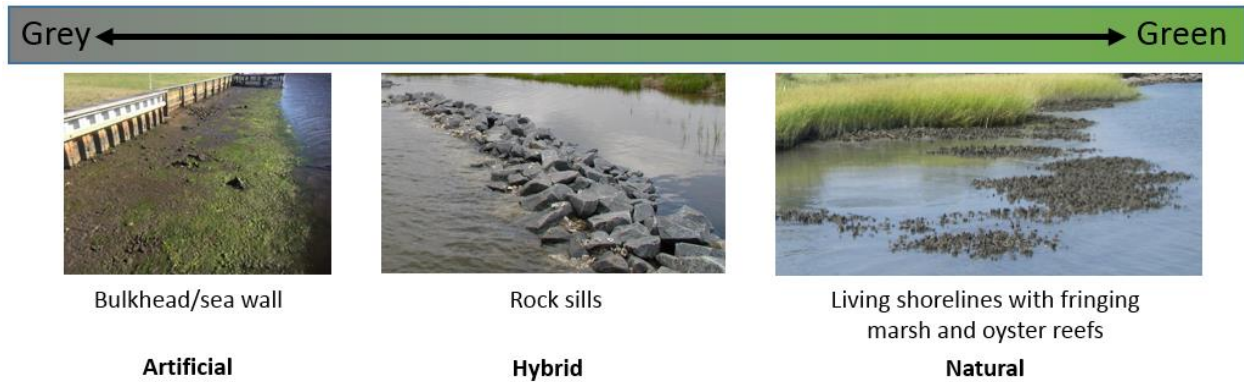


Fig. 1. Shoreline infrastructure. Coastal shorelines are often composed of a mix of "grey-to-green" infrastructure.

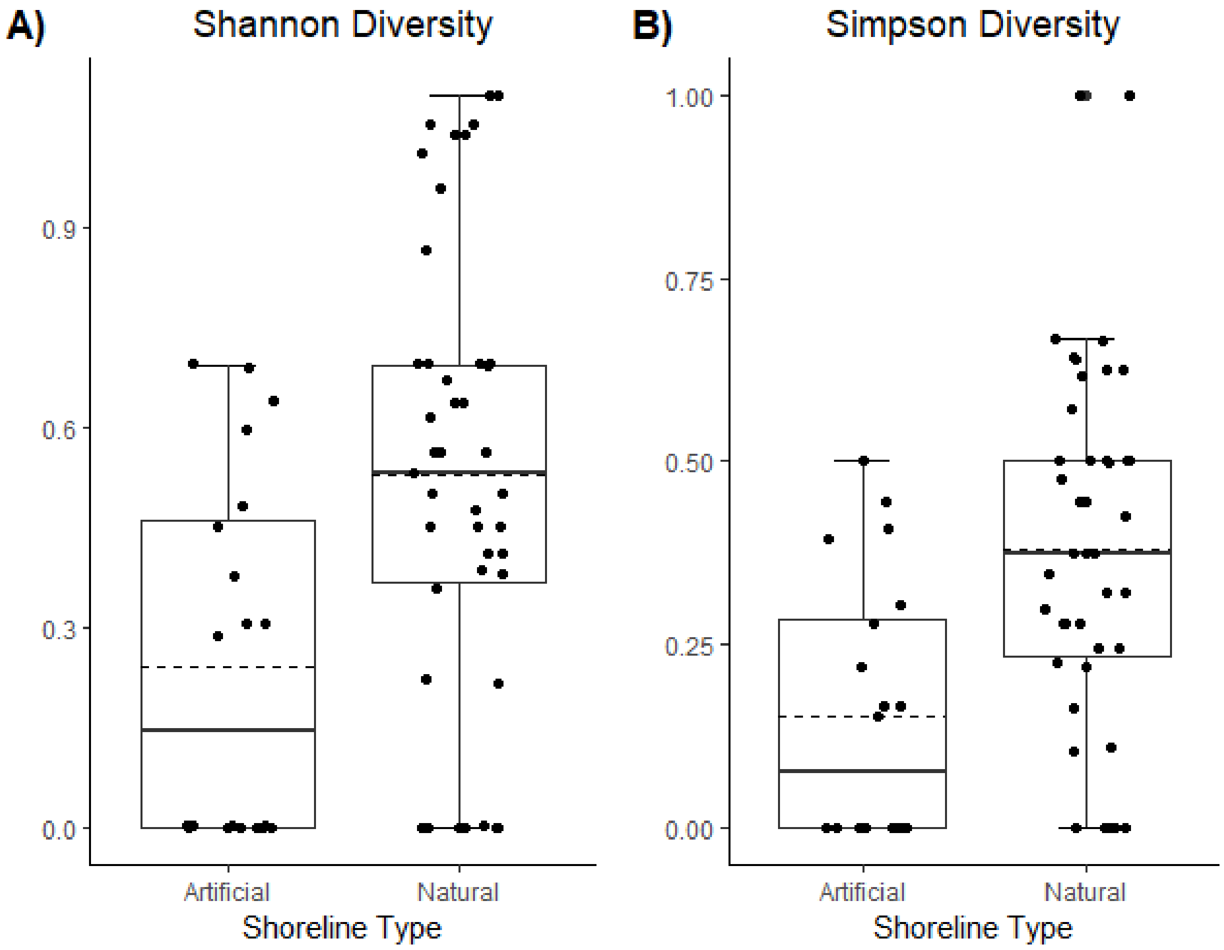


Fig. 2. Shannon (A) and Simpson (B) diversity of snail trematodes sampled from artificial and natural shoreline sites. Shannon diversity was higher in natural shorelines (W=229.5, p=0.003). There was a moderate effect size difference in diversity between artificial (n=20) and natural (n=44) shoreline sites (r=0.38, CI low=0.18, CI high=0.57). Simpson diversity was likewise higher in natural shorelines (W=198, p=0.0006). There was a moderate effect size difference (r=0.44, CI low=0.21, CI high=0.61).

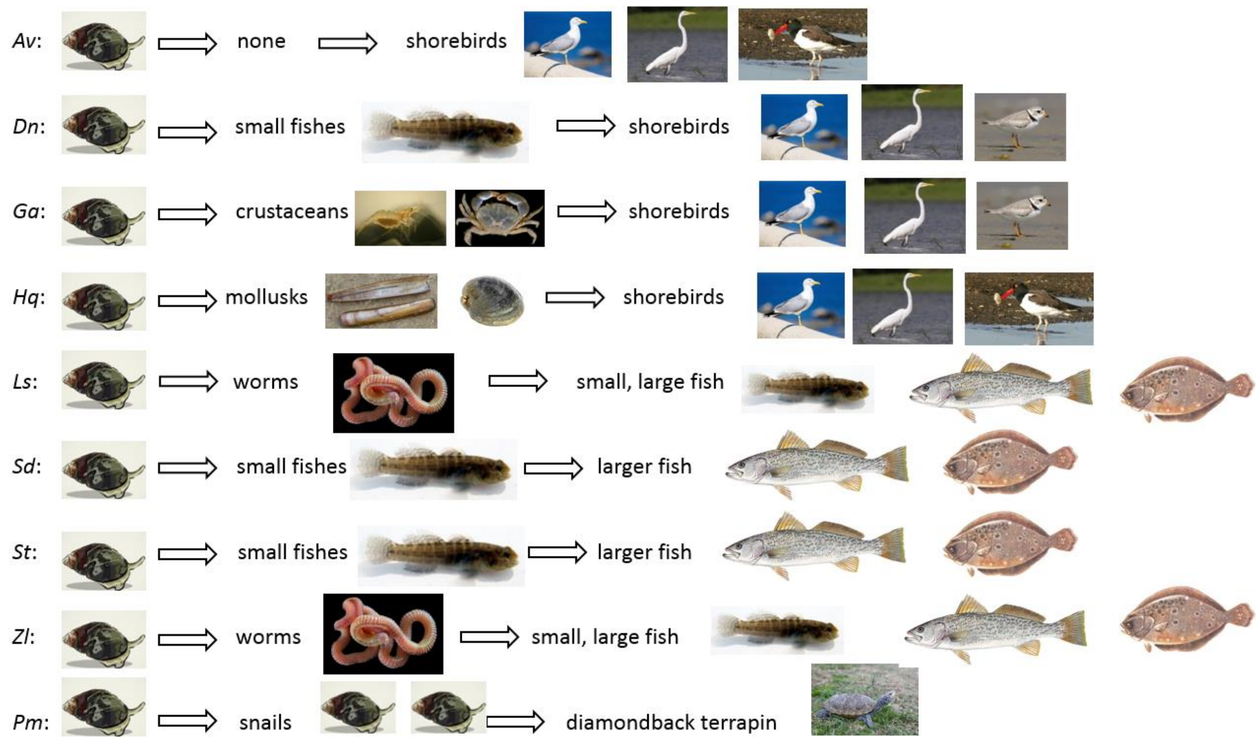


Fig. 3. Community of downstream hosts. This figure is a placeholder for one being designed by Kristen Orr. We want to create something that illustrates how the diversity of snail-trematodes at artificial and natural shoreline sites can indicate the presence of additional host taxa.

Supplementary Figures

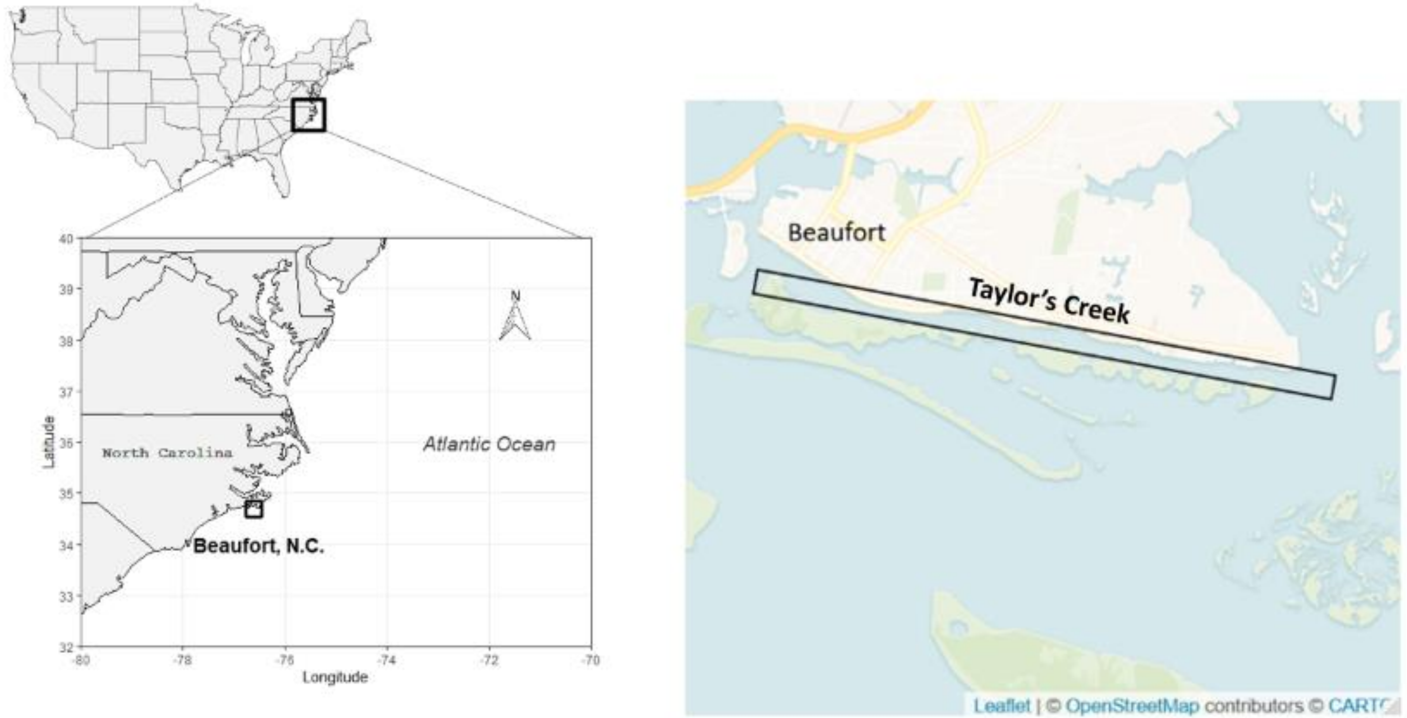


Fig. S1. Taylor's Creek (longitude=-76.614046, latitude=34.70690) is in the town of Beaufort, which is located along the central coast of North Carolina.

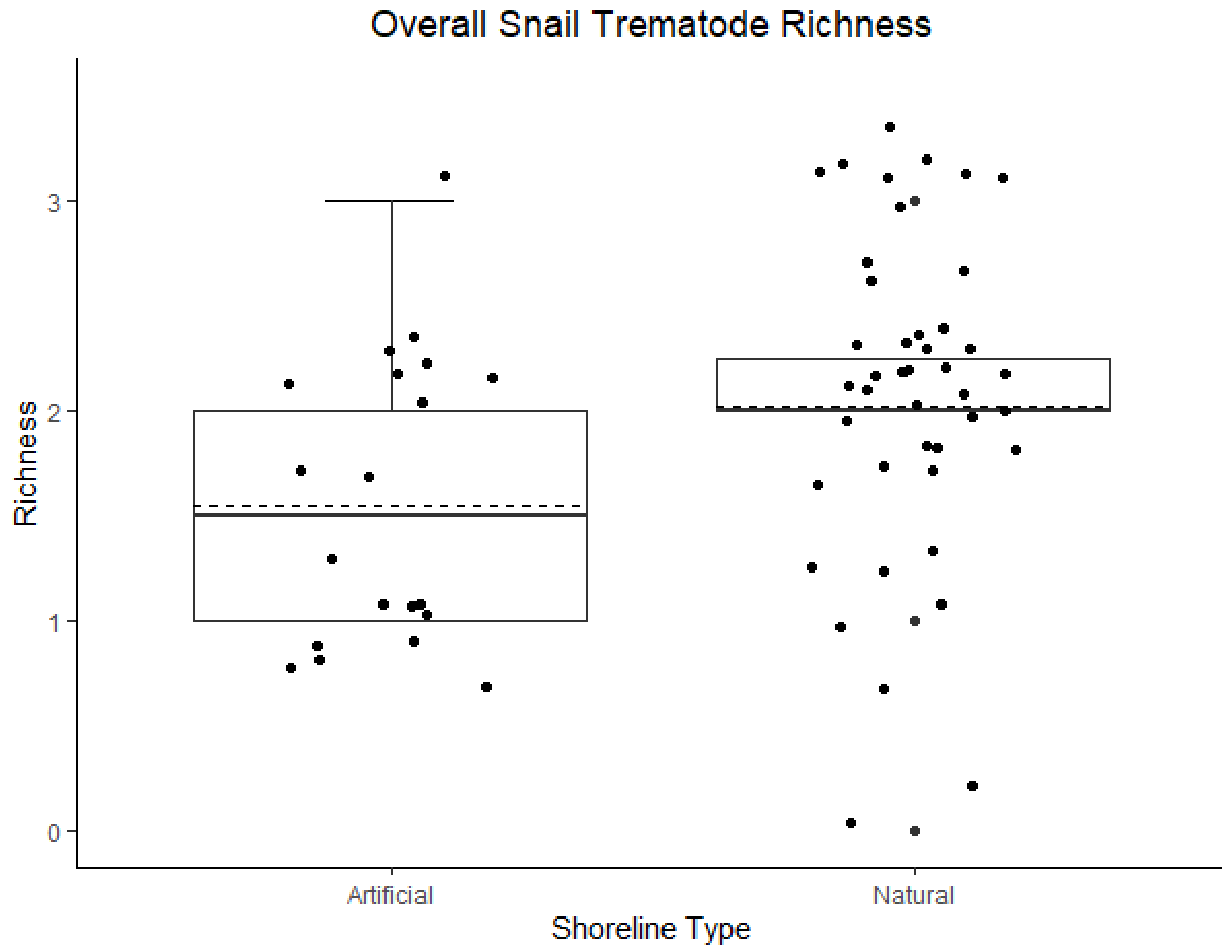


Fig. S2**.** Overall snail trematode species richness in artificial and natural shorelines. Richness was greater in sites with natural shorelines (W=273, p=0.008). There was a moderate effect size difference in richness between artificial (n=20) and natural (n=44) shoreline sites (r=0.33, CI low=0.1, CI high=0.54).

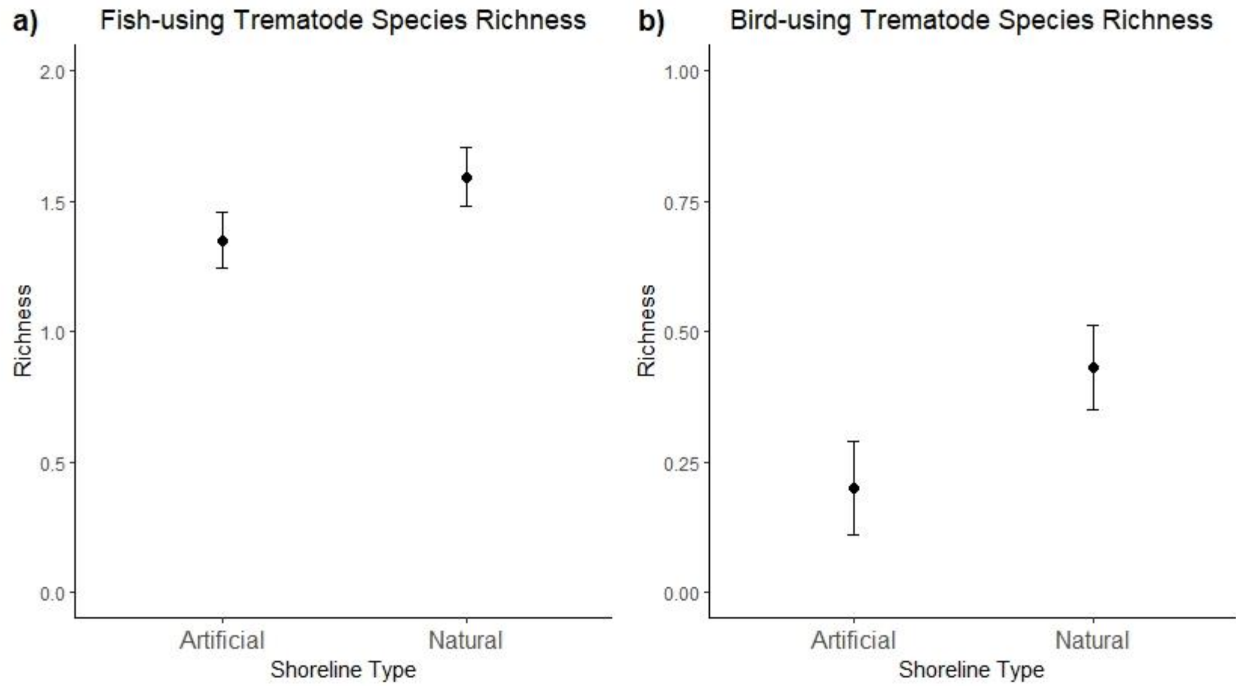


Fig. S3. Mean estimates (±1SE) of fish-using (a) and bird-using (b) trematode species. The richness of trematode species using fish as final hosts did not differ between sites with artificial and natural shorelines, nor did the richness of trematode species using birds as final hosts.

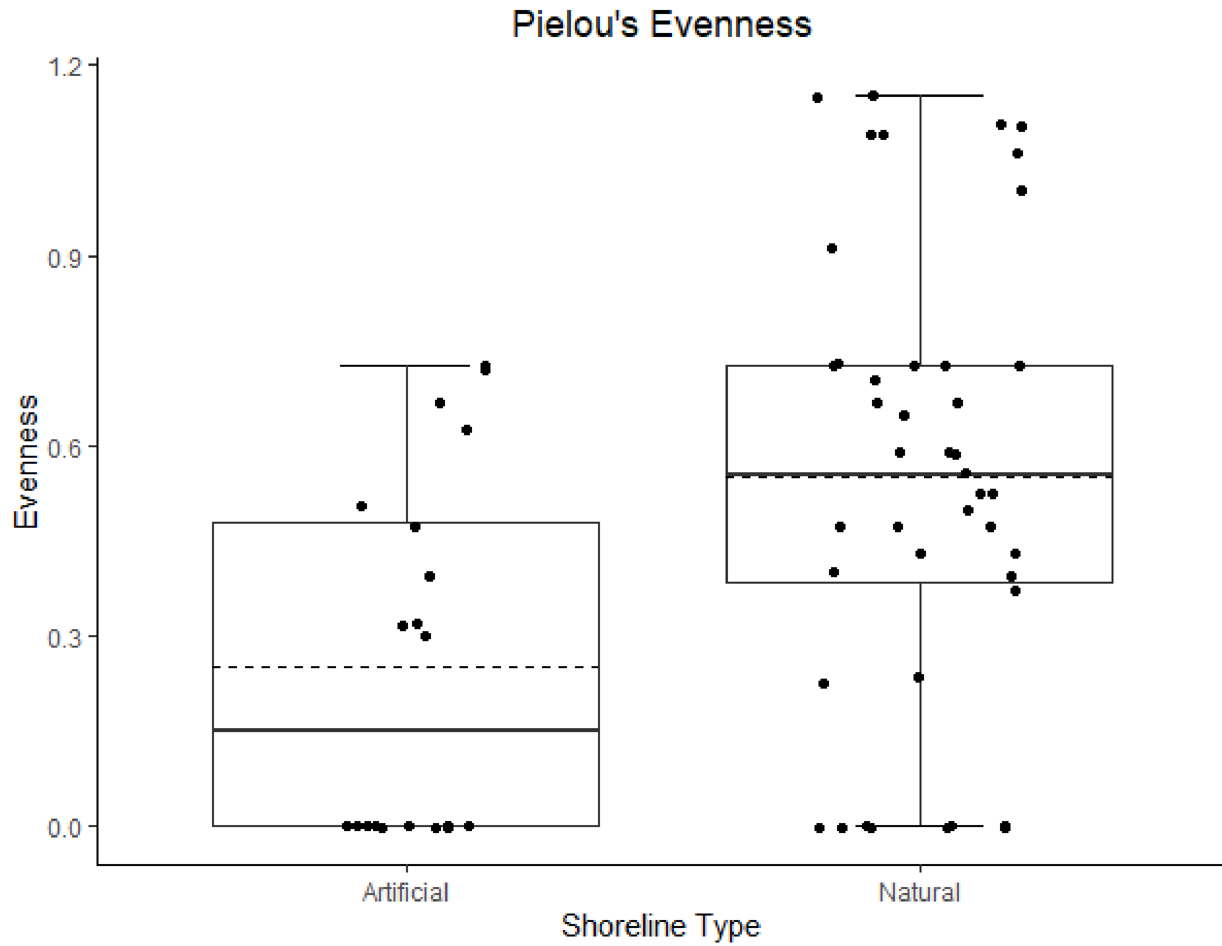
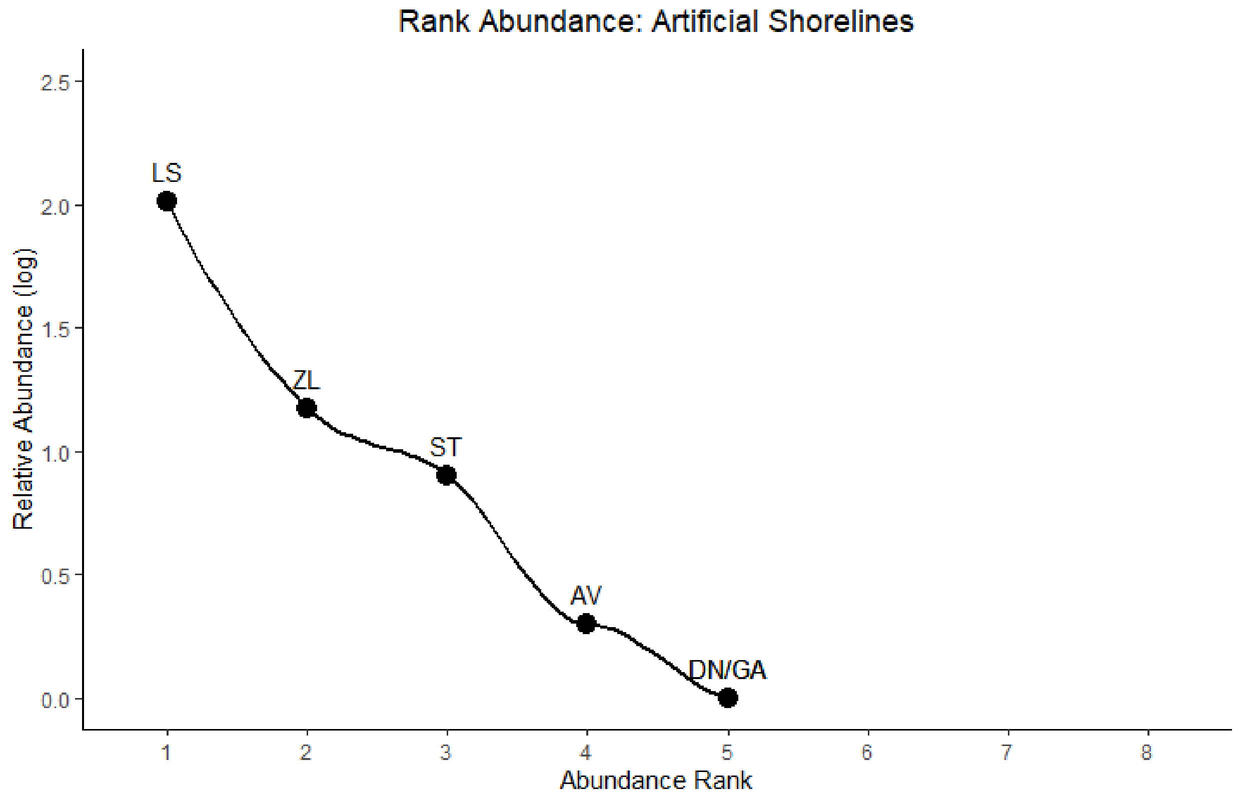


Fig. S4. Pielou's evenness of trematode abundance data in snails sampled from artificial (A) and natural (B) shorelines. Evenness was greater in snails sampled from natural shoreline sites (W=229.5, p=0.003). There was a moderate effect size difference in evenness between artificial (n=20) and natural (n=44) shoreline sites (r=0.38, CI low=0.16, CI high=0.58).



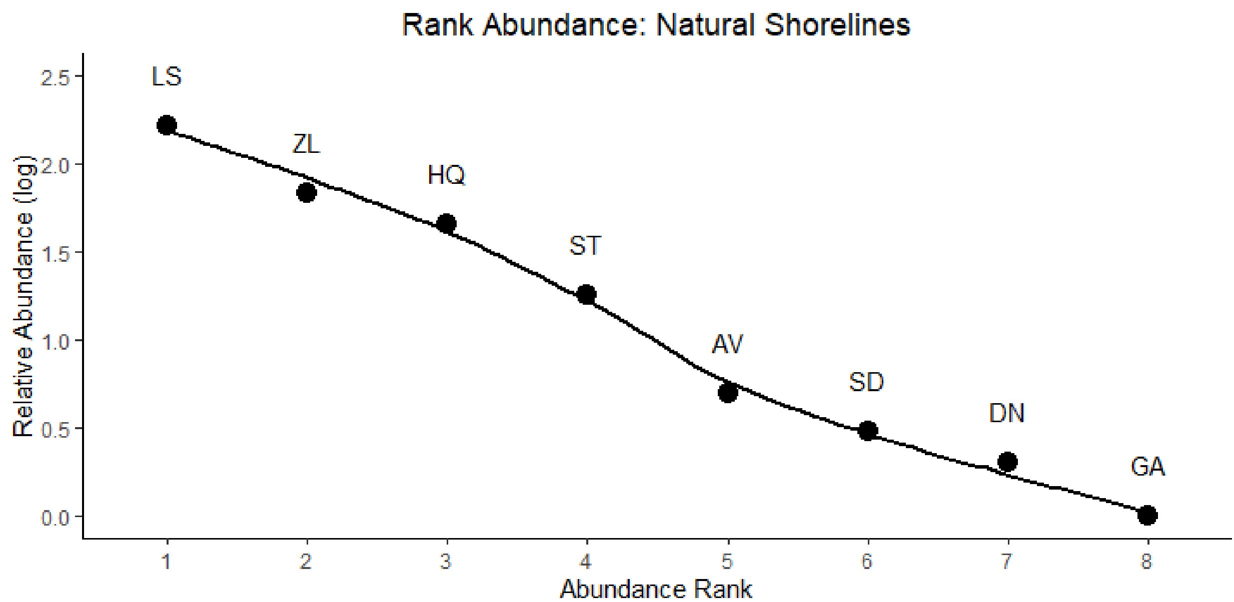


Fig. S5. Rank-abundance curves depicting ranked abundance (x-axis) and log-transformed abundance (y-axis) at Artificial (n=6 species) and Natural (n=8) shoreline sites for the n=8 total species of trematodes sampled from Taylor's Creek. A steeper slope equates to a more uneven and less diverse community. Note that there was only a single collection record for both DN and GA at sites with artificial shorelines, which is why they share the same abundance rank.

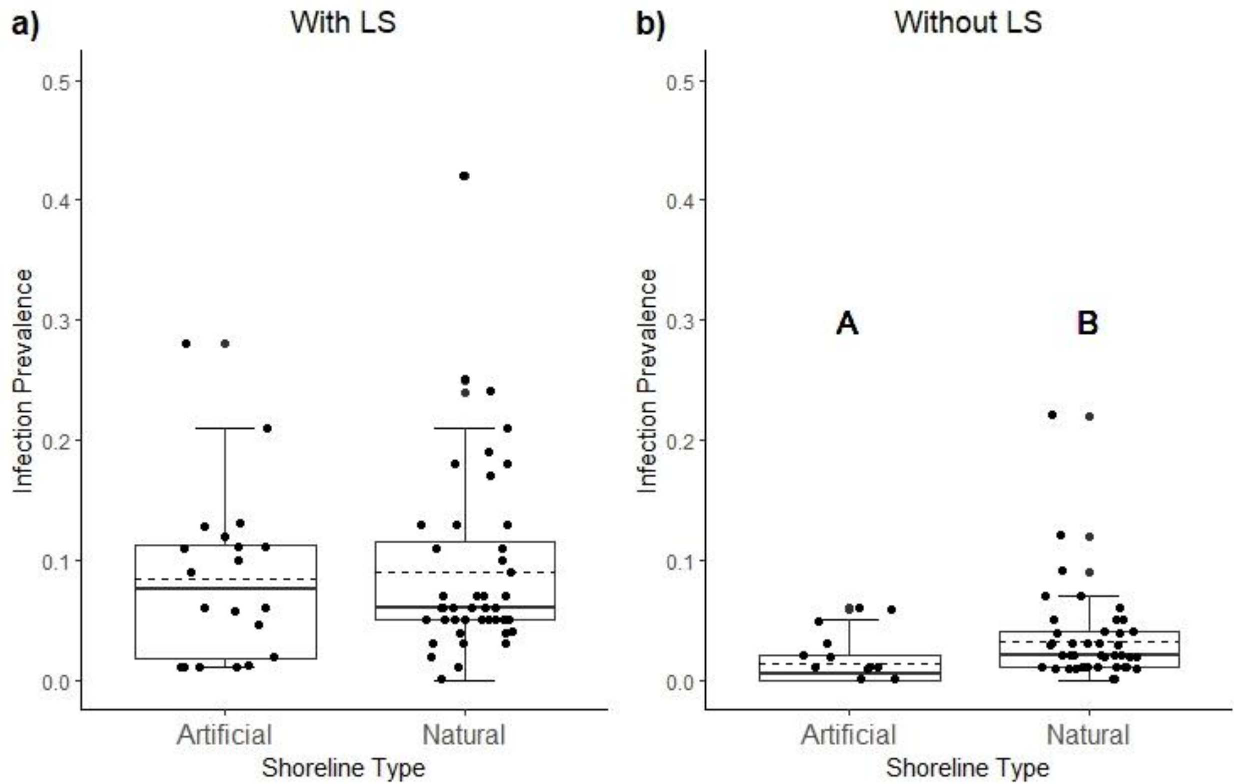


Fig. S6. Total infection prevalence with the trematode LS (a) and without the trematode LS (b). This species of trematode is highly abundant in both artificial and natural shorelines. However, it is the dominant species present in artificial shoreline trematode communities. Without LS, infection prevalence is greater in natural shorelines (W=242, p=0.004). The magnitude of difference likewise increases between communities with LS (a) [r=0.02, CI low=0.005, CI high=0.3, magnitude=small] and without LS (b) [r=0.36, CI low=0.11, CI high=0.58, magnitude = moderate].

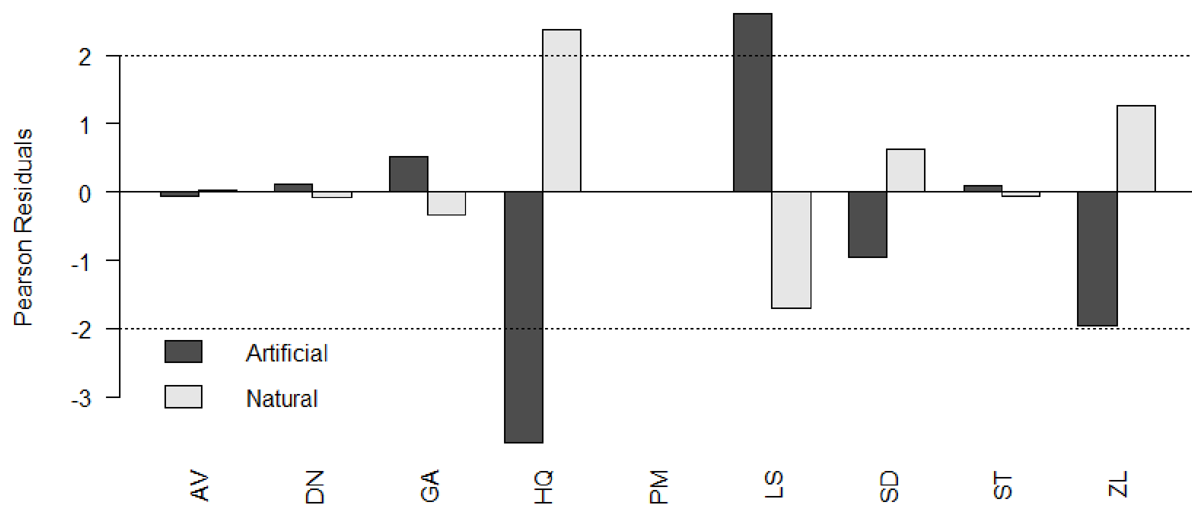


Fig. S7. Plot of Pearson residuals based on Chi-square analysis of raw trematode abundance data from sites with artificial and natural shorelines. An absolute value of 2 indicates that species are positively or negatively associated with either artificial or natural shorelines.

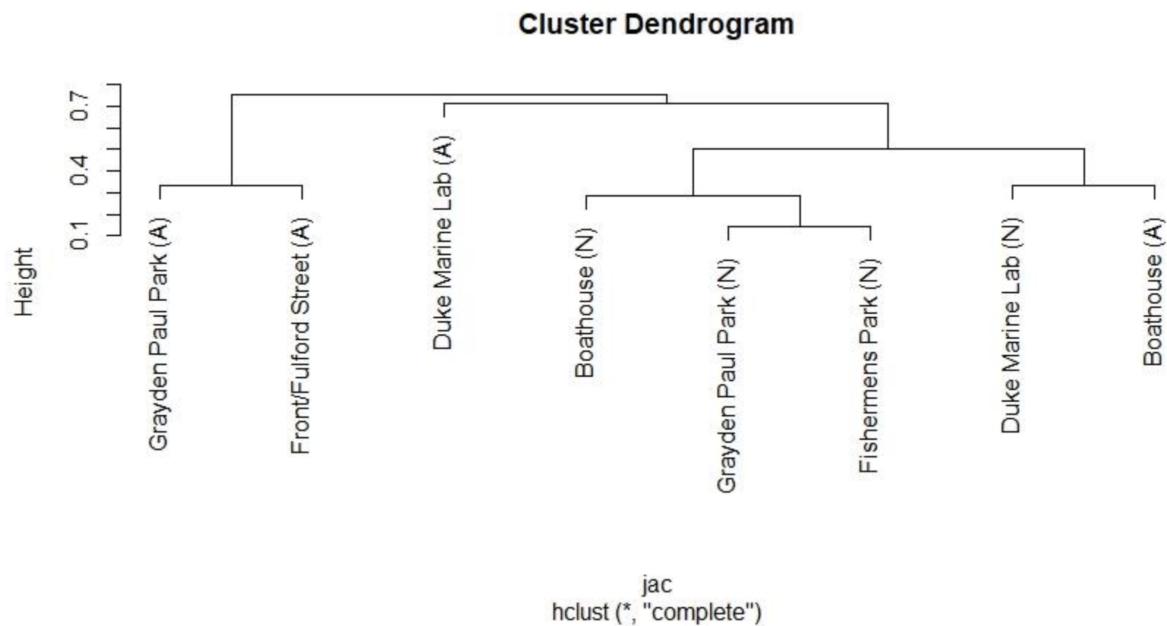


Fig. S8. Cluster dendrogram based on Jaccard dissimilarity matrix depicting snail-trematode data from all sites. Site-level data cluster by shoreline type (Artificial vs. Natural), except for the site Boathouse (A), which tends to cluster with data from natural shorelines.

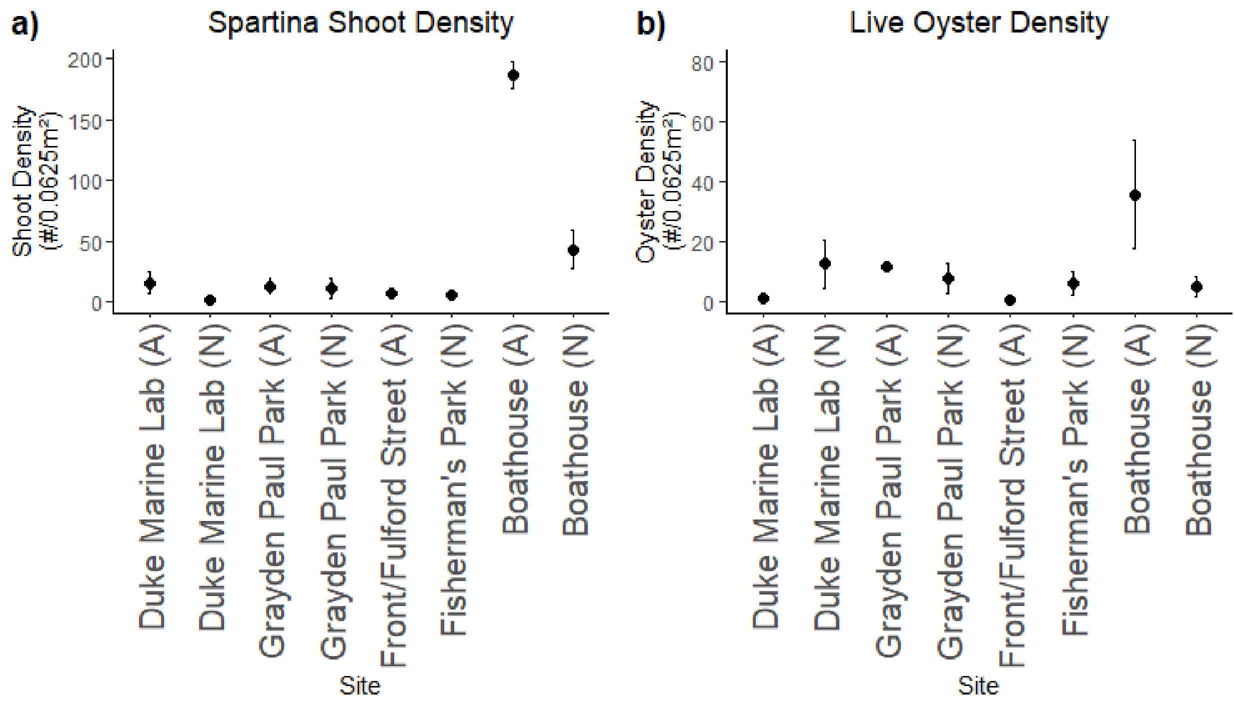


Fig. S9. Mean estimates (±1SE) of *Spartina* shoot density (a) and live oyster density (b) across all sites. Sites are paired (A vs. N) to better visualize site-specific differences in habitat. Overall, there were no differences in *Spartina* shoot density ($\chi^2 = 13.2$, df=7, p=0.07) or live oyster density (χ^2=11.3, df=7, p=0.13) across all sites. At the Boathouse site, *Spartina* shoot density (W=12, p=0.05) and live oyster density (W=12, p=0.05) were greater at the Artificial shoreline.

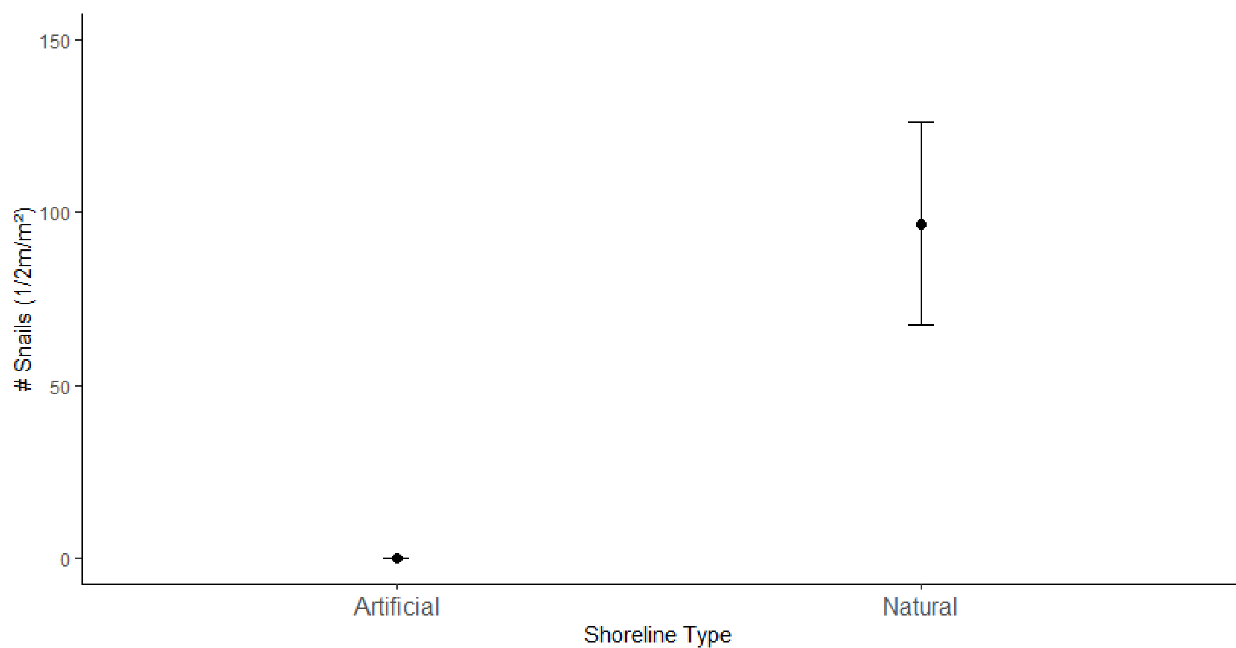


Fig. S10. Mean estimates of snail density (±1SE) at artificial and natural shoreline sites along Taylor's Creek. Densities of mudsnails were greater at sites with natural shorelines (W=0, n=4, p=0.03) with a large effect size detected (r=0.84, n=4, p=0.03). Sample size was too small to be able to extract confidence intervals. Raw data are provided in Table S4.

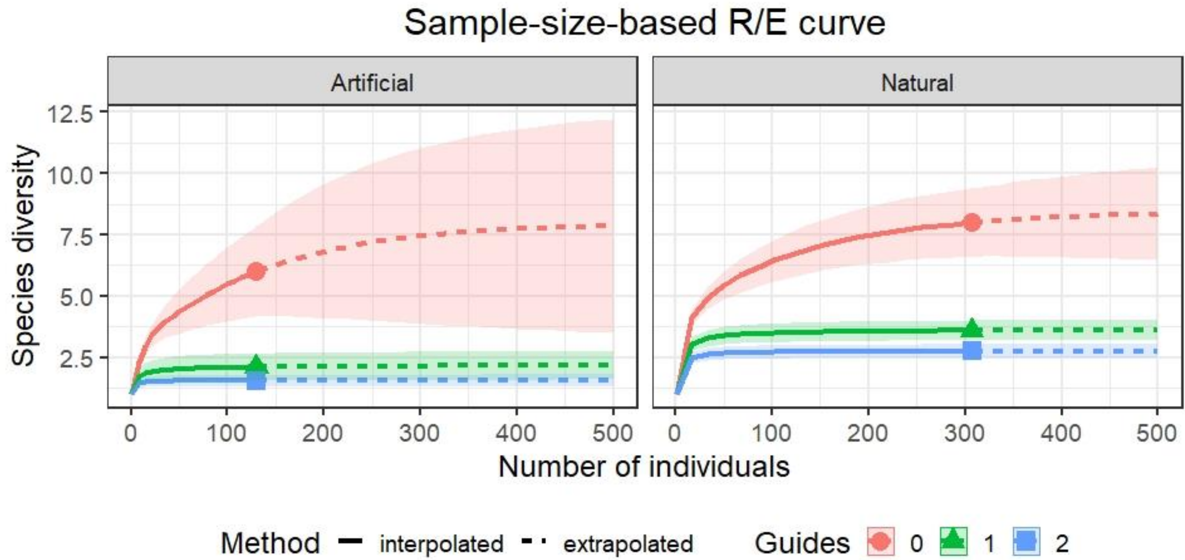


Fig. S11a. Conventional rarefaction/extrapolation estimator based on sample size. Species richness = red; Shannon diversity = green; Simpson diversity = blue. Shaded areas represent the 95% confidence interval for each fitted curve.

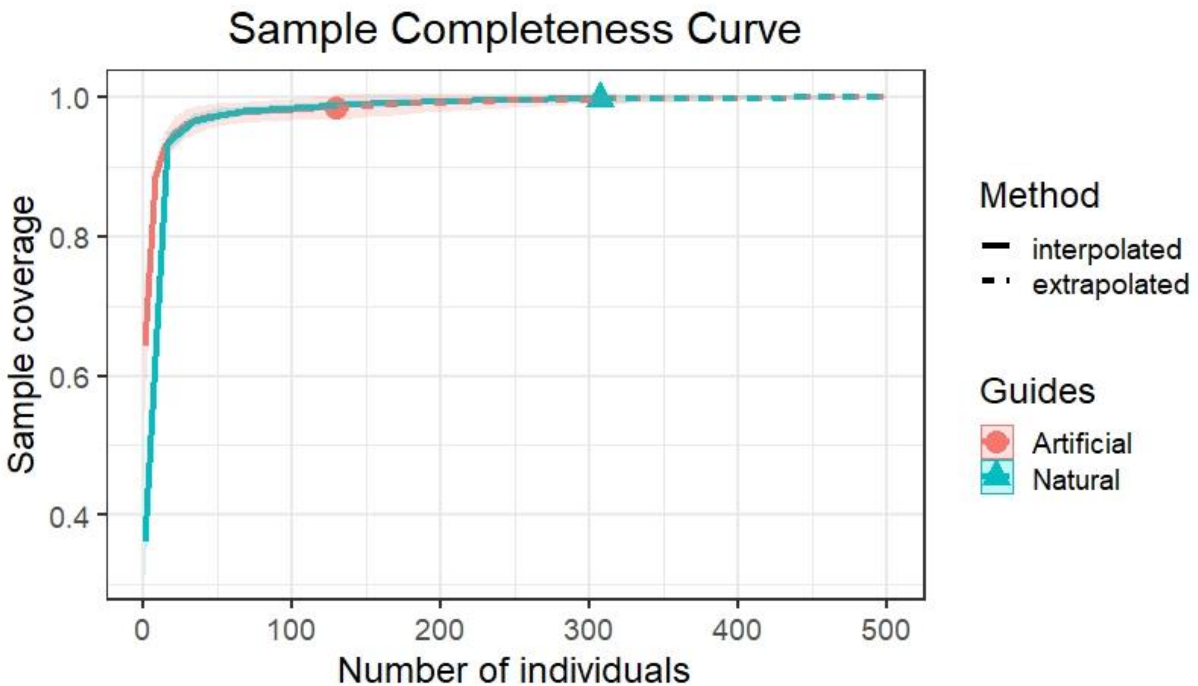


Fig. S11b. Rarefaction/extrapolation estimator based on sample completeness. Species richness = red; Shannon diversity = green; Simpson diversity = blue. Shaded areas represent the 95% confidence interval for each fitted curve.

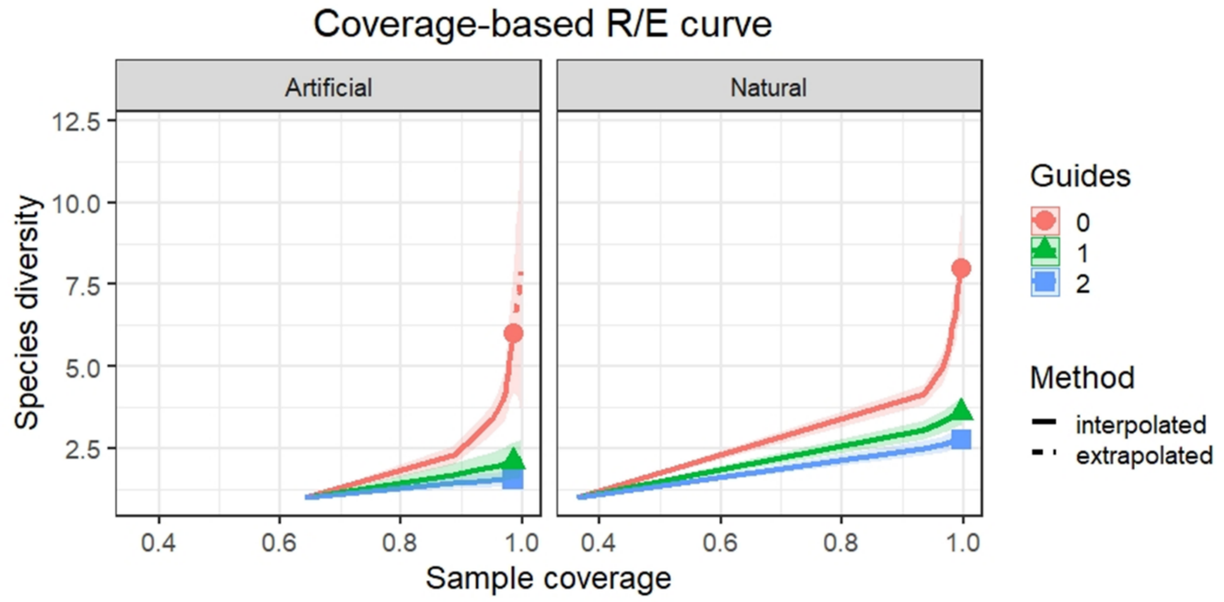


Fig. S11c. Rarefaction/extrapolation estimator based on sample coverage. Species richness = red; Shannon diversity = green; Simpson diversity = blue. Shaded areas represent the 95% confidence interval for each fitted curve.

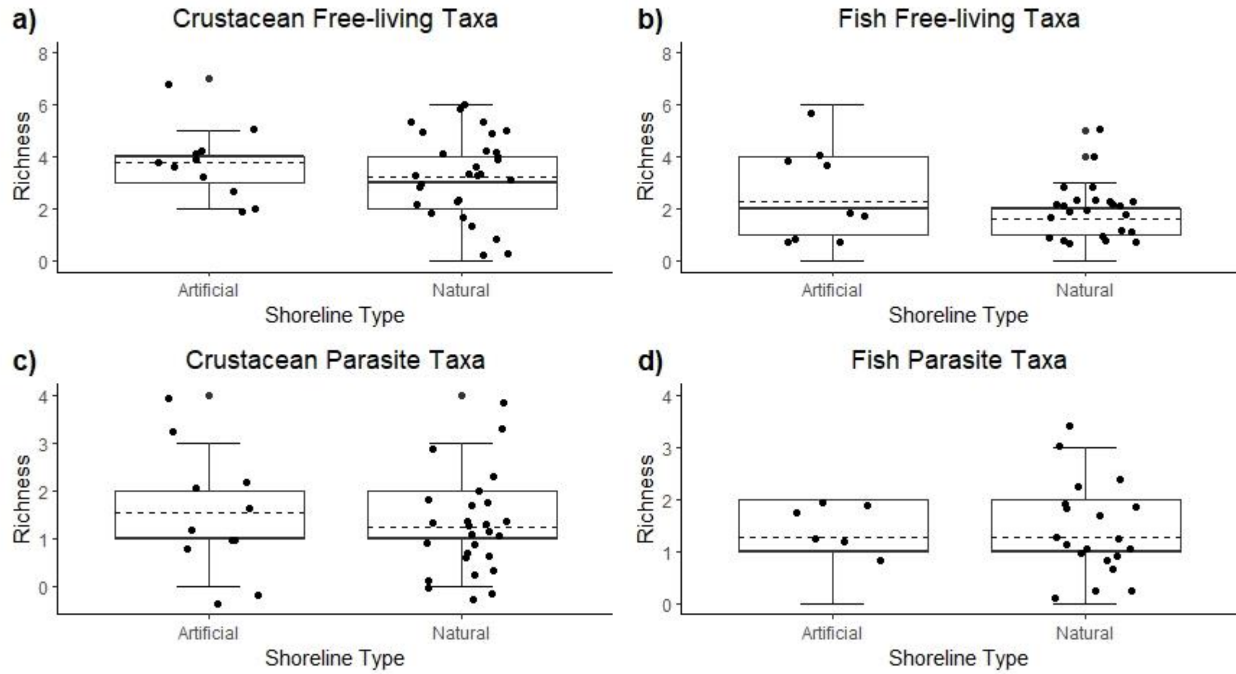


Fig. S12. There were no differences in crustacean and fish free-living taxa richness (a-b), or crustacean and fish parasite taxa richness (c-d) sampled from artificial and natural shoreline sites.

Supplementary Tables

Table S1. Digenetic trematode species parasitizing the eastern mudsnail *Ilyanassa obsoleta* and potential host species found in coastal North Carolina (based on Blakeslee et al. 2012).

Trematode Species	2nd Intermediate Host	Final Host
AV	none required	*Anas rubripes* (black duck, winter only), *Aythya affinis* (lesser scaup, winter only), *Branta Canadensis* (Canada goose, year-round), *Larus argentatus* (herring gull, year-round), *Larus atricilla* (laughing gull, year-round), *Larus delawarensis* (ring-billed gull, non-breeding resident), *Larus marinus* (great black-backed gull, non-breeding resident), *Melanitta deglandi* (white-winged scoter, winter only), *Mergus serrator* (red-breasted merganser, winter only), *Nycticorax nycticorax* (black-crowned night heron, year-round), *Phalacrocorax auritus* (double-crested cormorant, non-breeding resident)
DN	*Fundulus heteroclitus* (mummichog), *Mugil cephalus* (striped mullet)	Birds (unknown)
GA	*Americorchestia longicornis* (Atlantic sandhopper), *Talorchestia longico* (amphipod), *Uca pugilator, Uca pugnax, Uca minax* (fiddler crabs) *Eurypanopeus depressus* (black-fingered mudcrab), *Rhithropanopeus harrisii* (white-fingered mudcrab), *Panopeus herbstii* (Atlantic mudcrab), *Dyspanopeus sayi* (Say's mudcrab)	*Larus argentatus* (herring gull, year-round), *Larus atricilla* (laughing gull, year-round), *Ammospiza maritime maritima* (seaside sparrow, year-round), *Charadrius wilsoni* (Wilson's plover, breeding – common) *Calidris alba* (sanderling), *Opsanus tau* (oyster toadfish), *Sphoeroides maculatus* (northern puffer), *Procyon lotor* (raccoon), *Rhynchops nigra* (black skimmer, year-round), *Sterna hirundo* (common tern, year-round)
HQ	*Argopecten irradians* (bay scallop), *Cerastoderme edule* (common cockle), *Crepidula fornicata* (common slipper) *Cumingia tellinoides* (clam), *Ensis directus* (jackknife clam), *Geukensia demissa* (ribbed	*Larus argentatus* (herring gull, year-round), *Larus atricilla* (laughing gull, year-round), *Larus marinus* (great black-backed gull, non-breeding resident), *Nycticorax nycticorax* (black

	mussel), *Modiolus modiolus* (northern horse-mussel), *Mytilus edulis* (blue mussel), *Leitoscoloplos fragilis* (polychaete)	crowned night heron, year-round), *Sterna hirundo* (common tern, year-round)
LS	*Chaetozone setosa* (polychaete), *Childia groenlandica* (polychaete), *Chrysaora quinquecirrha* (sea nettle jellyfish), *Eteone longa* (polychaete), *Euplana gracilis,* (flatworm), *Foviella warreni* (flatworm), *Polydora ciliata* (polychaete), *Polydora cornuta* (polychaete), *Streblospio benedicti* (polychaete), *Stylochus ellipticus* (flat--worm)	*Pseudopleuronectes Americanus* (winter flounder)
SD	*Menidia menidia* (Atlantic silverside)	*Paralichthys dentatus* (summer flounder), *Sphoeroides maculatus* (northern puffer)
ST	*Anguilla rostrata* (eel), *Fundulus heteroclitus* (mummichog), *Menidia menidia* (Atlantic silverside)	*Menticirrhus saxatilis* (northern kingfish), *Morone saxatilis* (striped bass), *Opsanus tau* (oyster toadfish), *Sphoeroides maculatus* (northern puffer)
ZL	*Arabella Iricolor* (polychaete**)**, *Arbacia punctulatus* (purple sea urchin), *Bdelloura candida* (flat**-**worm), Hydroides spp. (tubeworm) *Leonereis culveri* (polychaete), *Lumbrineris hebes* (polychaete), *Scoloplos robusta* (polychaete)	*Anguilla rostrata* (eel), *Leiostomus xanthurus* (spot), *Trinectes maculatus* (hogchoker), *Menidia menidia* **(**Atlantic silverside), *Paralichtys dentatus* (summer flounder), *Opsanus tau* (oyster toadfish), *Tautoga onitis* (tautog)
PM	vegetation, hard surfaces	*Chelonia mydas* (green sea turtle, non-resident), *Malaclemys terrapin* (diamondback terrapin)

Table S2. Raw abundance data of trematode species sampled in *Ilyanassa obsoleta* snails from artificial and natural shoreline sites along Taylor's Creek. The sampling unit is the number of snails infected with a particular species of trematode parasite.

	Species of Trematode								
Shoreline	**AV**	**DN**	**GA**	**HQ**	**PM**	**LS**	**SD**	**ST**	**ZL**
Artificial	2	1	1	0	0	103	0	8	15
Natural	5	2	1	45	0	165	3	18	68

Table S3. Mean estimates (±1SD) of snail trematode species richness and infection prevalence at artificial and natural shorelines along Taylor's Creek.

	Artificial (n=20)	**Natural (n=44)**
Overall Trematode Species Richness (excludes immature-stage infections)		
Mean	1.55 (0.605)	2.02 (0.762)
Median [Min, Max]	1.50 [1.00, 3.00]	2.00 [0, 3.00]
Species Richness of Bird-using Trematodes		
Mean	0.200 (0.410)	0.432 (0.545)
Median [Min, Max]	0 [0, 1.00]	0 [0, 2.00]
Species Richness of Fish-using Trematodes		
Mean	1.35 (0.489)	1.59 (0.757)
Median [Min, Max]	1.00 [1.00, 2.00]	2.00 [0, 3.00]
Overall Trematode Infection Prevalence (includes immature-stage infections)		
Mean	0.0841 (0.0713)	0.0902 (0.0802)
Median [Min, Max]	0.0750 [0.0100, 0.280]	0.0600 [0, 0.420]
Overall Trematode Bird-using Prevalence		
Mean	0.0403 (0.116)	0.154 (0.276)
Median [Min, Max]	0 [0, 0.500]	0 [0, 1.00]
Overall Trematode Fish-using Prevalence		
Mean	0.799 (0.242)	0.627 (0.348)
Median [Min, Max]	0.902 [0.250, 1.00]	0.744 [0, 1.00]

Table S4. Raw abundance data used to estimate snail density at artificial and natural shoreline sites along Taylor's Creek.

Site	Shoreline	Quadrat 1	Quadrat 2	Quadrat 3	Quadrat 4	Quadrat 5
Boathouse	Artificial	0	1	0	0	0
Duke Marine Lab	Artificial	0	0	0	0	0
Front/Fulford Street	Artificial	0	0	0	0	0
Grayden Paul Park	Artificial	0	0	0	0	0
Boathouse	Natural	173	74	0	0	0
Duke Marine Lab	Natural	70	747	50	115	0
Fisherman's Park	Natural	0	3	186	128	0
Grayden Paul Park	Natural	0	0	0	0	390

Table S5a. Summary statistics mean (±1SD) of abiotic data collected from artificial and natural shorelines along Taylor's Creek.

Abiotic Data from Taylor's Creek		
	Artificial	**Natural**
	(N=45)	(N=45)
Salinity (ppt)		
Mean	29.7 (4.28)	29.9 (4.10)
Median [Min, Max]	29.4 [20.1, 38.0]	29.3 [21.0, 38.1]
Temperature (°C)		
Mean	21.8 (7.75)	21.8 (7.78)
Median [Min, Max]	22.3 [8.10, 31.2]	22.5 [8.10, 32.4]
Dissolved Oxygen (mg/L)		
Mean	7.84 (1.37)	7.83 (1.36)
Median [Min, Max]	7.72 [5.34, 10.5]	7.48 [5.37, 10.3]

Table S5b. Summary statistics (±1SD) of abiotic data stratified by site and shoreline.

	Artificial			
	Boathouse	**Duke Marine Lab**	**Front/Fulford Street**	**Grayden Paul Park**
	(N=12)	**(N=9)**	**(N=12)**	**(N=12)**
Salinity (ppt)				
Mean	29.3 (4.38)	29.9 (4.75)	29.5 (4.45)	30.0 (4.16)
Median [Min, Max]	29.1 [21.0, 37.9]	29.4 [22.8, 38.0]	29.2 [20.1, 37.9]	30.0 [23.2, 37.9]
Temperature (°C)				
Mean	21.3 (8.46)	23.4 (7.26)	21.3 (8.06)	21.6 (7.89)
Median [Min, Max]	22.2 [8.10, 31.2]	26.9 [8.80, 30.4]	22.5 [8.60, 31.1]	22.6 [9.20, 30.8]
Dissolved Oxygen (mg/L)				
Mean	8.10 (1.44)	7.48 (1.23)	7.89 (1.51)	7.79 (1.38)
Median [Min, Max]	7.92 [5.34, 9.98]	7.34 [5.93, 9.61]	7.77 [5.49, 10.5]	7.48 [5.69, 10.2]

	Natural			
	Boathouse	**Duke Marine Lab**	**Fisherman's Park**	**Grayden Paul Park**
	(N=12)	**(N=9)**	**(N=12)**	**(N=12)**
Salinity (ppt)				
Mean	29.2 (4.43)	29.8 (4.67)	30.2 (4.16)	30.3 (3.72)
Median [Min, Max]	28.8 [21.0, 38.1]	29.0 [23.3, 37.8]	29.8 [22.0, 37.9]	30.4 [22.9, 37.9]
Temperature (°C)				
Mean	21.3 (8.54)	23.5 (7.26)	21.3 (8.09)	21.5 (7.90)
Median [Min, Max]	22.2 [8.10, 32.4]	27.1 [8.70, 30.8]	22.4 [8.60, 30.9]	22.3 [8.90, 30.7]
Dissolved Oxygen (mg/L)				
Mean	7.90 (1.50)	7.43 (1.23)	7.97 (1.39)	7.90 (1.38)
Median [Min, Max]	7.61 [5.37, 9.96]	7.00 [5.83, 9.58]	7.81 [5.51, 10.3]	7.70 [5.65, 10.2]

Table S6a. Mean abundances (±1SD) of site-resident (R) and transient (T) nekton collected from artificial and natural shorelines along Taylor's Creek.

	Artificial (N=30)	**Natural (N=30)**
Atlantic mud crabs (*P. herbstii*) (R)		
Mean	4.43 (4.60)	4.30 (4.14)
Median [Min, Max]	3.00 [0, 20.0]	3.50 [0, 19.0]
Say's mud crab (*D. sayii*) (R)		
Mean	4.67 (6.19)	4.93 (8.39)
Median [Min, Max]	2.00 [0, 22.0]	0 [0, 33.0]
Flat-backed mud crab (*E. depressus*) (R)		
Mean	0.567 (1.25)	0.567 (1.28)
Median [Min, Max]	0 [0, 6.00]	0 [0, 6.00]
White-fingered mud crab (*R. harrissi*) (R)		
Mean	1.00 (2.35)	0.633 (1.25)
Median [Min, Max]	0 [0, 11.0]	0 [0, 5.00]
Unknown mud crabs (juv. Panopeidae) (R)		
Mean	0.733 (1.78)	0.600 (1.25)
Median [Min, Max]	0 [0, 8.00]	0 [0, 5.00]
Blue Crabs (T)		
Mean	0.233 (1.10)	0.267 (1.46)
Median [Min, Max]	0 [0, 6.00]	0 [0, 8.00]
Stone crabs (R)		
Mean	0.800 (1.27)	0.500 (0.820)
Median [Min, Max]	0 [0, 6.00]	0 [0, 3.00]
Spider Crabs (T)		
Mean	0.100 (0.305)	0 (0)
Median [Min, Max]	0 [0, 1.00]	0 [0, 0]
Porcelain Crabs (R)		
Mean	0 (0)	0.0333 (0.183)
Median [Min, Max]	0 [0, 0]	0 [0, 1.00]
Spiny Lobsters (T)		
Mean	0 (0)	0.0333 (0.183)
Median [Min, Max]	0 [0, 0]	0 [0, 1.00]
Grass Shrimp (T)		
Mean	1.07 (2.78)	0.967 (1.83)
Median [Min, Max]	0 [0, 14.0]	0 [0, 8.00]
Snapping Shrimp (R)		
Mean	1.30 (2.05)	1.70 (2.10)

	Artificial (N=30)	Natural (N=30)
Median [Min, Max]	0 [0, 7.00]	0.500 [0, 8.00]
Naked Gobies (R)		
Mean	0.633 (1.56)	1.03 (2.20)
Median [Min, Max]	0 [0, 7.00]	0 [0, 9.00]
Striped Blennies (R)		
Mean		
Median [Min, Max]	0 [0, 6.00]	0 [0, 1.00]
Feather Blennies (R)		
Mean	0.500 (0.974)	1.00 (1.74)
Median [Min, Max]	0 [0, 3.00]	0 [0, 7.00]
Oyster Toadfish (R)		
Mean	0.900 (1.09)	0.733 (0.785)
Median [Min, Max]	0.500 [0, 4.00]	1.00 [0, 2.00]
Mummichogs (T)		
Mean	0.133 (0.571)	0.167 (0.747)
Median [Min, Max]	0 [0, 3.00]	0 [0, 4.00]
Sheepshead (T)		
Mean	0.100 (0.403)	0.0333 (0.183)
Median [Min, Max]	0 [0, 2.00]	0 [0, 1.00]
Darter Gobies (T)		
Mean	0.0667 (0.254)	0.0333 (0.183)
Median [Min, Max]	0 [0, 1.00]	0 [0, 1.00]
Rock Seabass (T)		
Mean	0.0333 (0.183)	0 (0)
Median [Min, Max]	0 [0, 1.00]	0 [0, 0]
Croaker (T)		
Mean	0.0333 (0.183)	0 (0)
Median [Min, Max]	0 [0, 1.00]	0 [0, 0]
Grey Snapper (T)		
Mean	0.133 (0.571)	0.0667 (0.254)
Median [Min, Max]	0 [0, 3.00]	0 [0, 1.00]
American Eels (T)		
Mean	0 (0)	0.0333 (0.183)
Median [Min, Max]	0 [0, 0]	0 [0, 1.00]
Banded Killifish (T)		
Mean	0 (0)	0.0333 (0.183)
Median [Min, Max]	0 [0, 0]	0 [0, 1.00]
Pinfish		

	Artificial (N=30)	**Natural (N=30)**
Mean	0 (0)	0.0333 (0.183)
Median [Min, Max]	0 [0, 0]	0 [0, 1.00]

Table S6b. Mean abundances (±1SD) of site-resident (R) and transient (T) nekton stratified by site and shoreline.

	Artificial				**Natural**			
	Boathouse	**Duke Marine Lab**	**Front/Fulford Street**	**Grayden Paul Park**	**Boathouse**	**Duke Marine Lab**	**Fisherman's Park Natural**	**Grayden Paul**
	(N=8)	**(N=6)**	**(N=8)**	**(N=8)**	**(N=8)**	**(N=6)**	**(N=8)**	**(N=8)**
Atlantic mud crabs (*P. herbstii*) (R)								
Mean	6.00 (6.12)	5.50 (6.80)	3.38 (1.85)	3.13 (2.59)	7.13 (5.46)	2.50 (2.59)	3.25 (3.33)	3.88 (3.44)
Median [Min, Max]	4.00 [0, 20.0]	3.50 [0, 18.0]	3.00 [1.00, 6.00]	2.50 [0, 7.00]	6.00 [1.00, 19.0]	2.00 [0, 6.00]	2.50 [0, 8.00]	3.50 [0, 10.0]
Say's mud crab (*D. sayii*) (R)								
Mean	1.63 (3.29)	6.67 (8.57)	7.63 (7.71)	3.25 (3.11)	0 (0)	3.00 (6.00)	10.4 (11.7)	5.88 (7.99)
Median [Min, Max]	0 [0, 9.00]	2.50 [0, 19.0]	5.50 [0, 22.0]	2.00 [0, 9.00]	0 [0, 0]	0 [0, 15.0]	7.50 [0, 33.0]	3.50 [0, 23.0]
Flat-backed mud crab (*E. depressus*) (R)								
Mean	1.25 (2.05)	0.167 (0.408)	0 (0)	0.750 (1.04)	1.38 (2.20)	0 (0)	0.125 (0.354)	0.625 (0.744)
Median [Min, Max]	0.500 [0, 6.00]	0 [0, 1.00]	0 [0, 0]	0.500 [0, 3.00]	0 [0, 6.00]	0 [0, 0]	0 [0, 1.00]	0.500 [0, 2.00]

	Artificial				Natural			
	Boathouse	**Duke Marine Lab**	**Front/Fulford Street**	**Grayden Paul Park**	**Boathouse**	**Duke Marine Lab**	**Fisherman's Park Natural**	**Grayden Paul**
	(N=8)	**(N=6)**	**(N=8)**	**(N=8)**	**(N=8)**	**(N=6)**	**(N=8)**	**(N=8)**
White-fingered mud crab (*R. harrissi*) (R)								
Mean	0.125 (0.354)	0 (0)	3.13 (3.87)	0.500 (0.756)	0 (0)	0.333 (0.816)	1.25 (1.91)	0.875 (1.13)
Median [Min, Max]	0 [0, 1.00]	0 [0, 0]	2.00 [0, 11.0]	0 [0, 2.00]	0 [0, 0]	0 [0, 2.00]	0 [0, 5.00]	0.500 [0, 3.00]
Unknown mud crabs (juv. Panopeidae) (R)								
Mean	0.500 (0.756)	1.33 (3.27)	0.625 (1.77)	0.625 (1.19)	0 (0)	0.500 (0.837)	1.63 (2.00)	0.250 (0.463)
Median [Min, Max]	0 [0, 2.00]	0 [0, 8.00]	0 [0, 5.00]	0 [0, 3.00]	0 [0, 0]	0 [0, 2.00]	1.00 [0, 5.00]	0 [0, 1.00]
Blue Crabs (T)								
Mean	0 (0)	0.167 (0.408)	0 (0)	0.750 (2.12)	0 (0)	1.33 (3.27)	0 (0)	0 (0)
Median [Min, Max]	0 [0, 0]	0 [0, 1.00]	0 [0, 0]	0 [0, 6.00]	0 [0, 0]	0 [0, 8.00]	0 [0, 0]	0 [0, 0]
Stone crabs (R)								

	Artificial				Natural			
	Boathouse	**Duke Marine Lab**	**Front/Fulford Street**	**Grayden Paul Park**	**Boathouse**	**Duke Marine Lab**	**Fisherman's Park Natural**	**Grayden Paul**
	(N=8)	**(N=6)**	**(N=8)**	**(N=8)**	**(N=8)**	**(N=6)**	**(N=8)**	**(N=8)**
Mean	0.625 (0.916)	1.83 (2.23)	0.375 (0.744)	0.625 (0.744)	0.125 (0.354)	1.33 (1.21)	0.125 (0.354)	0.625 (0.744)
Median [Min, Max]	0 [0, 2.00]	1.50 [0, 6.00]	0 [0, 2.00]	0.500 [0, 2.00]	0 [0, 1.00]	1.50 [0, 3.00]	0 [0, 1.00]	0.500 [0, 2.00]
Spider Crabs (T)								
Mean	0.125 (0.354)	0.167 (0.408)	0.125 (0.354)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Median [Min, Max]	0 [0, 1.00]	0 [0, 1.00]	0 [0, 1.00]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 0]
Porcelain Crabs (R)								
Mean	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.125 (0.354)	0 (0)
Median [Min, Max]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 1.00]	0 [0, 0]
Spiny Lobsters (T)								
Mean	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.125 (0.354)	0 (0)
Median [Min, Max]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 1.00]	0 [0, 0]
Grass Shrimp (T)								

	Artificial				Natural			
	Boathouse	**Duke Marine Lab**	**Front/Fulford Street**	**Grayden Paul Park**	**Boathouse**	**Duke Marine Lab**	**Fisherman's Park Natural**	**Grayden Paul**
	(N=8)	**(N=6)**	**(N=8)**	**(N=8)**	**(N=8)**	**(N=6)**	**(N=8)**	**(N=8)**
Mean	0.250 (0.463)	1.17 (2.40)	0.875 (1.46)	2.00 (4.87)	1.75 (2.92)	0.667 (1.21)	0.750 (1.16)	0.625 (1.41)
Median [Min, Max]	0 [0, 1.00]	0 [0, 6.00]	0 [0, 4.00]	0 [0, 14.0]	0 [0, 8.00]	0 [0, 3.00]	0 [0, 3.00]	0 [0, 4.00]
Snapping Shrimp (R)								
Mean	1.13 (1.64)	1.00 (2.00)	0.500 (0.926)	2.50 (2.93)	0.875 (1.25)	3.17 (1.94)	1.88 (2.85)	1.25 (1.75)
Median [Min, Max]	0 [0, 4.00]	0 [0, 5.00]	0 [0, 2.00]	1.50 [0, 7.00]	0 [0, 3.00]	3.00 [0, 6.00]	0.500 [0, 8.00]	0 [0, 4.00]
Naked Gobies (R)								
Mean	1.00 (2.45)	0.667 (0.816)	0 (0)	0.875 (1.73)	1.50 (1.69)	1.17 (2.86)	1.25 (3.15)	0.250 (0.707)
Median [Min, Max]	0 [0, 7.00]	0.500 [0, 2.00]	0 [0, 0]	0 [0, 5.00]	1.50 [0, 5.00]	0 [0, 7.00]	0 [0, 9.00]	0 [0, 2.00]
Striped Blennies (R)								
Mean	1.38 (2.07)	0 (0)	0 (0)	0 (0)	0.125 (0.354)	0 (0)	0 (0)	0 (0)
Median [Min, Max]	0.500 [0, 6.00]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 1.00]	0 [0, 0]	0 [0, 0]	0 [0, 0]

	Artificial				Natural			
	Boathouse	Duke Marine Lab	Front/Fulford Street	Grayden Paul Park	Boathouse	Duke Marine Lab	Fisherman's Park Natural	Grayden Paul
	(N=8)	(N=6)	(N=8)	(N=8)	(N=8)	(N=6)	(N=8)	(N=8)
Feather Blennnies (R)								
Mean	0.375 (0.744)	1.33 (1.51)	0 (0)	0.500 (0.926)	0.375 (1.06)	3.17 (2.79)	0.625 (0.744)	0.375 (0.518)
Median [Min, Max]	0 [0, 2.00]	1.00 [0, 3.00]	0 [0, 0]	0 [0, 2.00]	0 [0, 3.00]	2.50 [0, 7.00]	0.500 [0, 2.00]	0 [0, 1.00]
Oyster Toadfish (R)								
Mean	0.875 (0.991)	1.33 (0.816)	0.625 (1.19)	0.875 (1.36)	0.625 (0.744)	0.833 (0.753)	0.625 (0.744)	0.875 (0.991)
Median [Min, Max]	0.500 [0, 2.00]	1.50 [0, 2.00]	0 [0, 3.00]	0.500 [0, 4.00]	0.500 [0, 2.00]	1.00 [0, 2.00]	0.500 [0, 2.00]	0.500 [0, 2.00]
Mummichogs (T)								
Mean	0.125 (0.354)	0.500 (1.22)	0 (0)	0 (0)	0.500 (1.41)	0.167 (0.408)	0 (0)	0 (0)
Median [Min, Max]	0 [0, 1.00]	0 [0, 3.00]	0 [0, 0]	0 [0, 0]	0 [0, 4.00]	0 [0, 1.00]	0 [0, 0]	0 [0, 0]
Sheepshead (T)								
Mean	0.250 (0.707)	0.167 (0.408)	0 (0)	0 (0)	0 (0)	0.167 (0.408)	0 (0)	0 (0)
Median [Min, Max]	0 [0, 2.00]	0 [0, 1.00]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 1.00]	0 [0, 0]	0 [0, 0]

	Artificial				Natural			
	Boathouse	**Duke Marine Lab**	**Front/Fulford Street**	**Grayden Paul Park**	**Boathouse**	**Duke Marine Lab**	**Fisherman's Park Natural**	**Grayden Paul**
	(N=8)	**(N=6)**	**(N=8)**	**(N=8)**	**(N=8)**	**(N=6)**	**(N=8)**	**(N=8)**
Darter Gobies (T)								
Mean	0 (0)	0 (0)	0.125 (0.354)	0.125 (0.354)	0 (0)	0 (0)	0.125 (0.354)	0 (0)
Median [Min, Max]	0 [0, 0]	0 [0, 0]	0 [0, 1.00]	0 [0, 1.00]	0 [0, 0]	0 [0, 0]	0 [0, 1.00]	0 [0, 0]
Rock Seabass (T)								
Mean	0 (0)	0.167 (0.408)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Median [Min, Max]	0 [0, 0]	0 [0, 1.00]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 0]
Croaker (T)								
Mean	0 (0)	0 (0)	0 (0)	0.125 (0.354)	0 (0)	0 (0)	0 (0)	0 (0)
Median [Min, Max]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 1.00]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 0]
Grey Snapper (T)								
Mean	0 (0)	0.167 (0.408)	0.375 (1.06)	0 (0)	0.125 (0.354)	0.167 (0.408)	0 (0)	0 (0)
Median [Min, Max]	0 [0, 0]	0 [0, 1.00]	0 [0, 3.00]	0 [0, 0]	0 [0, 1.00]	0 [0, 1.00]	0 [0, 0]	0 [0, 0]

	Artificial				Natural			
	Boathouse	**Duke Marine Lab**	**Front/Fulford Street**	**Grayden Paul Park**	**Boathouse**	**Duke Marine Lab**	**Fisherman's Park Natural**	**Grayden Paul**
	(N=8)	**(N=6)**	**(N=8)**	**(N=8)**	**(N=8)**	**(N=6)**	**(N=8)**	**(N=8)**
American Eels (T)								
Mean	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.125 (0.354)
Median [Min, Max]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 1.00]
Banded Killifish (T)								
Mean	0 (0)	0 (0)	0 (0)	0 (0)	0.125 (0.354)	0 (0)	0 (0)	0 (0)
Median [Min, Max]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 1.00]	0 [0, 0]	0 [0, 0]	0 [0, 0]
Pinfish								
Mean	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.125 (0.354)
Median [Min, Max]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 1.00]

Chapter 3: If you build it, they will come: restoration positively influences free-living and parasite diversity in a restored tidal marsh

Abstract

Ecological monitoring studies have shown that surrogate species can provide good predictive power for the community diversity of other taxa. In our study, we sampled for resident and transient fauna in several newly created oyster reefs. We also sampled for common multi-host parasites in these organisms, as parasite abundance can act as a surrogate for trophic complexity in structured habitat. The restoration consisted of three treatments: control plots (no restoration), stacked bags of oyster shell, and reefs constructed from a novel design made of concrete and burlap. We also included three previously restored loose-shell reefs for comparison. Over 16 months, we quantified parasite diversity in mudsnails, crustaceans, and benthic fishes, as well as crustacean and fish free-living diversity. Taxa richness increased post-restoration for most free-living and parasite groups, regardless of restoration design, and communities shifted toward reef-resident species assemblages. Shell bags showed the greatest positive change in richness one-year post-restoration—at 2x that of control plots on average. The relative abundance of trematodes also increased with time, particularly those using fish as final hosts, and nematodes, cestodes, and entoniscid isopods were also more abundant post-restoration. Habitat complexity and abiotic factors (e.g., temperature and dissolved oxygen) were also positively correlated with shifts in host and parasite diversity. Our study demonstrates that free-living and parasite responses to restoration can occur rapidly and are sensitive to restoration design and site-specific environmental characteristics. Parasites are thus a promising tool for quantifying biodiversity and ecosystem health, particularly in systems subject to frequent monitoring

Introduction

Habitat destruction is the single greatest threat to biodiversity worldwide (Brooks et al. 2002; MEA 2005; Hanski 2011; Maxwell et al. 2016). Coastal zones are particularly vulnerable to environmental degradation and habitat loss, as population density has rapidly expanded in these areas (Small & Nicholls 2003; Halpern et al. 2008; Waycott et al. 2009; Barbier 2014; Neumann et al. 2015). While some ecosystems are capable of partially regenerating without human interference (Palmer et al. 2016), restoration intervention is often required when natural systems have degraded to the point where self-sustaining recovery is not possible (Lotze et al 2011; Suding 2011). Increasingly, however, researchers are calling for a more proactive approach to conservation, one that incorporates both protection and restoration (Possingham et al. 2015; Abelson et al. 2016). In this sense, a "fully restored" ecosystem (i.e, Palmer et al. 2016) is not just biologically diverse; it is also resilient and able to recover from natural and human-induced stressors (SER 2004; Montoya et al. 2012).

Restoration outcomes can vary widely (Suding 2011), and complete recovery often depends upon restoring natural biological communities and the abiotic processes that sustain them (Montoya et al. 2012; Naeem 2016). Biodiversity is essential to several important ecosystem services (Delegates of the World Conference on Marine Biodiversity, 2008), including enhanced water quality (Lamb et al. 2017), resistance to invasion by non-native species (Stachowicz et al. 2007), fisheries productivity (Worm et al. 2006), and aesthetic enjoyment (Sandifer et al. 2015; Sutton-Grier et al. 2018). As a goal or driver of ecological restoration "success" (i.e., Naeem 2016), biodiversity thus represents more than just a measure of taxonomic richness (Wortley et al. 2013; Palmer et al. 2014). Instead, biodiversity should be viewed as a "multidimensional construct" (Naeem 2016) that also incorporates the trait-based

diversity of organisms (Cadotte et al. 2011). In other words, a degraded landscape is missing more than a variety of species—it is also deficient in the structural and functional complexity of a resilient ecosystem.

However, for restoration to be fully incorporated into conservation planning (*sensu* Abelson et al. 2016), functional metrics and other indicators of ecosystem performance must be carefully defined (Palmer & Filoso 2009). It is therefore important to establish realistic criteria for evaluating restoration success (Ellison 2000; Bell et al. 2008; 2014; Baggett et al. 2015), especially when considering whether restoration can enhance the secondary productivity of supported fauna (Powers et al. 2009; Angelini et al. 2011; Derksen-Hooijberg et al. 2017). To be truly effective, restoration projects need to incorporate a monitoring program designed to collect data that can be used to inform future work, i.e., "adaptive monitoring" (Lindenmayer & Likens 2009; 2018). Yet in most ecological restoration projects, there is often little or no post-project monitoring of restored habitat (Lindenmeyer & Likens 2018). Long-term monitoring programs can be challenging to administer for a variety of reasons. First, surveying for multiple target organisms is often impractical, and monitoring budgets are usually hampered by inadequate funding and other constraints (Bates et al. 2007). Second, monitoring must be conducted routinely if it is to function as an early warning system for evaluating threats to biodiversity (Yoccoz et al. 2001). Third, some habitats may be sensitive to disturbance, and repeated monitoring can be destructive depending on the number of personnel required or the equipment used (Bates et al. 2007). Many monitoring programs are therefore limited in scope, often focusing on target species or threatened habitats (Henry et al. 2008). As a result, there is a clear need for novel approaches that can account for the limitations imposed by traditional survey methods while offering a systematic way to survey biodiversity at the landscape scale. For these

reasons, "surrogate species" are frequently used in environmental monitoring—organisms that can reliably predict the presence of other, more elusive taxa (Rodrigues & Brooks 2007; Lindenmeyer et al. 2015).

Over the years, many organisms have been identified as indicator species, providing evidence of specific environmental impacts across a variety of conservation-related questions and environmental scales (Caro 2010). However, indicator species that confer information across taxa (i.e., cross-taxon surrogates) are most effective at serving as proxies for overall community diversity. Trophically transmitted parasites are valuable cross-taxon surrogates because their indirect life cycles often incorporate multiple invertebrate and vertebrate taxa—thus enhancing connectivity and interactions between organisms in food webs (Fig. 1) (Huspeni et al. 2005; Lafferty et al. 2006; Dunne et al. 2013). Indeed, trophically transmitted parasites have proven effective in monitoring the recovery of salt marsh and other habitats following significant human-mediated disturbance (Huspeni & Lafferty 2004; Bass & Weis 2008; Anderson & Sukhdeo 2013). More specifically, the abundance of trophically transmitted parasites in intermediate hosts has been found to reflect patterns of land use and overall animal biodiversity (Hechinger et al. 2008) in organisms as diverse as shorebirds (Hechinger & Lafferty 2005), diamondback terrapins (Byers et al. 2011), and small fishes and benthic invertebrates (Hechinger et al. 2007). This fundamental relationship between host and parasite diversity has been validated by multiple meta-analyses (Kamiya et al. 2014; Wood & Johnson 2016), which have documented a consistently positive correlation between host and parasite species richness. Also, these interactions are long-lasting, as many host-parasite relationships remain stable over time (Byers et al. 2008; 2016). Trophically transmitted parasites can thereby function as cross-taxon surrogates for the presence of their hosts. In coastal estuaries, target hosts often include abundant

and easily-collected organisms like mollusks, mud crabs, and small resident fishes (e.g., gobies, blennies, toadfish)—all of which are relatively immobile and exhibit strong site-fidelity (Curtis & Hurd 1983; Toscano et al. 2014; Harding et al. 2019). Because of the strong positive correlation between host and parasite abundance, high parasite diversity in these hosts indicates that the other hosts required by the parasite are also present in the system (Hechinger & Lafferty 2005; Hechinger et al. 2007; 2008; Byers et al. 2011).

Host-parasite interactions are also strongly influenced by habitat complexity (Sousa & Grosholz 1991; Froeschke et al. 2013; Johnson et al. 2016), particularly in communities with overlapping heterogeneous habitat types (Rossiter & Sukhdeo 2014). Thus, adding structurally complex habitat to a degraded environment would be expected to strengthen trophic links as new hosts recruit to the area. For example, at a series of restored saltmarsh sites, Anderson and Sukhdeo (2013) found that stable communities of host organisms were required for parasite life cycles to persist over time. Interestingly, the parasite community in a newly restored marsh (up to a year) was found to have only *directly transmitted* parasite species. Parasites with direct lifecycles require only a single host, whereas trophically transmitted parasites require multiple hosts (Rohde 2005). A site with only directly transmitted parasites would thus be deficient in community structure and functional diversity because the additional hosts needed for parasite life cycle completion are not present in the system. To some extent, other researchers have used trophically transmitted parasites to evaluate habitat restoration and have demonstrated a positive correlation between trematode parasite diversity and time post-restoration; however, in all cases, parasite diversity (and only that of trematodes) was used in a post-hoc analytical framework (e.g., Huspeni & Lafferty 2004; Bass & Weis 2008).

Restoration projects in coastal systems of eastern North America and the Gulf of Mexico have widely incorporated an important habitat-forming species, the eastern oyster (*Crassostrea virginica*), at a variety of spatial scales (Kennedy 1996; Altieri & Koppel 2014; Hernandez et al. 2018). Oyster reefs offer a wealth of ecosystem services (Coen et al. 2007; Grabowski & Peterson 2007; Grabowski et al. 2012), including habitat provisioning (Coen et al. 1999; Tolley & Volety 2005; Humphries et al. 2011), water filtration (Peterson et al. 2003; Piehler & Smyth 2011), carbon sequestration (Fodrie et al. 2017), and stabilization of erosion-prone salt marsh shorelines (Piazza et al. 2005). However, these services are imperiled by the drastic decline in oyster abundance over the last few decades (Beck et al. 2011). Structurally complex oyster-reef habitat has declined up to 85% relative to historical baselines (zu Ermgassen et al. 2012), with some areas in North Carolina experiencing losses > 90% since the early 1900s (Jackson et al. 2001). In response, states along the U.S. East and Gulf coasts have invested heavily in "living" shorelines that use oyster reefs and other nature-based features to enhance biodiversity and mitigate erosion (Bilkovic et al. 2017). Since the mid-2000s, the pace of such restoration projects has escalated (Hernandez et al. 2018), with practitioners increasingly incorporating foundation species like oysters into restoration planning (Byers et al. 2006; Kennedy et al. 2011; Gedan et al. 2014). These foundation or "habitat-forming" species (sensu Dayton 1972) can enhance biodiversity and ecosystem multi-functionality at both the patch and landscape-scale (Angelini et al. 2015), making them ideally suited for use in locations where foundation species placement would maximize the delivery of ecosystem services.

In our study, we incorporated traditional surveys of free-living biodiversity with novel parasite-surrogate approaches to evaluate the success of oyster reef restoration. Here, we define "success" to mean the creation of self-sustaining biogenic habitat that offers provisioning for

associated free-living and parasite taxa across multiple trophic levels. We posed the following questions in our study: (1) How does restoration influence free-living and parasite diversity over time and in response to seasonal changes (i.e., pre-restoration, 3–6 months post-restoration, and 9–12 months post-restoration)? (2) Will restoration design (i.e., traditional versus novel restoration materials) differentially affect biodiversity and habitat complexity when compared to a no-restoration control? To answer these questions, we surveyed resident free-living and parasite surrogate taxa 3-5 months prior and 12 months following the addition of habitat. To our knowledge, our study is the first to *a priori* incorporate parasites into the experimental design of a restoration project, providing a more powerful, multi-species approach for evaluating the impact of restoration on biodiversity and trophic complexity.

Materials and Methods

Study System

Our study took place in the Rachel Carson National Estuarine Research Reserve (NERR), a protected landscape of sounds and dune-forest habitat located along the central coast of North Carolina in Beaufort, NC, United States (34∘42′14.99′′N, 76∘37′25.41′′W) (NC DEQ 2020). Within the Reserve, we sampled an unnamed creek located along the south-facing shoreline of Carrot Island, which experiences high erosion (Riggs & Ames 2003) resulting from a large fetch and dominant south-southwest summer winds (Fig. 2, Pietrafesa et al. 1986; Theuerkauf et al. 2017). The creek has a maximum tidal exchange of ± 0.75 meters and is polyhaline with a salinity of 23.2—37.5 ppt. (mean 30.0 ppt) measured during this project. Sides of the creek are lined with *Spartina alterniflora* (Bortolus et al. 2019), and the pre-restoration substrate consisted mostly of bare mudflat mixed with low-density oyster shell and shell fragments (i.e., cultch) and scattered clumps of live oyster in the upper intertidal zone. We sampled a roughly 300 meter

section of the northern shoreline of the creek and one additional cultch reef on the southern shoreline (Fig. 2). We were unable to replicate natural reefs in our system because two of these reefs had been buried with sediment by the time our project began. We did, however, collect data from a single natural reef, which we include in a supplementary table (*see* Discussion).

Experimental Restoration Design

We used a block design to test the effects of adding two forms of oyster habitat to the creek shoreline along Carrot Island (Fig. 2). Habitat was added in the form of bags of oyster cultch (hereafter "shell bags," SB) and Oyster Catcher (OC) ™ structures. Oyster Catcher™ is a novel form of biogenic habitat that may be more resilient in high energy environments and incorporates only biodegradable materials such as burlap, concrete, and steel wire (Sandbar Oyster Company 2020). Each block thus consisted of the following three treatments: cultch shell-bag reefs; Oyster Catcher™ reefs; and control plots (CP) where no restoration occurred (Fig. 2; *see* also Fig. S1). We also sampled three areas that had been previously restored with loose oyster cultch in 2012 (*see* Keller et al. 2019). These were incorporated into the experimental design as cultch reefs (CR) 1, 2, and 3.

In August 2017 and April 2018, we collected baseline surface elevation data to determine appropriate locations for intertidal oyster reef restoration. We used a Trimble Real-Time Kinematic (RTK) Global Navigation Satellite System (0.5–1.0 cm horizontal and 1.0–4.0 cm vertical resolution) to identify locations adjacent (within 1 m) to the creek shoreline with surface elevations between -0.6 m and -0.3 m relative to the North American Vertical Datum of 1988 (NAVD88). These base elevations would allow us to build the reefs within the "optimal growth zone" for oyster reefs in this system (Ridge et al. 2015). We collected additional baseline-monitoring data in August and September 2017 and April 2018 for each site.

In January 2018, sections of shoreline were randomly assigned into blocks (1–4). However, because oyster cultch had previously been distributed in Block 2, that block was excluded from this study; thus our study examined Blocks 1, 3, and 4. Restoration occurred at the end of May 2018 under a General Permit (#69643) for marsh toe revetments from the NC Division of Coastal Management. SB reefs consisted of 159 ± 3 polyethylene oyster bags with 1.0 cm^2 mesh, filled with loose shells ranging from 5–15 cm. Each SB was 4 meters long × 1.5 m deep × 0.5 m tall and was constructed by volunteers with the North Carolina Coastal Federation. OC reefs consisted of a framework of concrete-burlap bars positioned laterally and at right angles to each other atop a series of Y-shaped pillars driven 1–1.5 meters into the sediment, elevating the oysters roughly 0.5 m above the substrate. They were each 4 meters long × 2 m deep × 0.25 m tall and were designed and constructed in partnership with Sandbar Oyster Company (Sandbar Oyster Co. 2020). The materials used to construct the OC and SB reefs were seeded via the settlement of oyster spat on Sandbar Oyster Co.'s lease in the Newport River, NC, beginning in August 2017.

Sampling for Free-living/Sessile Organisms

We sampled resident free-living diversity over two sampling events pre-restoration ("pre": March, May 2018), over three sampling events six months post-restoration ("post-6": August, September, November 2018), and over three sampling events 12 months post-restoration ("post-12": January, March, May 2019). Over the restoration trajectory of our project, crustaceans, fishes, and other mobile free-living organisms were collected using small plastic milk crates (19 × 22 × 16 cm) filled with autoclaved oyster shells. This is modeled on the successful methodology used by the Smithsonian Environmental Research Center over the past 20 years to sample for small benthic crustaceans (e.g., Roche & Torchin 2007) and is also

analogous to the use of shell-rubble trays, which are highly effective for sampling reef-resident fish (La Peyre 2019). Although organisms can freely move inside and outside the crates, they are attracted to the complex three-dimensional habitat that the shell provides. In our system, 2 replicate crates (n = 26 total) were zip-tied to 0.75 m wooden stakes in the shallow subtidal ~2 meters from each treatment. We were unable to place the collectors within the treatments themselves because this would have exposed them during low tide, preventing us from collecting resident and transient fishes.

Crabs and fish were collected by sorting the contents of each crate using a floating sieve (56 × 56 × 13 cm) with a 2 mm mesh. Due to the abundance of xanthid crabs in our system, crabs were collected for dissection from just one of the two replicate crates at each site, whereas fish were collected from both. We also documented the free-living diversity of other mobile taxa (e.g., blue crabs, shrimps, transient fishes) found within the sampling units at every site. Sampling took place every 6–8 weeks from March 2018 until May 2019 with a brief hiatus in June 2018 following the installation of restored habitat. Last, the influence of restoration on sessile invertebrate taxa was quantified using 13.5 cm^2 plastic fouling plates (Piedmont Plastics, Atlanta, GA), which were attached in September 2018 to all collecting units in our system. Plates were left for ~6 months and removed in March 2019. The contents of each plate were scraped into an aluminum foil wrapper and placed into a drying oven at 75°C for 72 hours. Samples were weighed on a digital scale (Mettler Toledo model ME54E), and the dry weight of each sample was recorded.

Sampling for Parasites

We quantified the relative abundance and taxa richness of endoparasites with complex, multi-host life cycles (e.g., nematodes, trematodes, acanthocephalans, cestodes, entoniscid

isopods) in a variety of common intermediate host organisms, which we partitioned into three host groups: fish, crustaceans, and snails. Host sampling was performed over the same period and using the same passive collectors described above (except for host snails—see below). Ectoparasites like monogeneans and copepods were not sampled because these organisms are easily dislodged during collection and transport. Though not a multi-host parasite, we did include the endoparasite *Loxothylacus panopaei* in our study because this parasitic barnacle is a castrator and can be highly prevalent in southeastern coastal estuaries, where it exerts considerable selective pressure on mud crab populations (Tepolt et al. 2020). Hosts retained for parasite dissections included: (a) the eastern mudsnail (*T. obsoleta*); (b) xanthid crabs: *Rhithropanopeus harrisii* (white-fingered mud crab), *Eurypanopeus depressus* (flatback mud crab), *Panopeus herbstii* (Atlantic mud crab), *Dyspanopeus sayi* (Say's mud crab), and *Menippe mercenaria* (stone crab); and (c) small benthic fishes: blennies *Chasmodes bosquianus*, *Hypsoblennius hentz*; naked gobies *Gobiosoma bosc*; and toadfish *Opsanus tau*. Mud crabs were identified to species using Williams (1984).

To examine parasites (specifically trematodes) in snail hosts, we focused on the eastern mudsnail, *Tritia* (=*Ilyanassa*) *obsoleta*. Snails were sampled on a slightly different schedule compared to crustacean and fish hosts: pre-restoration (January, March, May 2018); post-6 (June, August, September, November 2018); and post-12 (January, March, May 2019). Thus, compared to the crustacean and fish sampling (n = 8 collection events), we collected two additional samples of snails in January and June of 2018 (n = 10 collection events). Approximately 100 snails were collected haphazardly at each of our three blocks and from the previously restored reefs (CR 1 and CR 2-3 combined; see Fig. 2). Our sample size was based upon previous work showing it to be adequate for assessing trematode prevalence and species

richness in first-intermediate snail hosts (e.g., Blakeslee et al. 2012; Byers et al. 2016). Moreover, we sampled at the block-level because mudsnails tend to aggregate in groups (Scheltema 1964; Curtis 2002) and move in response to disturbance and currents (Levinton et al. 1995), although net movement in any one direction is limited (Curtis 2005; 2007a).

As hosts for parasites, mudsnails, crabs, and benthic fishes are all relatively immobile and display strong site-fidelity (Wilson et al. 1982; Curtis & Hurd 1983; Toscano et al. 2014; Harding et al. 2019), which is important because parasite diversity in these organisms should correlate with factors like habitat complexity and free-living diversity at the site-level (Bortone 2005). Moreover, to avoid problems with spatial autocorrelation, it is important to match the scale of a species' response to its environment (i.e., its mobility) to the scale of the analysis (i.e., biodiversity at the site-level) (Knegt et al. 2010). Only snails ≥ 11 mm, xanthid crabs ≥ 5 mm, and benthic fishes ≥20 mm were dissected for parasites because smaller individuals are immature and would have just recently become part of the community of competent hosts exposed to trophically transmitted parasites (McDonald 1982; Wilson et al. 1982; Curtis & Hurd 1983; Harding 1999; Blakeslee et al. 2012; D'Aguillo et al. 2014; Tepolt et al. 2020). However, even though mud crabs < 5 mm and resident fishes < 20 mm were not dissected for parasites, these individuals were still recorded as part of the overall taxonomic diversity associated with each site, as they provide an understanding of the recruitment of free-living taxa to the restoration treatments and controls.

All hosts were dissected using light microscopy at 4–10x (Zeiss stereo and compound microscopes), and parasites were identified using standard protocols and keys (e.g., Golvan 1969; Yamaguti 1971; Anderson 1975; Schmidt 1986; Khalil et al. 1994; Blakeslee et al. 2012; Blakeslee et al. 2015). Xanthid crabs and fish were checked for parasites using tissue squashes

and light microscopy. In crabs, all hepatopancreas tissue was removed and examined under low power (4x, 10x). In fish, the entire gastrointestinal tract was removed, and the stomach, liver, gallbladder, spleen, and intestine were checked for parasites under low power (4x, 10x). Also, the gut cavity of each fish was rinsed, and the wash water examined for any parasites that had been dislodged during dissection. Last, the head, body, and fins of each fish were checked for trematode cysts by viewing each fish under a dissecting microscope at low power (4x). For snail hosts, the length of each shell was first recorded (measuring from the siphon base to the aperture) using digital calipers (mm). The shell was then cracked using a hammer, and the soft tissue of the snail was removed and placed into a watch glass. The gonad tissue was then teased apart using forceps and seawater was added to the dish to observe swimming cercariae (see Fig. 1). Trematodes were identified to species level using Curtis (2007b), Blakeslee et al. (2012), and Phelan et al. (2016). Field collections were authorized by the North Carolina Division of Marine Fisheries (Scientific or Educational Permit #706671) and North Carolina Coastal Reserve (research permit #1-2018). Animal husbandry and dissection protocols were approved by East Carolina University's IACUC (AUP #D346).

Habitat Complexity Data

We collected the following habitat complexity data for each replicate treatment: live oyster density (per m^2), cultch (dead oyster shell) percent cover (per m^2), and surface elevation at the marsh edge. Habitat complexity sampling was conducted three times: August 2017 (nine months before restoration), September 2018 (two months following restoration), and August 2019 (thirteen months following restoration). Live adult and juvenile in situ oyster densities were determined for each treatment (OC, SB, CP) using a 0.0625-m^2 quadrat in October 2018 (three months following the addition of habitat) and again in September 2019 (fourteen months later).

Although we were unable to match our habitat complexity measures with our biodiversity sampling timeline, the habitat data we collected still provides a general understanding of habitat measures before restoration (before our biodiversity surveys); 6 months post-restoration (during our surveys); and 16 months post-restoration (after our surveys ended). Densities were calculated based on the number of juveniles and adults (both living and dead) divided by the total surface area available for recruitment and then standardized to per m^2. At CP sites, the density of adult (>25 mm) and juvenile (<25 mm) oysters were assessed using a 0.0625 m^2 quadrat placed in the center of the plot. In an attempt to make accurate inter-substrate comparisons for the SB and OC samples, density calculations were based upon their respective surface areas (and therefore, area available for oyster settlement and growth). At SB sites, one shell bag was removed from the midpoint of the reef and measured to calculate an average surface area based on the formula of a rectangular solid (in m^2). All juvenile and adult oysters within the sample were enumerated and their totals divided by the calculated surface area, accounting for the settlement of most oysters on the exterior of the bag. At each OC site, a 10-cm linear section was removed from one corner of the reef using a hacksaw. Juvenile and adult oyster density was calculated based on the number of individuals from each size class present in the average surface area (in m^2) of the cylindrically-shaped samples. To assess oyster lengths, in all samples we used a ruler to measure 10 randomly selected adult and juvenile oysters (cm), or all oysters present if there were fewer than 10.

Abiotic Data

Abiotic measurements included water temperature (^{o} C), dissolved oxygen (mg/L), and salinity (ppt). These data were collected using a handheld YSI (Pro-ODO model) at the block

level (i.e., a single set of measurements for all three treatments in a block) at the midpoint of each block. For the cultch reefs, abiotic data were collected at each of the three reefs.

Statistical Analyses and Data Visualization

To examine the influence of restoration treatment on free-living and parasite taxa richness over time, we used species accumulation curves in EstimateS 9.1.0 (Colwell 2019) to extrapolate the asymptote in taxa richness and predict the accumulation of species with sampling effort and time post-restoration. Extrapolation curves were rescaled to accumulated individuals to compare taxa richness in a standardized manner (Gotelli and Colwell 2001). Curves were analyzed for both combined and separated crustacean and fish free-living richness, parasite richness in crustacean hosts, parasite richness in fish hosts, and trematode richness in the snail host, *T. obsoleta*. Also, to determine how taxa richness may have changed 1-year post-restoration for each restoration treatment, we subtracted the pre-restoration extrapolated taxa richness from the final extrapolated taxa richness for each of our free-living and host-parasite groups, and across all taxa.

We examined the composition and structure of free-living crustacean and fish communities (abundance data), as well as crustacean and fish parasite communities (presence/absence data) using permutational analysis of variance (PERMANOVA) on Bray-Curtis similarity matrices, with restoration period (pre, post-6, post-12) and restoration treatment as fixed factors (Anderson 2005). We also examined the change in snail trematode communities across restoration periods (pre, post-6, post-12). Permutational dispersion (PERMDISP) was used to test for homogeneity of variances for each factor tested. Differences were significant at the 0.05 level. Non-metric multi-dimensional scaling (nMDS) was used to visualize differences in communities across restoration period and treatment, with measurements of physical habitat

complexity (cultch cover, oyster density) and parasite habitat complexity (free-living fish and crustacean abundance), overlaid on the nMDS plots. Habitat measures with Pearson correlation coefficients of greater than [0.3] were included on the nMDS plots. Finally, analysis of similarity (SIMPER) was used to assess which species, if any, were contributing to observed differences in community composition and structure. Analyses were conducted on raw abundance data for free-living fish and crustaceans because counts were generally low across species and we were specifically interested in understanding if one or more species were driving patterns in community composition. PERMANOVA, nMDS, and SIMPER analyses were conducted using PRIMER- E software 7 with PERMANOVA+ (Anderson 2005; Clarke et al. 2014).

Habitat complexity in all experimental treatment blocks was quantified by measuring adult oyster densities and lengths. Oyster densities (measured in individuals per m^2) were analyzed using linear models and data were log-transformed before analysis to satisfy model assumptions of normality. Models examined the effects of treatment, year, and block. We compared models using Akaike's Information Criterion (AIC) and reported results from the lowest-scoring model, which included treatment only. Tukey's post-hoc tests were performed to assess pairwise comparisons among reef treatments (CP, OC, SB). Differences were significant at the 0.05 level. Lengths of adult oysters were analyzed using Bayesian linear mixed effect models. Adult oyster lengths (mm) were log-transformed before analysis to satisfy model assumptions of normality. Models examined the fixed effects of treatment, year, and block. We compared models using Akaike's information criterion (AIC) and reported results from the full additive model, which had the lowest AIC score. We compared the differences between the significant factors of treatment and year using the Kenward Rogers degrees of freedom approximation. Contrasts are considered significant at the 0.05 level. All habitat complexity

analyses were performed in R (version 3.5.2 (2018-12-20)) using R core functionality, the *blme* (Chung et al. 2013), *lsmeans* (Lenth 2016), and *multcomp* (Hothorn et al. 2008) packages.

Finally, as past studies have shown that "host diversity begets parasite diversity" (e.g., Kamiya et al. 2014; Wood & Johnson 2016), we wanted to assess the strength of the correlation between hosts and their parasites in our system. To do so, we used linear regressions to look for significant and positive correlations between parasite and free-living taxa richness. We made the following comparisons: 1) taxa richness of free-living crustaceans and fish with trematode richness from *T. obsoleta* snails; 2) taxa richness of free-living crustaceans with crustacean parasite richness; and 3) taxa richness of free-living fish with fish parasite richness. Richness data were square root transformed and only positive values (i.e., zeros excluded) were included in regression analyses because we were interested in examining whether the presence and number of parasite species could be used to indicate the presence and number of free-living species. Linear regressions were performed using JMP Pro 14.1.

Results

Impacts of Restoration on Free-living and Parasite Taxa Richness and Sessile Invertebrate Biomass

To determine the influence of restoration design on resident free-living and parasite taxa richness in our system over time, we constructed rarefaction curves based on observed taxa richness and then extrapolated the asymptote in richness for each comparison (Figs. S2 and S3). Across all free-living and parasite groups, richness showed a positive change 1-year post-restoration on average (Fig. 3), although within groups there were exceptions based on restoration design. Notably, the change in free-living crustacean richness was positive for SB,

but not OC, CR, or the control plots (CP). Similarly, the greatest positive change in fish free-living richness post-restoration was associated with the SB treatment. Crustacean parasite richness increased post-restoration in all treatments, including the control. Fish parasite richness showed a negative change 1-year post-restoration for OC and SB, while CR and CP showed little change (Fig. 3). In addition, snail trematode parasite richness showed a positive change 1-year post-restoration across all blocks. Across all taxa, there was no significant difference ($F_{3,19}=0.56$, $p=0.65$) in restoration treatment on taxa richness one-year post restoration; however, on average, the SB treatment demonstrated the greatest positive change—approximately two times that of the CP (Fig. 3).

In contrast, restoration design did have a significant ($F_{3,11} = 5.633$, $p = 0.023$) influence on the biomass of sessile invertebrates (primarily tunicates like *Molgula* spp.) that recruited to fouling plates following a 6-month exposure period. In this case, the OC treatment supported greater biomass of fouling organisms than CP and CR, with SB having the second highest biomass (Fig. S4).

Impacts of Restoration and Season on Community Structure and Assembly

Fish and crustacean (both free-living and parasite) community composition changed with time (pre-restoration post-6, and post-12), but did not differ among restoration treatments (OC, SB, CP, Table S1, $p> 0.05$ for restoration treatment and for restoration treatment by time interaction). Below we describe changes in community composition with time across all treatments.

The most abundant free-living fish species in the pre-restoration period (March and May 2018) was the naked goby, whereas feather blenny and oyster toadfish were the most abundant

species June–November 2018 (post-6), followed by the striped blenny from January–May 2019 (post-12, PERMANOVA posthoc pairwise tests for time: $p < 0.01$, Table S1). Thus, fish communities shifted from a single dominant species (naked goby) pre-restoration to a greater diversity of fish post-restoration. Some of these changes in fish communities were associated with seasonal changes in temperature and dissolved oxygen (Fig. 4A, Table S2). Further, salinity increased with distance from the creek mouth, and communities at sites farther from the creek mouth were more similar to one another than communities at sites closest to the creek mouth (Fig. 4A, Table S2).

Forty-nine percent of the free-living samples contained fish infected with parasites, with the most common parasite group being trematodes (larval metacercarial cysts and adult worms), followed by cestodes, and nematodes. Parasite communities using fish as hosts also changed with time, shifting from communities dominated by cestodes pre-restoration to communities dominated by trematodes 12 months after restoration (post-12) in all treatments (Fig. 4B, Table S1, pairwise tests pre vs. post-12, $p = 0.022$). These changes in parasite communities of fish were correlated with seasonal changes in temperature, with post-6 communities being more similar to pre-restoration communities (posthoc pairwise tests, $p = 0.621$) than to post-12 communities (Fig. 4B, $p=0.008$). The abundance of parasites in fish hosts, particularly larval cestodes, was most likely driven by striped blennies, as these were the dominant species of site-resident fishes collected across all of our treatments (Table S3). However, nematode presence may have been driven primarily by the abundance of oyster toadfish (*O. tao*) (Fig. 4B, Table S4). Notably, nematodes were not present in any samples after September 2018 (3 months post-restoration), while trematode cysts were only found in samples beginning eight months post-restoration across all treatments.

For free-living crustacean communities, the most abundant species before habitat addition (pre-restoration) were grass shrimp (*Palaemonetes* spp.) across all treatments (CP, OC, SB). *P. herbstii* and snapping shrimp (*Alpheus* spp.) became the most abundant species after restoration (Table S5**,** pre vs. post-6 vs. post-12, PERMANOVA posthoc pairwise tests $P<0.01$). Across the trajectory of restoration (pre- through post-12), *P. herbstii* and *D. sayi* were the two most abundant crustaceans sampled from our treatments (Table S3). The average number of *P. herbstii* was greatest in September 2018, whereas the mean abundance of *D. sayi* peaked in May of both 2018 and 2019 (Table S6). These changes in crustacean communities were correlated with seasonal changes in temperature and dissolved oxygen (Fig. 4C, Table S2). Similar to the trend for free-living fish species, salinity increased with distance from the creek mouth, with communities at sites farther from the creek mouth being more similar to one another compared to those closest to the creek mouth (Table S2).

Sixty-five percent of the crustacean host samples were infected with parasites, with the most common parasite group being larval trematodes (*Microphallidae* metacercarial cysts), followed by the entoniscid *Cancrion carolinus*, cestodes, nematodes, and finally the rhizocephalan barnacle *Loxothylacus panopaei*. Similar to free-living crustacean communities, parasite communities using crustacean hosts changed with time, with nematodes, cestodes, the entoniscid *C. carolinus*, and the rhizocephalan *L. panopaei* only found in post-restoration samples (Table S1, pairwise tests, pre vs. post-6 vs. post-12, $p < 0.03$). In contrast to trematodes in fish hosts, the trematodes in crustacean hosts were found both before and after restoration across all treatments. Parasite communities that included the entoniscid *C. carolinus* were positively associated with higher oyster densities and negatively associated with cultch percent cover (Fig. 4D, Table S2). More diverse crustacean parasite communities were positively

associated with *P. herbstii* abundance and negatively associated with dissolved oxygen levels (Fig. 4D, Tables S2 and S5).

Finally, trematode community composition in the snail host, *T. obsoleta*, changed with time, with *Lepocreadium setiferoides* being more common pre-restoration and 12-months post-restoration, whereas immature infection stages and *Himasthla quissetensis* were more common 6-months post-restoration (Fig. S5, Table S2, post-6 vs. post-12, $p = 0.01$). Differences in trematode community composition were associated with seasonal shifts in temperature and dissolved oxygen, as well as differences in salinity and percent cover of cultch (Fig. S5). Snails were most often infected by three trematode species (*Lepocreadium setiferoides*, *Zoogonus lasius*, and *Stephanostomum tenue*), all of which require fish as final hosts (Phelan et al. 2016). Overall, fish-using trematodes accounted for 85% of the total trematode infections in snails, whereas bird-using trematodes made up <15% (Table S7).

Impacts of Restoration Design on Habitat Complexity

We analyzed adult oyster density and length among treatments (CP, OC, SB) to determine whether habitat complexity was influenced by restoration design. For both density and length of adult oysters, pairwise comparisons were made among measurements in each treatment using data collected in 2018 and 2019 (2 and 13 months post-restoration). Oyster densities differed significantly between treatments ($p = 0.0018$), but not experimental blocks (Table 1a). Density was greatest in the OC treatments, followed by the shell bags (Fig. 5). However, this difference in adult oyster densities between the two restoration substrates was not significant ($p = 0.1472$). Post-hoc comparisons showed that densities were significantly different between oysters in the OC and CP treatments ($p = 0.001$).

Differences in oyster lengths were significant at the treatment level ($p = <2.2 \times 10^{-16}$) and between collection events in 2018 and 2019 ($p = 0.0001391$) (Table 1b). Across all treatments, the mean oyster length increased from 2018 to 2019 (Fig. 5). However, the change in mean length from year-to-year differed among treatments. On average, the length of adult oysters associated with SB reefs did not change between years, while those growing on OC reefs were much larger in 2019 ($p < 0.0001$).

Correlations between parasite and free-living taxa richness

To determine if parasite richness could inform free-living richness, and therefore also provide information on community biodiversity, we regressed parasite taxa richness for our three major parasite groups with either crustacean or fish free-living taxa richness collected from our passive samplers (Fig. S6). We found weak ($R^2 < 0.2$) but significant ($p < 0.05$) correlations between snail trematode richness and fish free-living richness, crustacean parasite taxa richness, and crustacean free-living taxa richness, and fish parasite taxa richness and fish free-living taxa richness. There was no trend observed for snail trematode richness and crustacean free-living richness.

Discussion

Restoration projects are designed to promote ecosystem functionality, with enhanced biodiversity often cited as a key criterion of "success" (i.e., Naeem 2016). The restoration of biogenic oyster reef habitat, in particular, has been shown to enhance community biodiversity by creating structural complexity for a variety of sessile and mobile taxa, at all stages of development, and across numerous taxonomic and functional groups (Coen et al 1999). Because host-parasite interactions are sensitive to changes in habitat quality and land use, it follows that

adding habitat to enhance native biodiversity should lead to increases in both parasite and free-living biodiversity (Lafferty 2012). Moreover, as biodiversity "surrogates," parasites can provide evidence of their hosts' presence, even on an ephemeral basis (Busch et al. 1997; Esch et al. 2001), and are therefore useful in identifying habitat usage by more elusive or difficult to sample hosts (Byers et al. 2011). In the sections that follow, we discuss how adding structured habitat affected the host-parasite community response over time.

Community Changes Pre- and Post-Restoration and Seasonal Influences

For free-living taxa (crustaceans, fish) and their parasites, as well as the trematodes infecting *T. obsoleta*, we documented positive increases in richness 1-year post-restoration across all taxa (Fig. 3; Figs. S2-S3). Because pre- and post-restoration comparisons were performed within the same season (winter/spring 2018-to-winter/spring 2019), these results suggest that these positive changes we observed were due to restoration-stimulated increases in diversity among our free-living species and parasite surrogates. Surveying for parasites, therefore, provided a more holistic understanding of the broader community response to habitat restoration. Past studies, notably Hechinger et al. (2007), have documented the importance of parasites in community investigations and their strong links to free-living biodiversity. Our study also demonstrates that parasites can provide key evidence for understanding changes in biodiversity following habitat alterations, such as adding habitat for restoration projects, and that these changes can be fairly rapid.

The greatest positive change in extrapolated richness across all treatments occurred among the free-living fish species (Fig. 3), with similar shifts documented by our community analyses (Fig. 4). We found that naked gobies (*G. bosc*) were more abundant in the pre-restoration period, but post-restoration, the fish community transitioned to reef-resident feather

blennies (*H. hentz)*, oyster toadfish (*O. tau)*, and striped blennies (*C. bosquianus)*—with the latter species becoming completely dominant 1-year following restoration. Compared to blennies and oyster toadfish, naked gobies are habitat generalists and are often found in both mudflats and structured benthic habitats (Able & Fahay 2010; Moore et al. 2018), whereas reef-resident blennies exhibit strong site fidelity to specific reef-patches throughout their lives (Harding et al. 2019). A multi-year tagging study by Harding et al. (2019) demonstrated that blennies were recaptured within 1-meter of their original location as much as 50% of the time, while the recapture rate for naked gobies was 9%. A high abundance of blennies can thus serve as an indicator of habitat complexity at small scales (Harding et al. 2019).

Moreover, the shift in species composition in our system—from goby-to-blenny abundance—suggests that a reef-resident fish community had developed in response to the increased availability of structured habitat (Fig. 5). In addition, seasonal influences on fish communities were identified in our community analyses. For example, striped blennies migrate to deeper areas of mudflat reefs in winter (Kells & Carpenter 2011), which is perhaps why they were most abundant in our sub-tidal collectors in March 2018 and January 2019 (Table S6), whereas the abundance of feather blennies peaked during the September/November sampling events (Table S6). Both species spawn in summer over oyster reefs (Kells & Carpenter 2011), and there would likely be competition for nesting habitat that would reinforce partitioning of reef-associated niche space (Tremont 2014).

For the free-living crustaceans, *P herbstii* remained abundant throughout the study period. However, shrimp species shifted over time, with grass shrimp, *Palaemonetes* spp., dominating pre-restoration totals across all treatments, and snapping shrimp (*Alpheus* spp.) becoming more abundant post-restoration. Snapping shrimp are more characteristic of oyster

reefs and other structured habitats (Williams 1984). On the other hand, grass shrimp are generalists and can be found in many estuarine environments (Eggleston et al. 1998). Previous studies have documented species-specific residency patterns of *P. herbstii* and *E. depressus* in oyster reef microhabitat in response to restoration (Meyer & Townsend 2000; Gregalis et al. 2009; Hadley et al. 2009). *Panopeus herbstii* is often associated with loose oyster shells at the base of reefs (McDonald 1977; 1982; Meyer 1994), which is perhaps why it was so frequently sampled in our sub-tidal collectors. Compared to *P. herbstii*, the flatback mud crab *E. depressus* is more often found in elevated sections of the reef (Gregalis et al. 2009) amidst smaller shell clusters (Hadley et al. 2009). In our study, *E. depressus* was the least abundant mud crab sampled (Table S3), which we may be due to our use of sub-tidal collectors as opposed to shell-rubble trays lodged within the reef itself (i.e., Gregalis et al. 2009; Hadley et al. 2009). Among crustaceans, seasonal habitat partitioning is another important feature of functionally complex oyster reef habitat (Meyer 1994; Grabowski 2004; Grabowski & Powers 2004; Tolley & Volety 2005; Hughes & Grabowski 2006), and we documented alternating patterns of residency among some of the most abundant species in our system. For example, the average number of *P. herbstii* was greatest in September 2018, while the mean abundance of *D. sayi* peaked in May of both 2018 and 2019 (Table S6), which may suggest that there is seasonal partitioning of reef-associated refuge space between these two species.

Differential use and occupancy of reef habitat would likewise have implications for parasite transmission, particularly under changing abiotic conditions. Factors like temperature, dissolved oxygen, and salinity were correlated in our models with shifts in community composition for all free-living hosts and their parasites (Fig. 4A-D). This is noteworthy because trematode larvae (e.g., egg and cercarial stages; Fig. 1) are vulnerable to environmental stressors

(Rohde 2005), as are larval cestodes (Pietrock & Marcogliese 2003). For example, temperature extremes can compromise egg development (Morley & Lewis 2017) and the ability of these parasites to infect a wide variety of second-intermediate hosts (Upatham et al. 1984; Sawabe & Makiya 1995).

However, not all potential hosts in a multi-host community will contribute equally to parasite transmission, particularly for trematodes (Fenton et al. 2015). Multiple studies (e.g., Byers et al. 2008; 2016; Thieltges 2016) have concluded that final hosts are more important for stabilizing trematode communities over time and contributing to hot spots of infection. However, in addition to final host abundance, Song & Proctor (2020) found that the time available to hosts for colonization was more important for explaining parasite prevalence in crustacean intermediate hosts than the abundance of hosts themselves. Similar to Song & Proctor (2020), we found that time was a significant factor for all investigated taxa (Table S1). Some studies have shown a lag in the response of parasite communities and other taxa to recruit to restored habitat, perhaps because of the initial disturbance imposed by restoration itself (Huspeni & Lafferty 2004; Morley & Lewis 2007; Henson et al. 2009; Anderson & Sukhdeo 2013). As such, the age of the habitat (i.e., time available for host colonization) would be expected to play a major role in structuring parasite communities (Song & Proctor 2020), as hosts would require time to colonize the area post-restoration. It is also reasonable to expect that some hosts, particularly mudsnails, which are more abundant on mudflats as opposed to structured habitat (Scheltema 1964; Curtis 2002), would have been infected before our pre-restoration monitoring. As first-intermediate hosts for larval trematodes, snails are infected for life (Curtis 2002), and infrapopulations of trematodes in mudsnails can persist unchanged for long periods (Curtis & Tanner 1999). Moreover, past work has demonstrated significant positive increases in trematode diversity

approximately 5-6 years following a saltmarsh restoration project (Huspeni & Lafferty 2004), suggesting that it may require additional time for clear patterns to emerge in snail-trematode systems following habitat changes. While our study showed positive increases in trematode richness in the treatment blocks after 1-year of monitoring (Fig. 3), longer-term monitoring may reveal more substantial changes associated with restoration. In contrast, parasite prevalence and richness may be more indicative of season-to-season changes or other ecosystem-level interactions, such as habitat use, for hosts and their parasites (Esch et al. 2001). In our study, trematode cysts in second-intermediate fish and crustacean hosts were only found in samples beginning 8-months post-restoration; thereafter, these parasites were increasingly prevalent in our data (Table S1). These patterns suggest that trophic complexity can rapidly increase following habitat restoration.

Restoration Design Differentially Affects Community Diversity and Habitat Complexity

Structurally complex habitat can be a major driver of biodiversity (Luckenbach et al. 2005; Kovalenko et al. 2012; Gittman et al. 2016), and we found that restoration design and associated habitat measures influenced host-parasite taxa richness and habitat complexity. For parasites, hosts are typically conceptualized as "habitat" (e.g., Esch et al. 2001), and "habitat heterogeneity" (i.e., host diversity) is often identified as the main driver of parasite diversity (e.g., Johnson et al. 2016). In theory, adding physical habitat to a system should have a *synergistic* effect on host-parasite diversity—enhancing niche diversity and/or resource availability would promote habitat use by a variety of species (Rosenzweig 1995), many of which also serve as "habitat" for parasites. For example, Rossiter & Sukhdeo (2014) found that habitat complexity was a strong predictor of parasite transmission in freshwater stream communities and also fostered higher-level trophic interactions. Also, while species richness

often positively correlates with habitat complexity (Diehl 1992; Kelaher 2003), trophic-mediated processes like predator-prey dynamics are also linked (Hughes & Grabowski 2006), and habitat complexity, in general, can modify trophic interactions on oyster reefs (Grabowski 2004). For example, in a study by Grabowski & Powers (2004), both foraging rates of mud crabs and predator foraging efficiency were higher in more complex reefs, implying that other trophic-mediated processes like parasite transmission should likewise increase in areas with complex habitat (Rossiter & Sukhdeo 2014).

For the crustacean and fish free-living taxa and the parasites of crustacean hosts, we observed a treatment effect 1-year post-restoration, with taxa richness being higher in the SB treatment versus the OC treatment, and across all taxa, the SB treatment was 3x greater than OC and 2x greater than CP on average (Fig. 3; Figs. S2-3). As habitat, shell bags offer a large and contiguous volume of refuge space. Taylor & Bushek (2008) observed that motile macrofauna "immediately" recruited to recently built shell bag reefs in a Delaware estuary, as did Rodney & Paynter (2006) in a similar study conducted in the Chesapeake Bay. Even so, this result was surprising considering that OC reefs were superior to the SB treatment in terms of habitat complexity (Fig. 5). However, this did not equate to a straightforward increase in host-parasite diversity within the time frame of our investigation. Dense patches of "clumped" habitat like shell bags can enhance larval settlement and recruitment of estuarine organisms relative to more dispersed habitat configurations (Morton & Shima 2013), which may explain why taxa richness was higher in the shell bags even though they were inferior to the OC reefs in terms of habitat complexity (Fig. 5). Also, recent work has shown that oyster density is a poor predictor of richness in intertidal reefs, but density can be a strong predictor in sub-tidal reefs (Tice-Lewis 2018). Our experimental reefs were constructed in the intertidal zone (*see* Methods), which may

explain why richness was lower in the OC reefs despite oyster density being much greater. Nevertheless, the OC treatment did recruit greater biomass of sessile, fouling organisms (particularly *Molgula* spp.) compared to the SB treatment (Figure S4). In the first 1-2 years post-restoration, this may suggest that metrics like oyster density and length in restored intertidal reefs are important as indicators of the fouling community—organisms that will subsequently provide resources for secondary taxa like crustaceans and fishes. Although the shell bags showed evidence of a treatment effect in the short term (< 15 months post-restoration), Oyster Catchers™ may outperform these structures over time, as they provide better substrate for fouling organisms and are more conducive to oyster growth and reef development relative to shell bag structures (Fig. S4). Alternatively, these results may indicate that taxa respond differently to restoration design features. Longer-term monitoring should be used in future studies to help understand these trends (i.e., Baggett et al. 2015). However, this also reinforces the need to carefully consider restoration goals (Baggett et al. 2015), as common performance indicators, like taxa richness, may not always be suitable for measuring "success."

In our model runs, percent cover of cultch was also found to be an important habitat metric, as it influenced snail and crustacean parasite richness as well as crustacean parasite community structure (Fig. 4, Table S2). This may explain why the control plots demonstrated positive changes in richness and shifts in community composition post-restoration (Fig. 3; Figs. S2-3). Pre-restoration, the controls were not completely devoid of habitat, as there were scattered and partially buried clumps of live oyster and cultch present. The controls had some of the largest adult oysters (Fig. 5), although these were few and far less dense compared to the OC and SB reefs (Fig. 5). Scattered shell would tend to concentrate motile organisms in the absence of

any other structure. However, the lack of smaller oysters in these plots would indicate that these areas are recruitment-limited due to the lack of suitable substrate.

Study Limitations and Future Work

Limitations to our study could come from several sources. First, as outlined in the *Methods*, we were unable to replicate natural reefs in our experimental design. The shoreline environment in the Rachel Carson Reserve is rapidly changing due to sea-level rise and wave energy from persistent southwest winds and boat wakes in the summer months. Further, the opening of Beaufort Inlet in 2016 resulted in two previously identified natural reefs (*see* Keller et al. 2019) being buried and/or eroded away prior to the onset of our project. Thus, we only collected data from a single natural reef, which we could not analyze using inferential statistics, although we present these data in a summary table (Table S3). Follow-up studies should include natural reefs within a similar spatial context to better understand how restoration methods compare to natural processes.

Second, while host and parasite richness were positively correlated in our system (Fig. S6), we were limited by a detailed understanding of the “supra community” (Bush et al. 1997) of hosts and their parasites (i.e., all developmental stages of parasites in all potential hosts in our system). This level of analysis is generally not feasible in most parasite ecology studies, which often work instead at the “component” level (i.e., all parasites at a specific life-stage in a single host) (Bush et al. 1997). By targeting key intermediate hosts like mudsnails, xanthid crabs, and abundant benthic fishes, we are assembling a picture of the larger community of hosts and parasites by focusing on its principal components. This provides insight into the “supra community” without needing to sample every taxonomic group in a system, particularly the

cryptic and elusive ones (Byers et al. 2011). Thus, even without a complete understanding of the host-parasite community, parasites can still serve as indicators of trophic complexity.

Finally, we recognize that the placement or size of habitat patches could be influencing other treatments in our study. In our experimental design, we followed the guidelines specified by Keller et al. (2019) for constructing reefs in low-energy "creek" environments. This work was in turn informed by earlier restoration studies (e.g., Grabowski et al. 2005; Fodrie et al. 2014) conducted in the same general area. Importantly, we made sure to account for the dispersal of host-taxa in our experimental design, and we limited our inference based on the movement ecology of these organisms (*see* Methods). This is meant to satisfy the criterion established by Knegt et al. (2010) for controlling for autocorrelation: namely, avoiding a mismatch between the scale of analysis and the scale of a species' response to its environment. Biodiversity is notoriously difficult to quantify because of issues with scale-dependence (*see* Chase et al. 2018). However, to control for these challenges, we purposefully accounted for the scale of species-environment relationships (i.e., movement patterns of hosts) in our sampling and analyses. Nevertheless, the lack of differences in community composition across treatments, which were located adjacent to one another, may be because taxa responded to an overall increase in structured habitat over time.

Our results add to a body of work spanning multiple habitats and geographic regions (e.g., Huspeni & Lafferty 2004; Morley & Lewis 2007; Henson et al. 2009; Anderson & Sukhdeo 2013), all of which demonstrate that parasites can be used as proxies for the presence of other host taxa in the broader community. Future studies should build upon these results by incorporating parasites into the design of a restoration project and monitoring for at least 2-3 years post-restoration – a period sufficient to account for multiple years of inter-annual variation

in monitoring data (Lindenmeyer & Likens 2018). In addition, sampling broadly for parasites and free-living taxa is labor-intensive, while snail-trematode communities have been argued as comparatively easy and can yield a wealth of valuable information on the greater community (Huspeni et al. 2005). From a practical standpoint, managers or trained volunteers could sample parasite communities for 1-2 years pre-restoration to obtain an understanding of which species are most abundant and what hosts they require (Esch & Fernandez 1994). Ideally, sampling would continue post-restoration for 2-3 years to monitor changes in the relative abundance of trematode species, as this would indicate that additional host taxa are recruiting to the system over time.

Conclusions

In sum, we found that host and parasite taxa rapidly recruited to structured habitat after restoration, although temporal and environmental factors were also important for explaining changes in the host-parasite community. In our study, the trajectory of the host-parasite response across multiple free-living and parasite taxa suggests that overall biodiversity had increased in response to habitat restoration, but that restoration design factored into community changes through time. Our work offers additional evidence of the important role of parasites in coastal communities. These diverse and fascinating organisms offer a tool for quantifying trophic complexity in restoration projects, particularly oyster reefs – perhaps making parasites the hidden links in healthy ecosystems.

Acknowledgments

Funding for this work was provided to Christopher Moore, in the lab of April Blakeslee, by a fellowship from North Carolina Sea Grant and the North Carolina Coastal Reserve (project #NA14OAR4170073); support and equipment additionally came from East Carolina University

(ECU) to April Blakeslee; a grant to Rachel Gittman and April Blakeslee from ECU's Natural Resources and the Environment Research Cluster; a grant to Rachel Gittman from the North Carolina Department of Environmental Quality (award # NA15NOS4190091); a grant to Rachel Gittman from the Southeastern Aquatic Resources Partnership (award #A19-0052-001); and a National Science Foundation Graduate Research Fellowship to Emory Wellman (award #213673-720101). We gratefully acknowledge the following individuals for their help with field work and sample processing: R. Barnard, T. Boyd, E. Edmonds, C. Erickson, M. Plafcan, S. Sutcliffe, K. Swanson, K. Walker, and C. Winkler. Special thanks to A. Rodrigues for help with figure preparation. We are grateful for the comments and suggestions provided by two anonymous reviewers and the handling editor, C. Layman.

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Figures

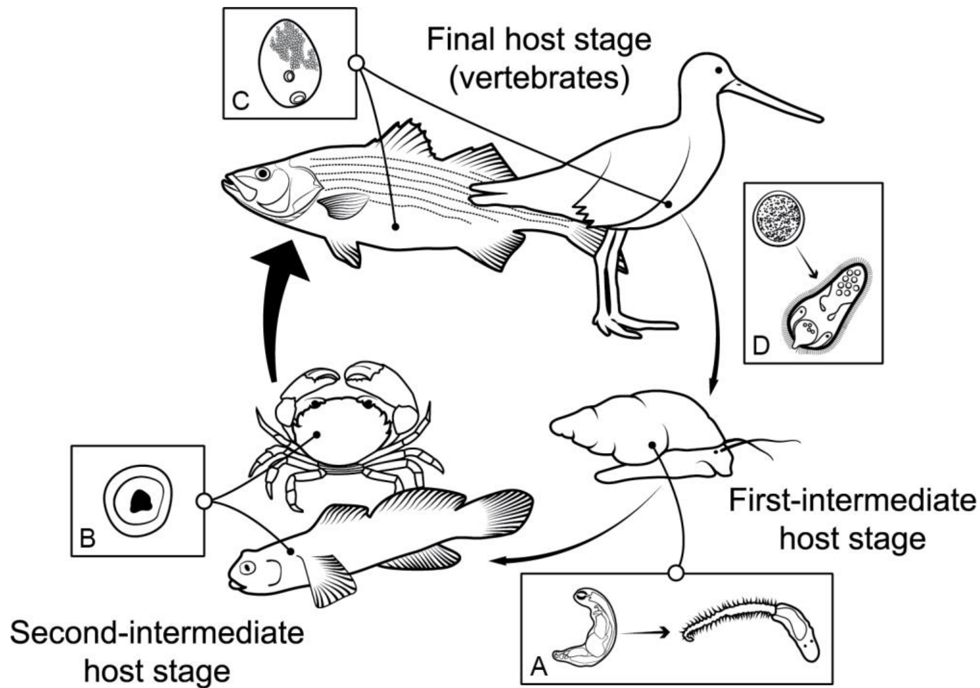


Fig. 1. Trematode lifecycle diagram. Trematodes are a common trophically transmitted parasite group that use multiple hosts, typically starting with (A) gastropods, where the trematode asexually reproduces, producing numerous free-swimming cercariae. (B) These cercariae are shed from the snail, where they may encyst as metacercariae in a second-intermediate host (e.g., crustaceans, mollusks, worms, fish). (C) The trematode's second host is ingested by a definitive vertebrate host, often a bird or fish, where sexual reproduction occurs. (D) The trematode's eggs are deposited into the marine environment with the feces of the final host. Grazing snails may ingest these eggs (A), and the cycle continues. Note: solid arrow from B-C denotes trophic transmission phase; other arrows (D-A, A-B) represent environmental transmission stages.

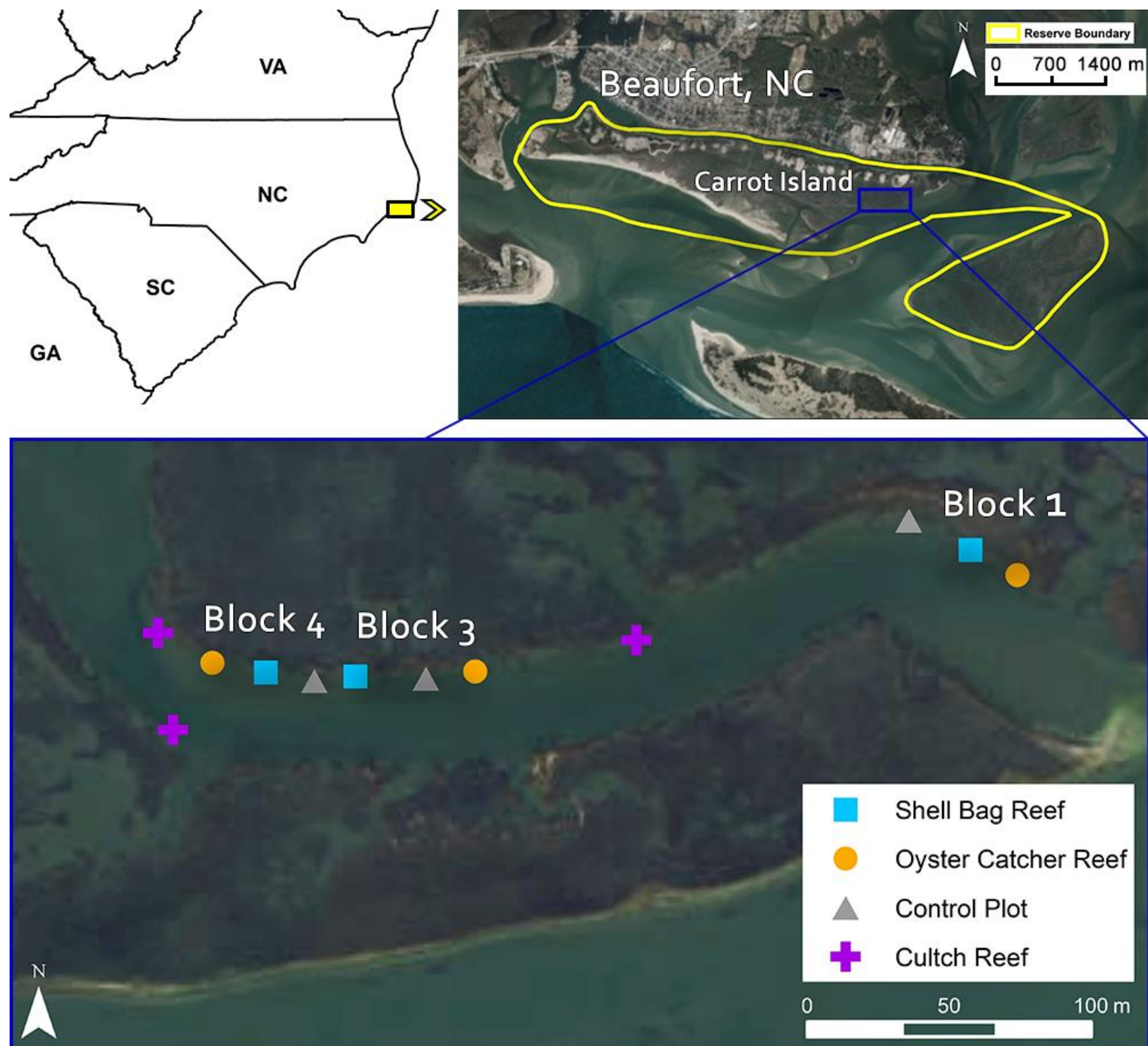


Fig. 2. The Rachel Carson National Estuarine Research Reserve, Beaufort, N.C., and sample area showing the experimental design within each of our 3 blocks. Reserve boundaries are outlined in yellow. The spatial arrangement of treatments within each block was broken down as follows (from creek mouth, right-to-left): Block 1 (CP, SB, OC); Block 3 (SB, CP, OC); and Block 4 (OC, SB, CP). Photo credit: N.C. Coastal Reserve and National Estuarine Research Reserve.

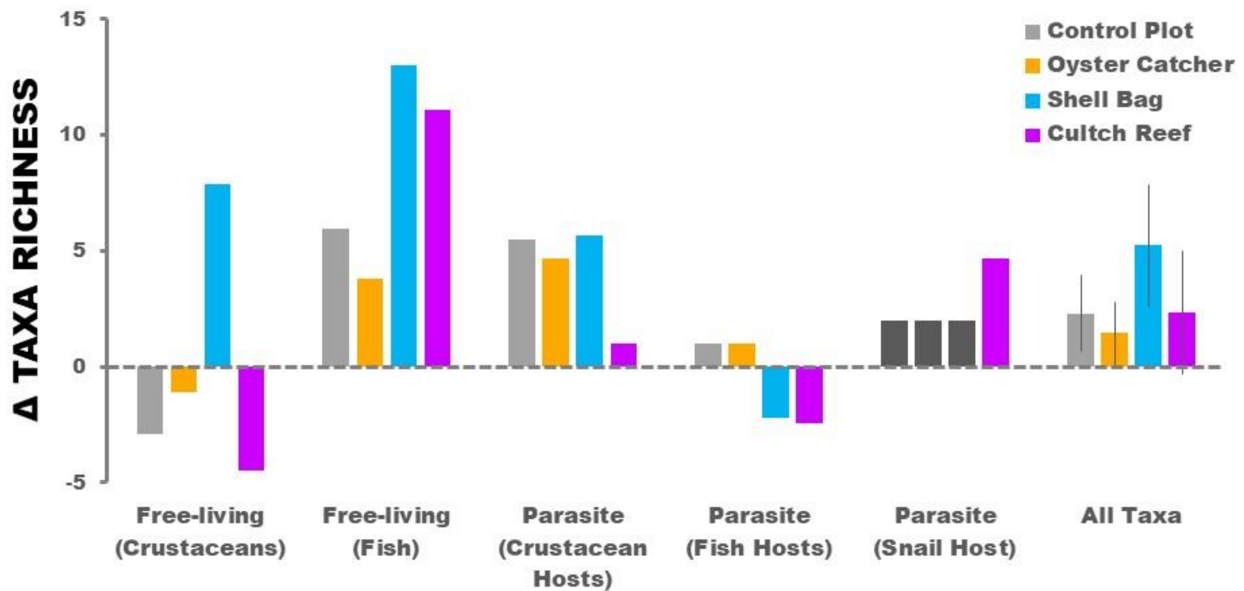
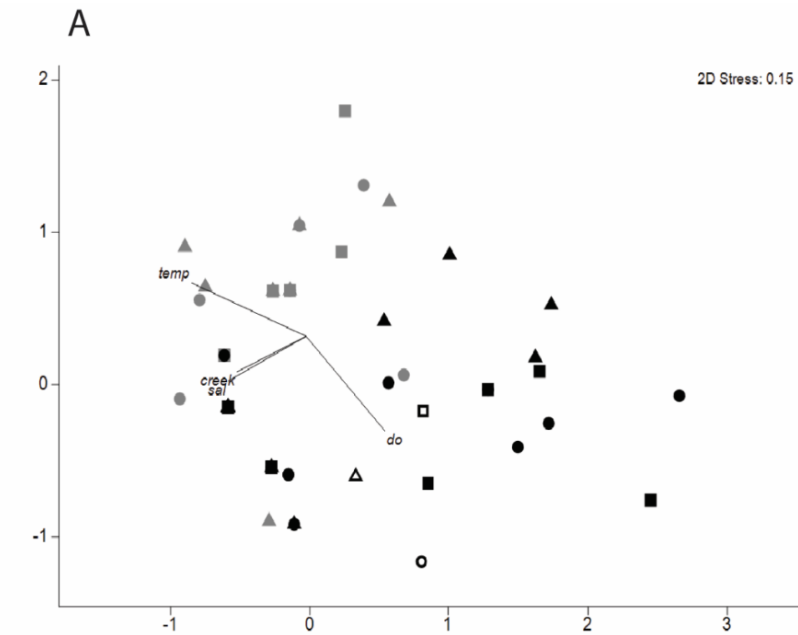
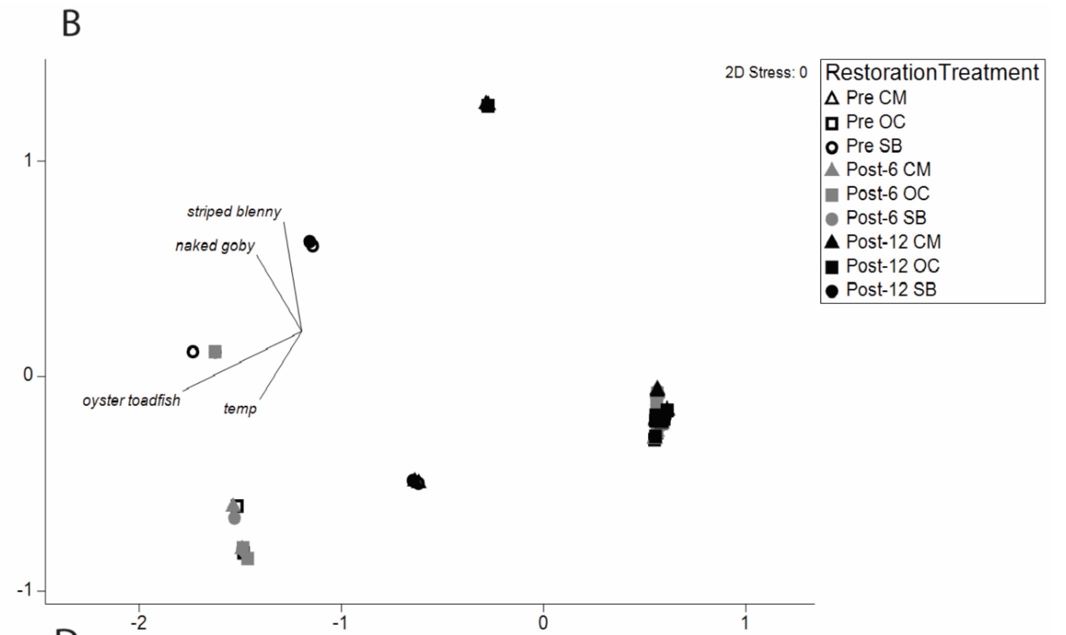
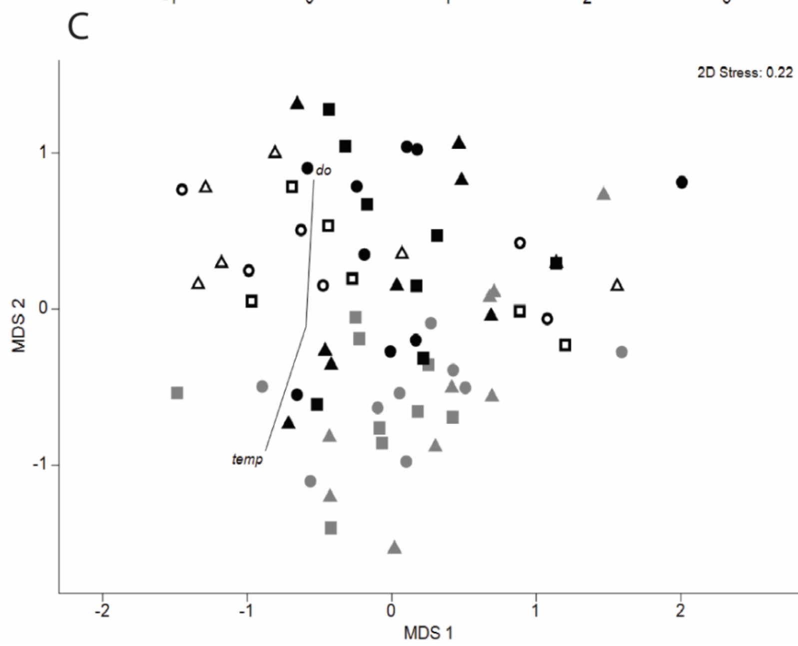


Fig. 3. The change in taxa richness from pre-restoration to post-restoration 1-year later. All bars above the x-axis represent a positive change in taxa richness for the specific free-living and parasite groups and the bars below the axis represent a negative change. The last figure represents a composite across all free-living and parasite taxa. Trematode parasites of the snail host, *Tritia obsoleta*, were analyzed at the block level rather than the treatment level, represented by the equivalent dark-gray bars for the three treatments (CP, OC, SB).


A
2D Stress: 0.15
temp
creek
sal
do


B
2D Stress: 0
RestorationTreatment
Pre CM
Pre OC
Pre SB
Post-6 CM
Post-6 OC
Post-6 SB
Post-12 CM
Post-12 OC
Post-12 SB
striped blenny
naked goby
oyster toadfish
temp


C
2D Stress: 0.22
do
temp
MDS 2
MDS 1

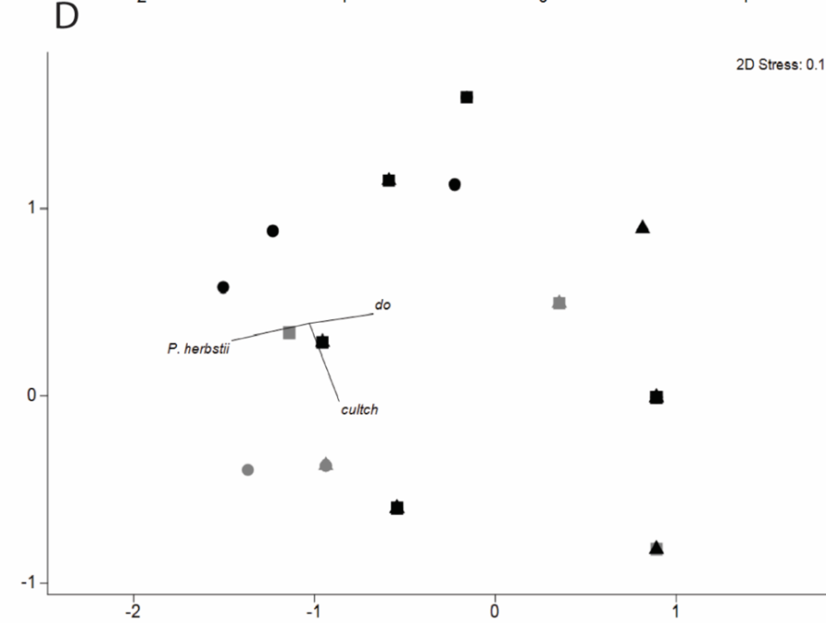

D
2D Stress: 0.1
do
P. herbstii
cultch

Fig. 4. Non-metric multidimensional scaling plots of (clockwise): A) free-living fish, B) parasites of fish hosts, C) free-living crustaceans, and D) parasites of crustacean hosts. Taxa were sampled pre-restoration (open symbols), 6 months post-restoration (gray symbols), and 12 months post-restoration (black symbols) from CP (triangles), OC (squares), and SB (circle) treatments. Overlaid factors represent environmental parameters (temperature, dissolved oxygen [DO], salinity, percent cover of cultch [cultch cover] and distance from the creek mouth [dist. creek]). In the case of parasite NMDS plots, biotic factors (raw abundance of fish: *O. tao*, *G. bosc, C. bosquianus*; raw abundance of crustaceans: *P. herbstii*) with Pearson correlation coefficients >0.3 with MDS1 (x-axis) or MDS2 (y-axis) are overlaid onto the nMDS plots (see Tables S1-2, S4-5).

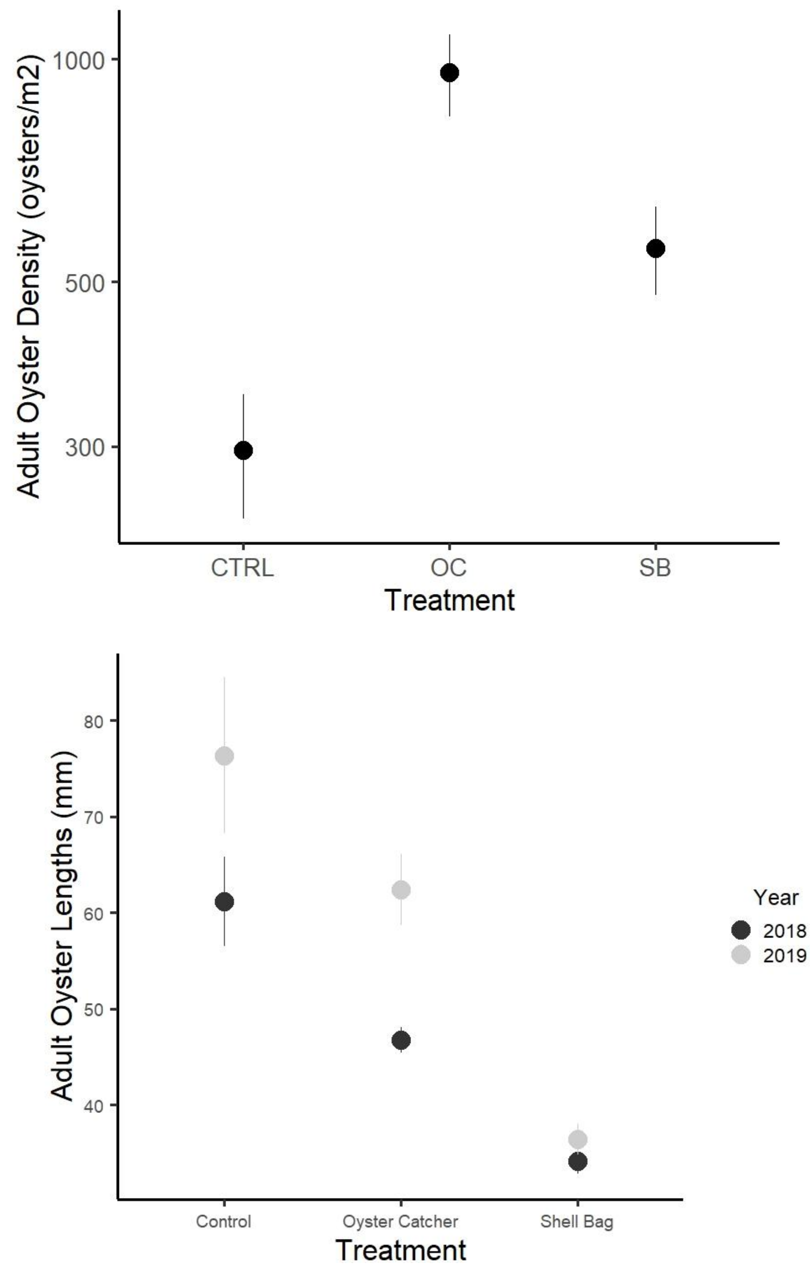


Fig. 5. Mean adult oyster density (+/- 1 SE) by treatment in 2018 and 2019, and mean adult oyster length (+/- 1 SE) by treatment in 2018 and 2019 (see Tables 1a-1b).

Tables

Table 1a. Analysis of Deviance table for adult oyster density model. ***=0.001, **=0.01

	Sum of Squares	df	F-value	*p*-value
Intercept	103.352	1	456.3780	1.651e-11***
Treatment	4.826	2	10.6558	**0.001821****
Block	0.367	2	0.8114	0.465517
Residuals	2.944	13		

Table 1b. Analysis of Deviance table for adult oyster length model. ***=0.001

	Chi Square	**df**	***p*-value**
Intercept	3932.586	1	**<2.2e-16*** **
Treatment	127.469	2	**<2.2e-16*** **
Year	14.515	1	**0.0001391*** **

Supplementary Figures

Fig. S1. Treatments in each block design

Pre-restoration (top) shoreline consisted mostly of mudflat and areas with scattered cultch and live oyster. Experimental treatment blocks consisted of (from left-to-right) no-restoration controls, shell bags, and Oyster Catcher™ structures (center = post-6 months restoration [December, 2018]; bottom = post-12 months [September, 2019]). The components of an Oyster Catcher™ (center-right; bottom-right) are initially tied together with bailing wire. Over time, the wire corrodes and the structure becomes self-reinforcing following growth and subsequent recruitment of juvenile oysters. Photo credits: Rachel Gittman.

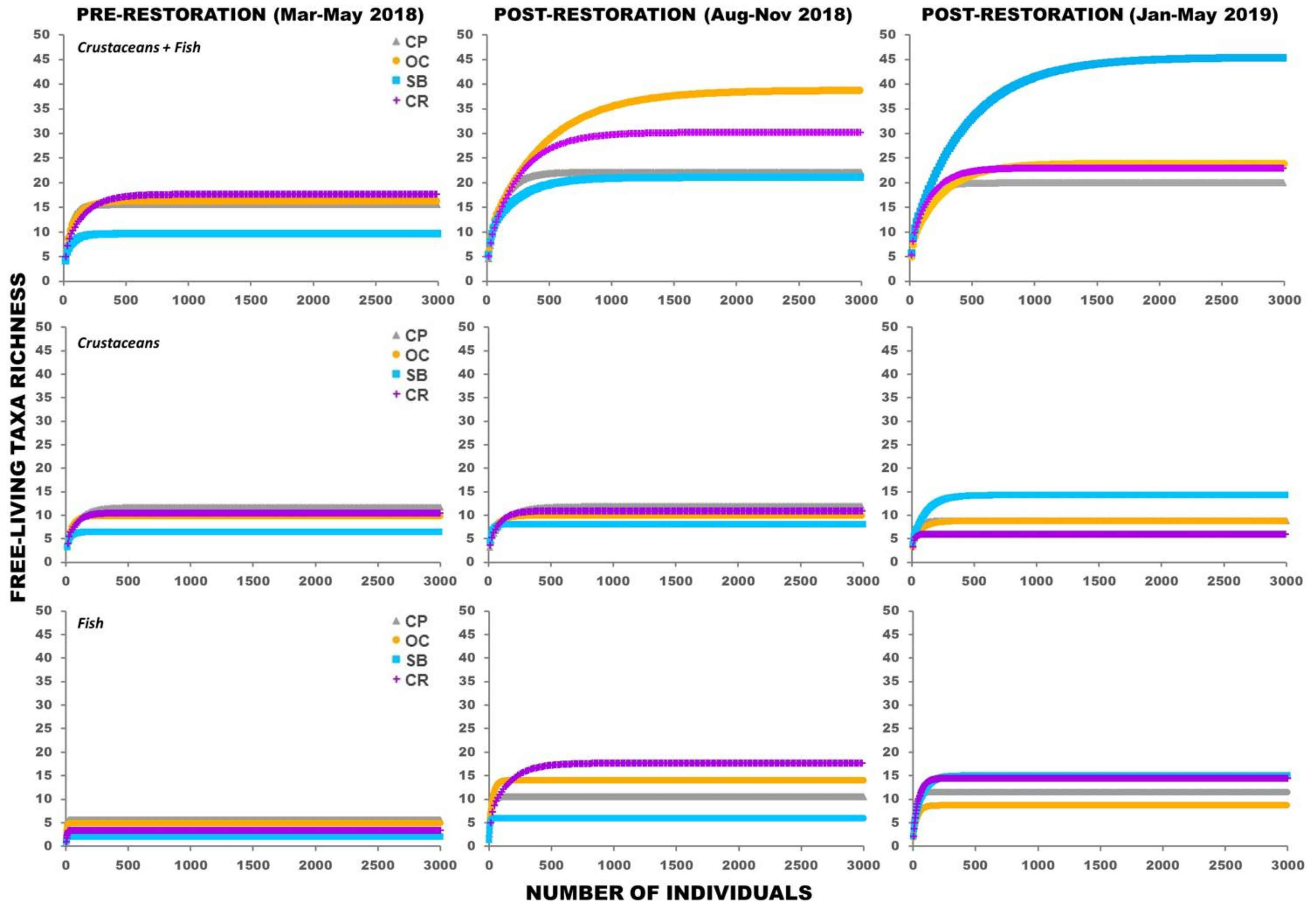


Fig. S2. Extrapolation curves of free-living taxa richness scaled by the number of individuals for different restoration periods (pre-restoration, post-restoration (3-6 months), and post-restoration (9-12 months). All free-living crustaceans and fish were examined in the top row, followed by crustaceans only, and fish only.

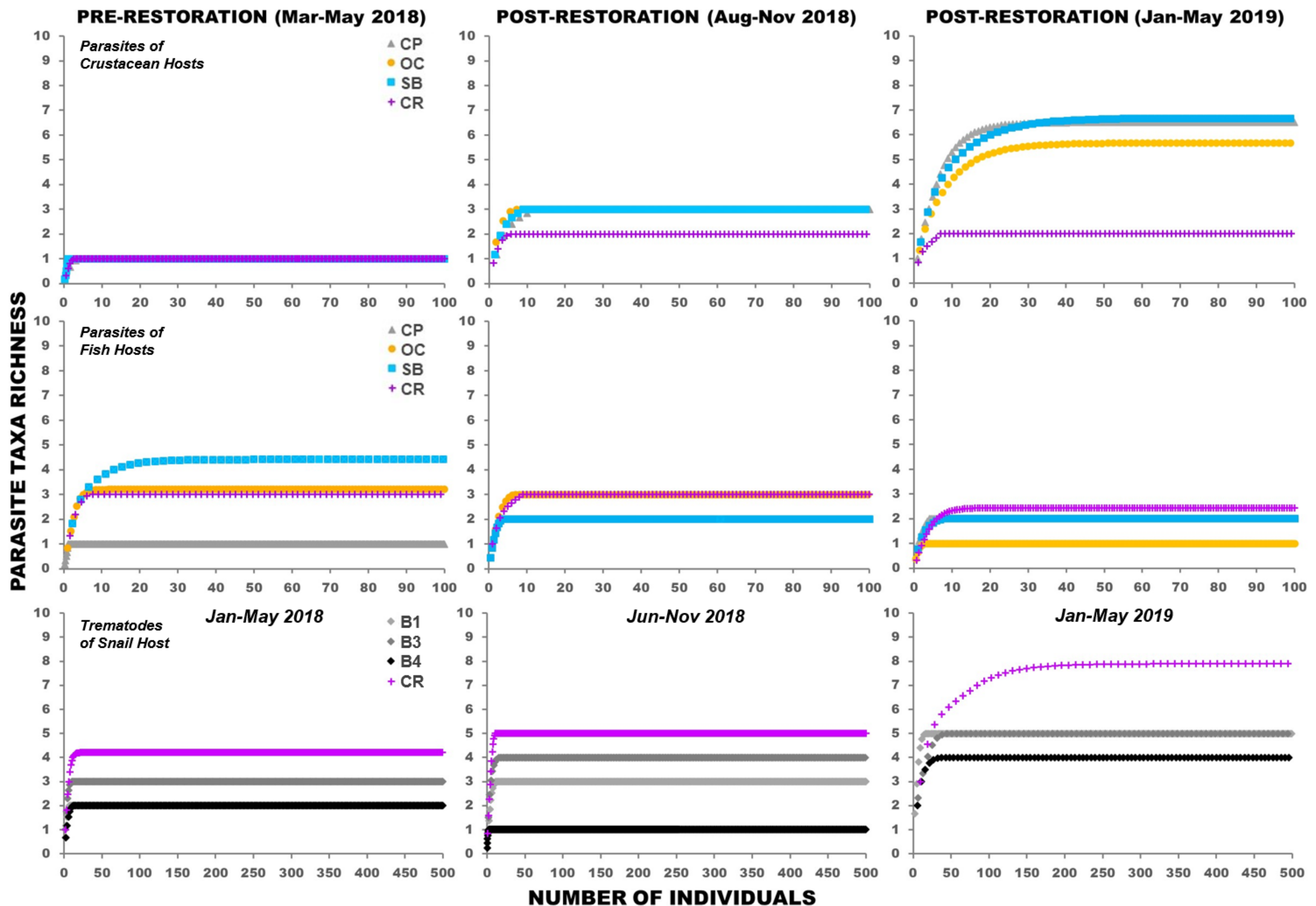


Fig. S3. Extrapolation curves of parasite taxa richness scaled by the number of individuals for the three restoration periods. The top row represents parasites of crustacean parasites; the middle row is parasites of fish hosts; and the bottom row is trematodes of the snail host, *Tritia obsoleta*. The latter parasite group was analyzed by block level; the upper two were analyzed at the treatment level.

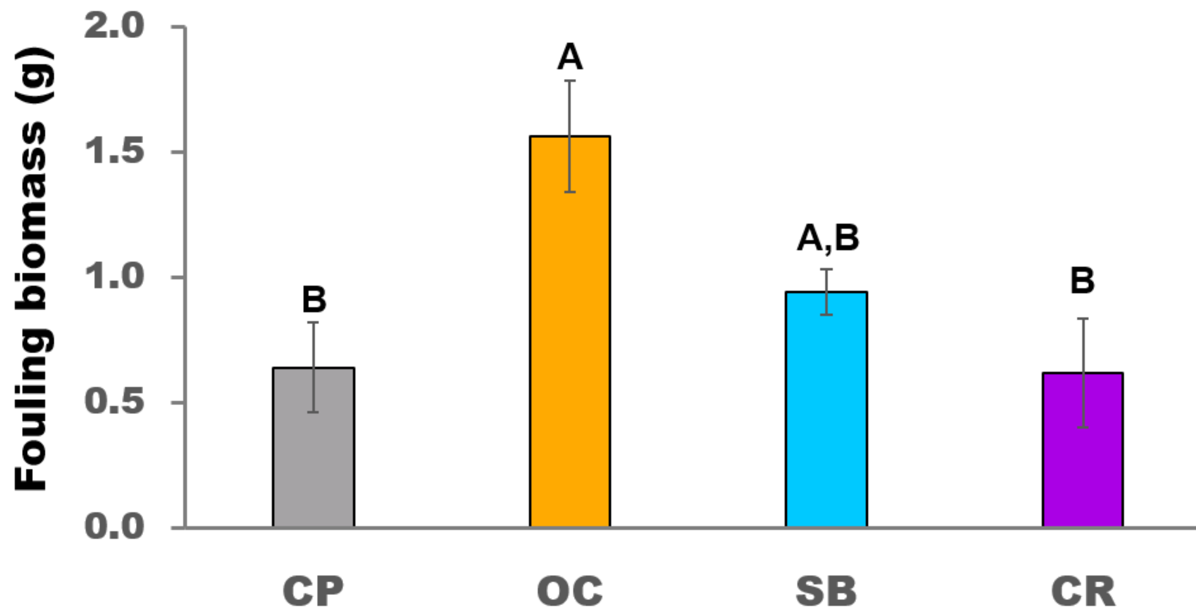


Fig. S4. Biomass in grams (g) of sessile, fouling organisms following six-months in the field at each of our sites. The x-axis represents the restoration treatments (CP=control plot; OC=Oyster Catcher; SB=shell bag) and the previously-restored cultch reef (CR). Letters above the bars represent significant differences ($p < 0.05$) among treatments in a post-hoc Tukey's test.

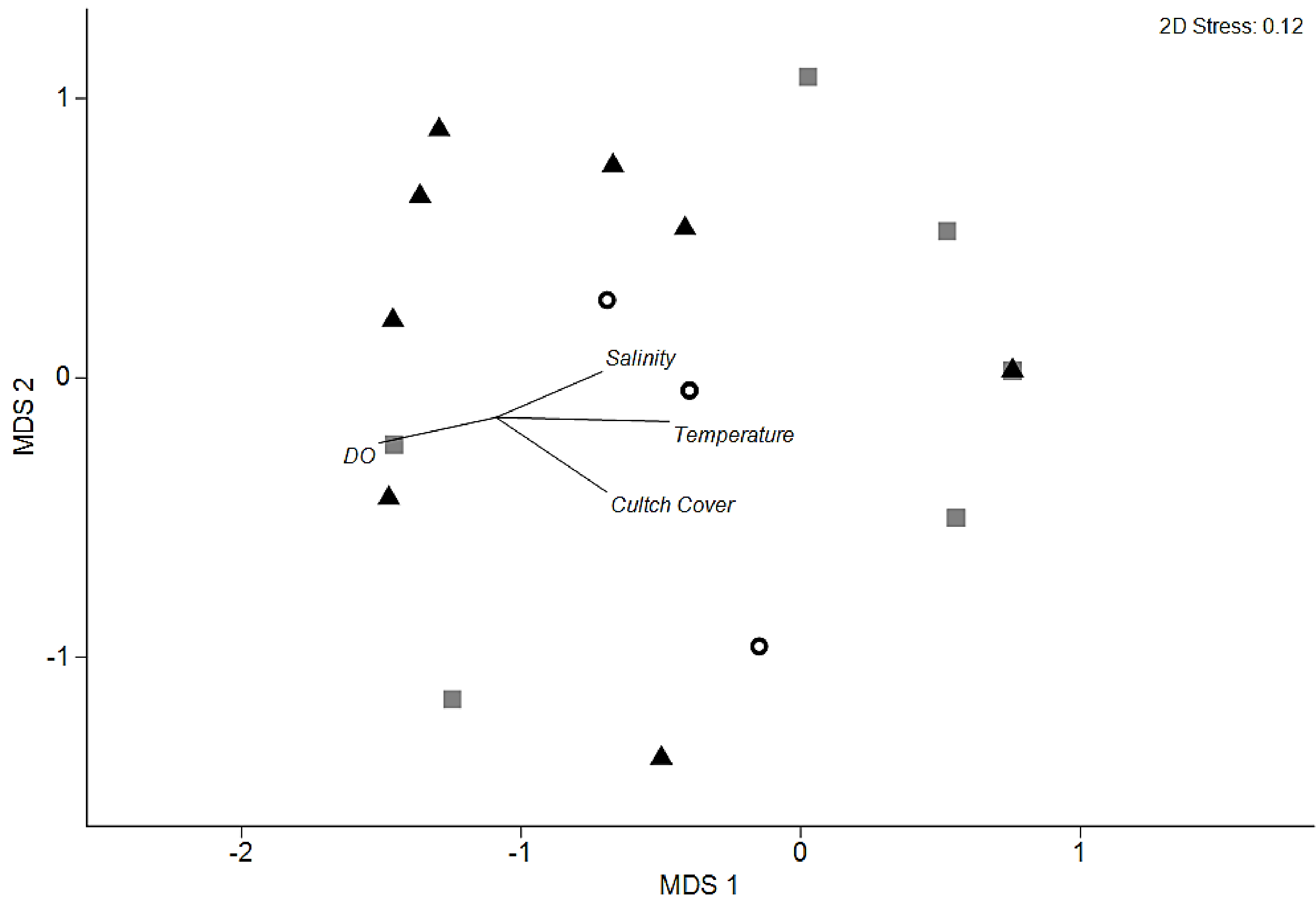


Fig. S5. Non-metric multidimensional scaling plots of snail trematode parasites sampled pre-restoration (open circle), 6 months post-restoration (gray square), and 12 months post-restoration (black triangle). Overlaid factors represent environmental parameters (temperature, dissolved oxygen [DO], salinity, and percent cover of cultch [cultch cover]) with Pearson correlation coefficients >0.3 with MDS1 (x-axis) or MDS2 (y-axis) (Table S2).

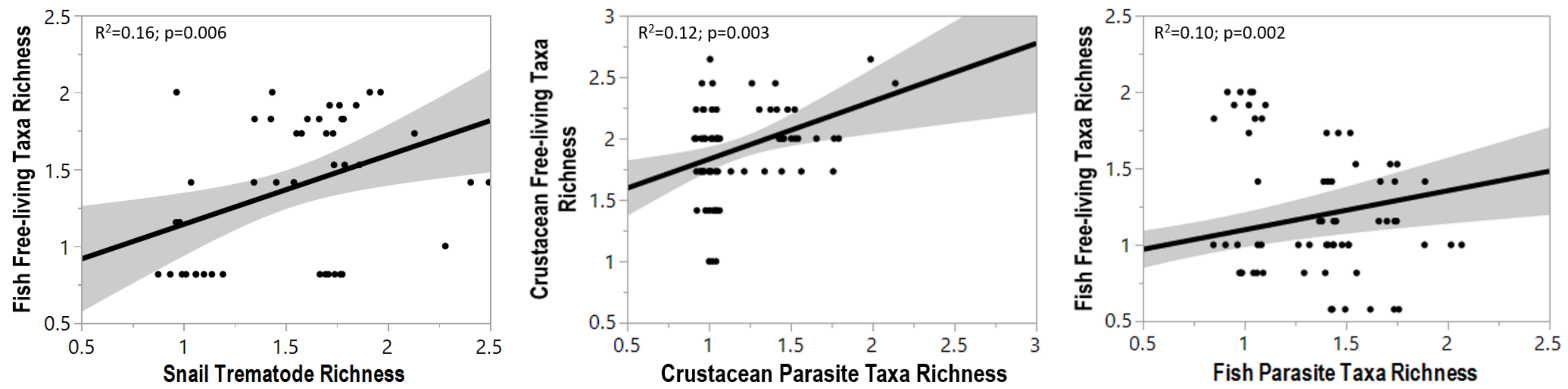


Fig. S6. Regressions of parasite richness with free-living richness in the three host-parasite groups (snail trematode, crustacean parasite, fish parasite). All three regressions demonstrate weakly positive significant correlations between parasite and free-living richness.

Supplemental Tables

Table S1. Results from two-factor, time (pre, post-6, and post-12) and restoration treatment (OC, SB, CP), PERMANOVA on Bray-Curtis similarity matrices for free-living fish abundance, fish parasite presence/absence, free-living crustacean abundance, and crustacean parasite presence/absence, and one-factor (restoration timing) PERMANOVA on Bray-Curtis similarity matrices for snail parasite presence/absence. P-values in **bold** are <0.05.

Community	Source	df	SS	MS	Pseudo-F	P (perm)	Unique permutations
Free-living fish	Time	2	16343	8171.6	5.8255	**0.001**	998
	Restoration Treatment	2	1541.8	770.9	0.54958	0.847	998
	Time × Treatment	4	4296.8	1074.2	0.7658	0.729	997
	Residuals	62	86969	1402.7			
	Total	70	1.0899E+05				
Parasites of fish hosts	Time	2	3503.5	1751.7	3.4031	**0.016**	998
	Restoration Treatment	2	1808.8	904.4	1.757	0.173	999
	Time × Treatment	4	2441	610.24	1.1855	0.319	999
	Residuals	62	31914	514.74			
	Total	70	39177				
Free-living crustaceans	Time	2	21922	10961	6.7914	**0.001**	997
	Restoration Treatment	2	1865.1	932.56	0.57782	0.843	999
	Time × Treatment	4	4674.9	1168.7	0.72416	0.813	999
	Residuals	62	1.0006E+05	1613.9			
	Total	70	1.2831E+05				
Parasites of crustacean hosts	Time	2	6679.8	3339.9	4.653	**0.003**	999
	Restoration Treatment	2	311.6	155.8	0.21705	0.889	999
	Time × Treatment	4	1263.7	315.93	0.44014	0.865	999
	Residuals	62	44503	717.79			
	Total	70	52656				
Snail parasites	Time	2	4670.4	2335.2	2.5714	**0.022**	999
	Residuals	27	24519	908.13			
	Total	29	29190				

Table S2. Pearson correlation coefficients for environmental variables associated with free-living fish and crustacean communities. Correlations are shown for each nMDS axis and coefficients with an absolute value of 0.3 or higher are shown on the nMDS plots (Fig. 4A and 4C).

Community	nMDS Axis	Distance from Creek Mouth	Salinity	Water Temperature	Dissolved Oxygen	Marsh Edge Elevation	Oyster Density	Cultch Percent Cover
Free-Living Fish	MDS1	**-0.35**	**-0.39**	**-0.58**	**0.40**	-0.27	0.15	-0.17
	MDS2	-0.16	-0.21	0.25	**-0.44**	0.11	0.11	0.21
Free-Living Crustaceans	MDS1	-0.06	-0.24	-0.21	0.04	0.12	0.02	0.06
	MDS2	-0.08	-0.24	**-0.58**	**0.69**	-0.22	-0.20	-0.09
Parasites of Fish Hosts	MDS1	0.16	-0.17	-0.20	0.18	0.02	0.02	-0.16
	MDS2	-0.05	-0.15	**-0.31**	0.18	-0.23	0.22	-0.21
Parasites of Crustacean Hosts	MDS1	-0.04	-0.25	-0.25	**0.30**	0.02	-0.26	0.14
	MDS2	-0.01	-0.03	-0.15	0.04	-0.10	0.23	**-0.36**
Trematode Parasites of T. obsoleta	MDS 1	-0.07	**0.32**	**0.53**	**-0.36**	0.18	-0.05	**0.34**
	MDS 2	0.22	0.14	-0.01	-0.08	-0.28	0.14	-0.23

Table S3. Mean abundances (and SE, n/treatment) of site-resident (R) and transient (T) fauna across treatments.

		Treatment					
Common Name	*Scientific Name*	*Natural Oyster Reef**	*Control Marsh*	*Oyster Catcher*	*Shell Bag*	*Cultch Reef*	*Mean***
Atlantic mud crab (R)	*Panopeus herbstii*	3.75 (0.49)	3.04 (0.49)	3.30 (0.49)	2.96 (0.48)	2.38 (0.48)	2.92
Grass shrimp (T)	*Palaemonetes pugio*	4.5 (2.44)	3.13 (1.25)	2.09 (0.51)	1.75 (0.46)	2.08 (0.64)	2.26
Snapping shrimp (R)	*Alpheus* spp.	1.38 (0.38)	2.17 (0.39)	1.91 (0.32)	1.83 (0.44)	1.67 (0.35)	1.90
Say's mud crab (R)	*Dyspanopeus sayii*	0.25 (0.25)	1.88 (0.51)	1.43 (0.48)	1.92 (0.50)	1.33 (0.50)	1.64
Unknown mud crabs (R)	Panopeidae (juv. < 5 mm)	1.38 (0.56)	0.83 (0.42)	1.22 (0.47)	0.75 (0.32)	1.46 (0.61)	1.07
Striped blenny (R)	*Chasmodes bosquianus*	1 (0.42)	0.54 (0.28)	1.04 (0.62)	1.04 (0.49)	1.08 (0.43)	0.93
White-fingered mud crab (R)	*Rhithropanopeus harrissi*	0.63 (0.26)	0.83 (0.28)	0.65 (0.20)	0.83 (0.22)	0.88 (0.23)	0.80
Peppermint shrimp (R)	*Lysmata* spp.	0.13 (0.13)	0.25 (0.11)	0.65 (0.40)	0.63 (0.32)	1.21 (0.57)	0.69
Naked goby (R)	*Gobiosoma bosc*	0.63 (0.33)	0.29 (0.12)	0.30 (0.10)	0.54 (0.20)	0.67 (0.21)	0.45
Feather blenny (R)	*Hypsoblennius hentz*	0.25 (0.25)	0.33 (0.11)	0.22 (0.11)	0.25 (0.12)	0.21 (0.13)	0.25
Oyster toadfish (R)	*Opsanus tau*	0.13 (0.13)	0.17 (0.10)	0.22 (0.09)	0.29 (0.11)	0.29 (0.09)	0.24
Sheepshead (T)	*Archosargus probatocephalus*	0.13 (0.13)	0.17 (0.10)	0.22 (0.12)	0.13 (0.07)	0.08 (0.06)	0.15
Pinfish (T)	*Lagodon rhomboides*	0	0.13 (0.07)	0.13 (0.07)	0.04 (0.04)	0.25 (0.18)	0.14
Flatback mud crab (R)	*Eurypanopeus depressus*	0.5 (0.27)	0.08 (0.06)	0.09 (0.06)	0.08 (0.06)	0.17 (0.10)	0.11

Grey snapper (T)	*Lutjanus griseus*	0	0	0.09 (0.09)	0.13 (0.07)	0.04 (0.04)	0.06
Stonecrab (R)	*Menippe mercenaria*	0.38 (0.18)	0.04 (0.04)	0.04 (0.04)	0.08 (0.06)	0.08 (0.06)	0.06
Blue crab (T)	*Callinectes sapidus*	0.13 (0.13)	0.04 (0.04)	0.04 (0.04)	0.04 (0.04)	0.04 (0.04)	0.04
Darter goby (R)	*Ctenogobius boleosoma*	0	0	0.04 (0.04)	0.08 (0.08)	0.04 (0.04)	0.04
Lane snapper (T)	*Lutjanis synagris*	0.13 (0.13)	0.08 (0.08)	0.04 (0.04)	0	0	0.03
Spider crab (T)	*Libinia emarginata*	0	0.04 (0.04)	0	0.04 (0.04)	0	0.02
Mantis shrimp (T)	*Squilla empusa*	0	0.04 (0.04)	0.04 (0.04)	0	0	0.02
Mummichog (T)	*Fundulus heteroclitus*	0.13 (0.13)	0	0	0.04 (0.04)	0.04 (0.04)	0.02
Skilletfish (R)	*Gobiesox strumosus*	0	0	0.04 (0.04)	0.04 (0.04)	0	0.02
Rock seabass (T)	*Centropristis philadelphica*	0	0.04 (0.04)	0	0	0.04 (0.04)	0.02
Belted sandfish (T)	*Serranus subligarius*	0	0	0	0	0.04 (0.04)	0.01
Slippery dick (T)	*Halichoeres bivittatus*	0	0.04 (0.04)	0	0	0	0.01
Spotted hake (T)	*Urophycis regia*	0	0.04 (0.04)	0	0	0	0.01
Red grouper (T)	*Epinephelus morio*	0	0	0	0	0.04 (0.04)	0.01

* denotes data with only 1 treatment replicate
** does not include Natural Oyster Reef

Table S4. Pearson correlation coefficients for free-living fish species sampled in conjunction with fish parasite communities. Correlations are shown for each nMDS axis and coefficients with an absolute value of 0.3 or higher are shown on the nMDS plots (Fig. 4A and 4C).

		MDS1	MDS2
Naked goby	*Gobiosoma bosc*	-0.22	**0.35**
Darter goby	*Ctenogobius boleosoma*	0.12	-0.05
Striped blenny	*Chasmodes bosquianus*	-0.09	**0.5**
Feather blenny	*Hypsoblennius hentz*	0.01	0.2
Oyster toadfish	*Opsanus tau*	**-0.58**	-0.27
Skilletfish	*Gobiesox strumosus*	0.03	0.14
Sheepshead	*Archosargus probatocephalus*	0	0.23
Mummichog	*Fundulus heteroclitus*	0.09	-0.03
Pinfish	*Lagodon rhomboides*	-0.11	0.04
Rock seabass	*Centropristis philadelphica*	0.09	-0.03
Gray snapper	*Lutjanus griseus*	0.16	-0.06
Lane snapper	*Lutjanis synagris*	0.11	-0.03
Belted sandfish	*Serranus subligarius*	0	0
Slippery dick	*Halichoeres bivittatus*	-0.04	0.25
Red grouper	*Epinephelus morio*	0	0
Spotted hake	*Urophycis regia*	-0.09	-0.1

Table S5. Pearson correlation coefficients for free-living crustacean species sampled in conjunction with crustacean parasite communities. Correlations are shown for each nMDS axis and coefficients with an absolute value of 0.3 or higher are shown on the nMDS plots (Fig. 4A and 4C).

		MDS1	MDS2
Atlantic mud crab	*Panopeus herbstii*	-0.37	-0.08
Flatback mud crab	*Eurypanopeus depressus*	0.1	-0.21
White-fingered mud crab	*Rhithropanopeus harrissi*	-0.17	0.07
Say's mud crab	*Dyspanopeus sayii*	-0.23	0.02
Unknown mud crabs (≤ 5.09 mm)		-0.11	-0.12
Blue crab	*Callinectes sapidus*	0.23	0
Stone crab	*Menippe mercenaria*	0.01	-0.11
Spider crab	*Libinia emarginata*	-0.26	0.03
Grass shrimp	*Palaemonetes pugio*	0.1	-0.16
Snapping shrimp	*Alpheus* spp.	-0.11	0.17
Peppermint shrimp	*Lysmata* spp.	-0.13	0.02
Mantis shrimp	*Squilla empusa*	0	-0.05

Table S6. Mean abundances (SE, n/month) of site-resident (R) and transient (T) fauna across sampling events.

Common Name	Scientific Name	*Sampling Months*							
		March, 2018	*May, 2018*	*August, 2018*	*September, 2018*	*November, 2018*	*January, 2019*	*March, 2019*	*May, 2019*
Atlantic mud crab (R)	*Panopeus herbstii*	1.85 (0.34)	2.31 (0.43)	3.00 (0.62)	5.92 (0.87)	3.69 (0.35)	2.00 (0.44)	1.23 (0.44)	3.92 (0.45)
White-fingered mud crab (R)	*Rhithropanopeus harrissi*	1.15 (0.3)	0.92 (0.30)	0.31 (0.13)	0.69 (0.25)	0.38 (0.23)	0.62 (0.30)	1.38 (0.44)	0.83 (0.33)
Flatback mud crab (R)	*Eurypanopeus depressus*	0.23 (0.12)	0.08 (0.08)	0	0.15 (0.15)	0.08 (0.08)	0.08 (0.08)	0.15 (0.1)	0.33 (0.18)
Say's mud crab (R)	*Dyspanopeus sayii*	0.08 (0.08)	4.15 (0.64)	0.69 (0.41)	0.92 (0.48)	0.77 (0.41)	0.69 (0.25)	0.77 (0.25)	4.42 (0.98)
Unknown mud crabs (R)	*Panopeidae*	0.46 (0.26)	0.15 (0.15)	4.23 (0.92)	3.54 (0.69)	0.23 (0.12)	0	0	0
Stone crab (R)	*Menippe mercenaria*	0.08 (0.08)	0	0.15 (0.1)	0.15 (0.1)	0.08 (0.08)	0.08 (0.08)	0	0.17 (0.1)
Snapping shrimp (R)	*Alpheus spp.*	0.69 (0.32)	1.31 (0.33)	1.38 (0.30)	1.54 (0.28)	3.54 (0.53)	3.15 (0.74)	1.69 (0.44)	1.5 (0.30)
Peppermint shrimp (R)	*Lysmata spp.*	0	0	1.31 (0.44)	3.38 (1.04)	0.23 (0.16)	0.08 (0.08)	0.08 (0.08)	0
Naked goby (R)	*Gobiosoma bosc*	1.23 (0.29)	0.62 (0.32)	0.23 (0.12)	0.15 (0.1)	0.08 (0.08)	0.92 (0.28)	0.23 (0.12)	0.25 (0.13)
Striped blenny (R)	*Chasmodes bosquianus*	0.85 (0.36)	0.08 (0.08)	0	0	0.23 (0.16)	5.08 (1.07)	1.00 (0.34)	0.17 (0.11)
Oyster toadfish (R)	*Opsanus tau*	0	0.08 (0.08)	0.46 (0.18)	0.46 (0.14)	0.15 (0.1)	0.15 (0.1)	0.15 (0.1)	0.42 (0.18)
Skilletfish (R)	*Gobiesox strumosus*	0	0	0	0	0.08 (0.08)	0.08 (0.08)	0	0
Darter goby (R)	*Ctenogobius boleosoma*	0	0	0	0	0	0.31 (0.17)	0	0
Feather blenny (R)	*Hypsoblennius hentz*	0	0	0.31 (0.20)	0.54 (0.18)	0.62 (0.28)	0.38 (0.13)	0.08 (0.07)	0.08 (0.08)

Blue crab (T)	*Callinectes sapidus*	0.15 (0.1)	0.08 (0.08)	0	0	0.08 (0.08)	0.08 (0.08)	0	0
Spidercrab (T)	*Libinia emarginata*	0	0	0	0.08 (0.07)	0	0	0	0.08 (0.08)
Grass shrimp (T)	*Palaemonetes pugio*	4.69 (1.46)	8.46 (1.69)	0.54 (0.23)	0.77 (0.35)	0.15 (0.1)	3 (0.62)	1.54 (0.70)	0.17 (0.16)
Mantis shrimp (T)	*Squilla empusa*	0	0	0	0.15 (0.1)	0	0	0	0
Mummichog (T)	*Fundulus heteroclitus*	0	0	0	0	0	0.23 (0.12)	0	0
Sheepshead (T)	*Archosargus probatocephalus*	0	0	0	0	0.23 (0.12)	0.54 (0.21)	0.38 (0.17)	0
Rock seabass (T)	*Centropristis philadelphica*	0	0.08 (0.07)	0	0.08 (0.07)	0	0	0	0
Pinfish (T)	*Lagodon rhomboides*	0	0	0.62 (0.30)	0	0.08 (0.08)	0.15 (0.1)	0.15 (0.15)	0
Grey snapper (T)	*Lutjanus griseus*	0	0	0	0	0.46 (0.18)	0	0	0
Lane snapper (T)	*Lutjanis synagris*	0	0	0	0	0.31 (0.17)	0	0	0
Belted sandfish (T)	*Serranus subligarius*	0	0	0	0	0	0.08 (0.08)	0	0
Slippery dick (T)	*Halichoeres bivittatus*	0	0	0	0	0	0.08 (0.07)	0	0
Spotted hake (T)	*Urophycis regia*	0	0	0	0	0	0	0.08 (0.07)	0
Red grouper (T)	*Epinephelus morio*	0	0	0.08 (0.07)	0	0	0	0	0

Table S7. Trematode species prevalence in *Tritia obsoleta* (N = 12,420). Trematode species are listed in order of decreasing prevalence. Immature infections are counted as part of the total infection prevalence (3.0%). Overall, our system is fish dominated, as trematodes using fish as final hosts accounted for 85% of trematode species present. Bird-using species (14%) were less commonly encountered, as was a single species of trematode (PM) that uses the diamondback terrapin as a final host (< 1%).

Trematode Species	# Snails Infected	Prevalence	Final host
Lepocreadium setiferoides (LS)	146	0.012	Fish
Zoogonus lasius (ZL)	50	0.004	Fish
Stephanostimum tenue (ST)	48	0.004	Fish
Himasthla quissitensis (HQ)	24	0.002	Shorebirds
Stephanostomum dentatum (SD)	11	0.001	Fish
Diplostomum nassa (DN)	9	0.001	Shorebirds
Austrobilharzia variglandis (AV)	8	0.001	Shorebirds
Pleurogonus malaclemys (PM)	2	0.0001	Diamondback terrapin
Gynaecotyla adunca (GA)	1	0.00008	Shorebirds
Immature infections (not identifiable to species)	72	0.006	---
Grand total: trematode parasites (includes immature)	371	0.030	
Fish-using Trematodes (LS, ZL, ST, SD)	**255**	**0.853**	
Bird-using Trematodes (HQ, DN, AV, GA)	**42**	**0.140**	

Chapter 4: Parasites, not free-living taxa, indicate trophic complexity and faunal succession in restored oyster reefs

Abstract

Successional states and other dynamic processes within ecosystems are among the most intensely discussed topics in ecology. Recently, ecological restoration has emerged as a tool for testing questions related to community assembly, as practitioners often seek to restore whole ecosystems using foundation species. For example, the eastern oyster (*Crassostrea virginica*) is capable of creating complex habitat for organisms across multiple trophic levels. Historic declines in oyster abundance have prompted decades of restoration efforts. However, we still do not understand how long it takes for restored reefs to resemble the trophic complexity of natural reefs. We examined community succession in natural oyster reefs and restored reefs ranging from 5 to 22 years old in coastal North Carolina by surveying both free-living taxa and parasite communities. Trophically transmitted parasites can serve as valuable biodiversity surrogates, sometimes providing greater information about a system or question than their free-living counterparts. We found that the diversity of free-living taxa was highly variable and did not differ among New-Restored (<10 years), Old-Restored (>20 years), and Natural reefs. Conversely, parasite diversity increased with elapsed time post-restoration, and parasite communities in older restored reefs were more similar to those found in natural reefs. Our study also revealed that oyster toadfish (*Opsanus tau*) act as a key host species capable of facilitating parasite transmission and trophic ascent in oyster reefs food webs, which we illustrate by mapping parasite diversity in toadfish across all reefs in our system. Overall, our results suggest that trophic complexity in restored oyster reefs requires at least 10 years to resemble that found

in natural reefs. This work adds to a growing body of evidence demonstrating how parasites can serve as conservation tools and cross-taxon surrogates of biodiversity.

Introduction

Studies on the processes that mediate community succession following natural or anthropogenic disturbances are foundational to ecology (Paine 1966; Odom 1969). Such studies have generally attempted to understand long-term mechanisms using short-term investigations or experiments (Dierssen 2000; but see Dale et al. 2005). Instead, it can take decades to obtain a holistic knowledge of the drivers of ecosystem function (Dierssen 2000). In the past, hurricanes and volcanic eruptions have provided natural "laboratories" for investigating the long-term response of communities to disturbance (Hibbs 1983; Franklin and MacMahon 2000). Recently, however, large-scale restoration efforts have afforded new opportunities for natural experiments that enhance our understanding of theoretical processes (Doyle and Drew 2008). Such studies can also reveal "ecological surprises" – unexpected yet transformative discoveries about the natural world (Lindenmayer and Likens 2018). For example, ecologists documented the effects of landscape-scale trophic cascades while monitoring the reintroduction of wolves to Yellowstone National Park (Ripple and Beschta 2012). Similarly, completion of the world's largest dam removal project on the Elwha River enabled researchers to study vegetative community succession in the dewatered reservoir (Prach et al. 2018). By reinstating ecological and evolutionary processes, restoration can serve as a tool for understanding basic ecological principles, like community succession, while also conserving and enhancing biodiversity.

In coastal environments, restoration efforts often focus on recreating structurally complex habitat using biogenic or "habitat-forming" organisms like seagrasses, mangroves, and oysters (Lotze et al. 2006). These and other foundation species have suffered drastic declines (Altieri and

von de Koppel 2014), prompting calls by the United Nations for the current decadal focus on ecological restoration (U.N. 2021). Oyster reefs are among the most degraded coastal habitats (Beck et al. 2011) and have been the focus of restoration efforts for over fifty years (Hernandez et al. 2018). However, despite significant investment in restoration, only an estimated 4.5% of reef area has been recreated relative to historical baselines (Hernandez et al. 2018). While these gains are important, it remains difficult to assess the long-term persistence and ecological function of restored oyster reefs given the short-term (1-3 years) monitoring typical of many projects (Baggett et al. 2015). In addition, from a practical standpoint, it can be challenging to quantify biodiversity and trophic complexity in oyster reefs (La Peyre et al. 2019). While there are many types of sampling gear, most methods have species-specific limitations and efficiencies (Stone and Brown 2018), complicating efforts to obtain a holistic knowledge of the diverse invertebrate and vertebrate communities found in reef communities (La Peyre et al. 2019).

Rather than monitor entire ecosystems, ecologists often rely on "surrogate species" for assessing biodiversity: species that are representative of their associated community (Wiens et al. 2008). Typically, researchers use free-living species as proxies for other taxa (Lindenmayer et al. 2015). However, *parasites* can serve as indicators of underlying complexity because they are "cross-taxon surrogates" (Caro 2010) for the presence of multiple invertebrate and vertebrate hosts (Hechinger and Lafferty 2005; Hechinger et al. 2007). While free-living surrogates are often assumed to represent their target organisms through a shared preference for microhabitat or functional group status (Cushman et al. 2009), trophically transmitted parasites require specific hosts from different taxonomic groups across trophic levels, making them excellent surrogates for overall community diversity. Moreover, host diversity is often strongly correlated with parasite diversity (Kamiya et al. 2014; Wood and Johnson 2016), and evidence suggests that the

abundance of trophically transmitted parasites is positively associated with predator-prey interactions and other higher-level trophic processes in food webs (Rossiter and Sukhdeo 2014). Parasites thus provide a critical monitoring tool, as increased parasite diversity correlates with greater overall biodiversity across taxonomic groups (Moore et al. 2020).

In our study, we examined a time-series of restored oyster reefs constructed 3, 8, 19, and 22-years prior to our sampling (i.e., 2016, 2011, 2000, and 1997) and natural reefs in the same comparable environment. Space-for-time substitution is often used in ecological studies for inferring how temporal processes like succession, community assembly, and species richness correlate with sites of different ages (reviewed in Wogan and Wang 2018). This method is ideal for working with time-series data from multiple sites, provided that inference is made to ecological processes operating at the same spatial scale (Damgaard 2019). We hypothesized that older restored reefs would exhibit greater host-parasite diversity compared to more recently restored reefs that would have accrued fewer species and trophic interactions. Reef age is clearly important for mediating community assembly, as multiple studies have documented faunal succession in restored reefs (La Peyre et al. 2019). However, environmental and methodological variation among studies often precludes drawing firm conclusions about the role of time itself in the succession process.

Materials and Methods

Study Site and Experimental Design

Our study was conducted in the Middle Marsh portion of the Rachel Carson Estuarine Research Reserve (34˚ 41′ 32″ N, 76˚ 37′ 16″ W), located along North Carolina's central coast (Fig. S1). Middle Marsh is polyhaline and experiences a semidiurnal tidal exchange of 0.9 meters (Ridge et al. 2017). Over the past 22 years, researchers have built nearly 100 intertidal

oyster reefs in the Reserve, approximately 50 of which remain. These restored reefs vary in proximity to other landscape features such as sandflats, salt marshes, seagrass beds, and natural oyster reefs (Zeigler et al. 2018). Importantly, all reefs in our system are in close physical proximity (<1 km), thus ensuring relatively similar abiotic conditions. Further, all sampled reefs had a clearly defined footprint and were originally restored using loose, weathered oyster shell shaped into uniform rectangular piles (~3 m wide x 5 m long x 0.30 m tall) using hand-tools and a modified oyster dredge. Additional details on site-placement and methods can be found in Grabowski et al. 2005 (1997/2000 reefs) and Fodrie et al. 2014 (2011/2016 reefs). We sampled a subset of n=3 reefs restored in 1997, 2000, 2011, and 2016, along with n=3 natural reefs that likely formed within the last 200 years (Ridge et al. 2017), for a total of 15 reefs sampled.

Sampling for Oyster Reef Fauna

Reef-resident fauna were sampled using passive collectors: small plastic milk crates (19 x 22 x 16 cm) filled with approximately 3.8 pounds of autoclaved oyster shell (Moore et al. 2020). Collecting units such as these provide a standardized volume of habitat for use in monitoring patch reefs of different sizes (La Peyre et al. 2019). Two crates were deployed at each replicate treatment making for a total of n=30 crates (2 crates x 3 replicates x 5 treatments). Crates were deployed in the shallow sub-tidal approximately 1-meter from the base of each reef to avoid desiccation at low tide. Because of the abundance of crustaceans in our system, all shrimps and crabs were collected from only one crate at each reef, which was selected randomly. Resident fishes were less common and were sampled from both crates. All organisms were placed in labeled bags and identified to species or genus-level. We also used unbaited minnow traps (1/4-inch mesh; 1-inch openings) to sample transient fauna during periods of inundation. At mid-rising tide 1-day prior to sampling, two minnow traps were deployed on opposite ends of each

reef. Twenty-four hours later, the contents of both traps were checked along with those of the plastic crates. Plastic crates were initially deployed in June 2019 and sampled approximately every 4 weeks until late October, after which all equipment was removed. During each sampling event, salinity (ppt), temperature (°C), and dissolved oxygen (mg/L) were measured using a handheld YSI (Pro-ODO).

Host Dissection and Parasite Identification

Xanthid crabs and small benthic fishes were dissected for parasites, as these are reef-resident organisms and known hosts for parasites in our system (Moore et al. 2020). Xanthid crab hosts included the stone crab *Menippe mercenaria* and four species of panopeid mud crabs, *Rhithropanopeus harrisii*, *Eurypanopeus depressus*, *Panopeus herbstii*, and *Dyspanopeus sayii*. Fish hosts included gobies *Ctenogobius boleosoma*, *Gobiosoma ginsburgi*; blennies *Chasmodes bosquianus*, *Hypsoblennius hentz*, *Hypleurochilus geminatus*; *Parablennius marmoreus*; and oyster toadfish *Opsanus tau*. Generally, these crab and fish species display strong site-fidelity to specific reefs (Toscano et al. 2014; Harding et al. 2019). This is important because parasite diversity should correlate with free-living diversity at each site (Moore et al. (2020). Moreover, to avoid problems associated with spatial autocorrelation, it is necessary to match the scale of a species' response to its environment (i.e., mobility) to the scale of the analysis (i.e., biodiversity at the site-level) (Knegt et al. 2010). In all hosts, we identified common trophically transmitted endoparasites including nematodes, digenetic trematodes, acanthocephalans, and cestodes. These macroparasite taxa develop within their hosts and require multiple invertebrate and vertebrate hosts for lifecycle completion (Poulin 2007). We did not sample ectoparasites like monogeneans and copepods because these organisms are easily dislodged in the process of collection and

transport. For panopeid mud crabs only, we also quantified *Loxothylacus panopaei,* a direct-developing endoparasite.

Crabs were measured (carapace width, mm), sexed, and dissected by separating the upper and lower carapace. Tissue squashes of hepatopancreas and gonad tissue were scanned for parasites at low power (4-10x) using a compound microscope (Zeiss AxioScope A1). Fish were measured (TL, mm), sexed, and dissected by removing the entire gastrointestinal tract (stomach, liver, gallbladder, spleen, intestine) and scanning for parasites at low power. The gut cavity of each fish was then rinsed and the wash examined for parasites that had been dislodged during dissection. Lastly, the head, body, and fins were checked for subcutaneous trematode cysts by viewing each fish at low power under a stereomicroscope (Zeiss Stemi 508). Only xanthid crabs ≥5 mm, and benthic fishes ≥20 mm were dissected for parasites because the macroparasites in our system primarily infect adult individuals (Moore et al. 2020). All parasites were identified using standard protocols and keys (e.g., Yamaguti 1971). Although smaller mud crabs and fish were not dissected for parasites, they were still included as part of our free-living diversity analyses. We also counted and identified other mobile taxa (e.g., blue crabs, shrimps, transient fishes) found within the crates and minnow traps at each site. Field collections were authorized by the North Carolina Division of Marine Fisheries (Scientific or Educational Permit #706671), and by the North Carolina Coastal Reserve (Permit #13-2019). Animal husbandry and dissection protocols were approved by East Carolina University's IACUC (AUP #D346).

Oyster Reef Habitat Parameters

We assessed habitat complexity in July-August 2019 by measuring multiple structural features in all reefs: reef area (m^2), reef height (m), maximum reef height (m), fringe elevation (m), crest elevation (m), crest peak elevation (m), oyster shell dead total weight (g), live oyster

count (n), live oyster weight (g), and live oyster length (mm). Dimensional parameters (area/elevation) were quantified using a Trimble® R10 Global Navigation Satellite System (<1·5-cm vertical precision). Oyster counts/weights were quantified by excavating and collecting all material to a depth of 10 cm from a 0.625m^2 quadrat at the crest and on the fringing slope of each reef (n=2 per reef). The total count of all live oysters >20mm and the length of up to 50 randomly selected live oysters were quantified for each sample. Correlation analyses revealed that most habitat parameters were either moderately or strongly correlated (coefficient ± 0.3). As a result, only reef area, reef height, and live oyster count were included in our analyses of habitat complexity because these variables were only weakly correlated (± 0.2). Moreover, all three of these variables were identified by Baggett et al. (2015) as key performance indicators of functioning oyster reefs.

Parasite Diversity and Trophic Complexity

We created food webs depicting how parasite diversity differed in a focal host species, the oyster toadfish, between New (<10 years), Old (>20 years), and Natural reefs. We used the toadfish because this species was the dominant fish host for parasites in our system. Moreover, an organism's location within a food web is an important predictor of its parasite diversity: fish species in the middle of food webs consume a variety of invertebrate and vertebrate prey, often harboring the greatest number of endoparasites (Marcogoliese 2002; Poulin and Leung 2011). Based on dietary studies, adult toadfish occupy an intermediate trophic level of 3.8 ± .04 (Froese and Pauly 2010). In our sampling, we captured juveniles and young adults (≤150 mm). We categorized toadfish as either juveniles (<100 mm) or adults (>100 mm) according to data from Wilson et al. (1982).

Parasite data from infected toadfish were used to construct food webs for New (<10 years), Old (>20 years), and Natural reefs by extrapolating the additional taxa required by these parasites for life cycle completion based on known toadfish predator-prey relationships (Linton 1905; Schwartz and Dutcher 1963; Wilson et al. 1982; Moore et al. 2020). In the Natural reefs, we documented a single toadfish infected with an adult acanthocephalan. Although we excluded this one observation from our multivariate analysis, we included it as part of our food web analysis. Taxa located in trophic levels 1, 2, and 3 are known hosts for trophically transmitted parasites (Moore et al. 2020). They are also common prey items of toadfish based on dietary studies (Wilson et al. 1982). Taxa located at trophic level 4 are putative hosts based on published data (Linton 1905) and the local abundance of these taxonomic groups in our system (Moore et al. 2020).

Statistical Analyses

To determine the drivers of diversity across reef age, we analyzed the following datasets: free-living crustacean host taxa (hereafter: free-living crustacean taxa), crustacean parasite taxa, free-living fish host taxa (hereafter: free-living fish taxa), and fish parasite taxa. For the free-living fish taxa, we combined data from the minnow traps and plastic collectors to create a single dataset of transient and reef-resident fishes. Parametric statistical tests were used when the data met the assumptions of a Gaussian distribution. Non-parametric tests were used on zero-inflated count data that did not meet the assumptions of normality and could not be transformed. Univariate analyses were conducted in R (version 4.0.3) using R core functionality. Abiotic data (salinity, temperature, DO) were not included in these statistical analyses because they were not collected at each site and thus could not be used in comparisons among reefs (Table S1).

We created box plots depicting taxa richness as a function of elapsed time since restoration. For each response variable, we tested for significant differences between reefs (2016, 2011, 2000, 1997, Natural) using one-way ANOVAs (free-living taxa) and non-parametric Kruskal-Wallis tests (parasite taxa). Post-hoc testing was performed using Tukey's post-hoc (free-living taxa) and Wilcoxon rank sum tests adjusted for multiple pairwise comparisons (parasite taxa). Effect sizes were calculated using Cohen's D (free-living taxa) and Wilcoxon effect size tests (parasite taxa) with the package *rstatix* (Alboukadel 2020). Welch's t-tests for unequal variance were used to compare the overall magnitude of effect between crustacean taxa vs. parasites and fish taxa vs. parasites. Shannon-Weiner diversity values were calculated using the *vegan* package (Oksanen et al. 2020). We also evaluated the strength of the correlation between host and parasite richness as a function of reef age across New (<10 years), Old (>20 years), and Natural reefs. For the response variables in these plots, the residuals were normally distributed and Pearson's correlation coefficients were fitted to the data. For our analysis of habitat, Kruskal-Wallis and Wilcoxon rank sum post-hoc tests were used to test for differences in reef area, height, and the number of live oysters.

Lastly, we used non-metric multidimensional scaling (nMDS) ordinations (Field et al. 1982) based on Bray-Curtis dissimilarity matrices in PRIMER v7 to visualize changes in host-parasite abundance during our project. The fish parasite data contained a single observation of an adult acanthocephalan parasite, which was removed prior to analysis. All abundance data were then 4th root transformed. Pearson correlation coefficient taxa overlays were established for each nMDS plot at a threshold ≥0.35. In addition, a PERMANOVA was conducted to examine whether host/parasite abundance differed between New (<10 years), Old (>20 years), and Natural reefs. We used a nested design with sampling event as a random effect within reef age to

avoid pseudo-replication. Pairwise comparisons were made among the data in different age groups via the unrestricted permutation of abundance data. Based on these results, a similarity percentage analysis (SIMPER) was performed on the fish parasite abundance data to determine which parasite taxa were responsible for observed differences between age groups.

Results

Comparison of Free-living and Parasite Diversity in Natural and Restored Reefs

There were no differences in free-living crustacean and fish taxa richness or diversity as a function of elapsed time (Fig. 1, A-B; Fig. S2, A-B). However, fish parasite richness in the Natural reefs was greater than richness in the 2016 reefs (Fig. 1, D, X^2=12.5, df=4, p=0.02), and there was a marginal increase in fish parasite diversity with time (Fig. S2, D, X^2=8.6, df=4, p=0.07). Effect size measurements of free-living taxa demonstrated no clear trend over time (Fig. 1, E), whereas the parasite taxa showed a consistent decrease in the magnitude of difference across all paired comparisons (Fig. 1, F). In addition, there were differences in the overall magnitude of effect between free-living taxa and parasites. The effect of reef age on diversity was greater for crustacean parasites compared to free-living crustacean taxa (t=-4.2, df=14.5, p=0.0008) and for fish parasites compared to free-living fish taxa (t=-4.5, df=13.7, p=0.0006) (Fig. S3, Table S2).

Correlation between Host/parasite Richness in New, Old, and Natural Reefs

The positive correlation between free-living crustacean taxa and crustacean parasite richness strengthened with time (Fig. 2, A-C). In Natural reefs, there was a strong, statistically significant positive relationship between free-living crustacean and parasite richness (R=0.68, t=3.07, df=11, p=0.01), while there was no significant correlation on New reefs (R=0.14, t=0.66,

df=22, p=0.52). However, a significant, positive relationship between free-living crustacean and parasite richness was observed for the Old reefs (R=0.44, t=2.49, df=26, p=0.02), which explained 44% of variance, slightly lower than the 68% of variance explained for Natural reefs. There was no correlation between free-living fish and parasite richness (Fig. 2, D-F).

Changes in Community Abundance through Time

We created nMDS plots depicting changes in the abundance of free-living taxa and their parasites during our project (Fig. S4). For the free-living taxa (A-B), there were clear seasonal differences in the relative abundance of crustacean and fish species sampled. However, there was no seasonal trend for crustacean and fish parasite abundance (C-D). Pearson correlation overlays depicted the free-living and parasite taxa most strongly associated with each sampling event (Table S3). A PERMANOVA with taxa abundance nested within sampling event showed that reef age [New (<10 years), Old (>20 years), Natural] was an important predictor of fish parasite abundance (Table S4, p=0.01). The abundance of fish parasites was different between the Natural and New reefs (Table S4, p=0.005) and between the Natural and Old reefs (Table S4, p=0.05). SIMPER analyses showed that these results were primarily driven by differences in the abundance of larval cestode (procerdoid) parasites (Table S5). Complete species composition and occurrence of all parasite taxa are available in Table S5. Mean abundances (±SD) of all free-living taxa are available in Table S6.

Oyster Reef Habitat Complexity

Mean estimates of reef habitat parameters are depicted in Figure S5. The area (mean ± SD) of Natural reefs (538 m^2 ± 293 m^2) was much greater than area in the Old (78.2 m^2 ± 12.7 m^2) (Table S7, $p=2.3x10^{-7}$) and New reefs (34.3 m^2 ± 13.9 m^2) (Table S7, $p=9.1x10^{-10}$). Old reefs

had an average height (0.431 m ± 0.0740 m) greater than both the Natural (0.338 m ± 0.0563 m) (Table S7, $p=0.0001$) and New reefs (0.308 m ± 0.0387 m) (Table S7, $p=4.4 \times 10^{-6}$). The average number of live oysters was not different among New (130 ± 110), Old (69 ± 42), and Natural reefs (90 ± 109) (Table S7).

Toadfish Food Web

Oyster toadfish comprised 72% of all parasitized fish (Table S8). Overall, parasitized toadfish were most abundant in the Natural reefs (n=32) followed by the Old (n=20) and New reefs (n=4) (Table S9). Trophic complexity, as represented by toadfish and their parasites, increased across the New, Old, and Natural reefs (Figure 3). The New reefs had the lowest overall trophic complexity, as there were only two major pathways present (cestodes, nematodes); moreover, juvenile toadfish were absent from these reefs (Table S8). However, complexity increased for the Old reefs with the addition of the trematode pathway and two out of the three life stages required for trematode life cycle completion. The Natural reefs had the greatest abundance of toadfish at different life stages, some of which were final hosts for trematodes due to trophic transmission resulting from predation and cannibalism (Figure 3).

Discussion

Ecological restoration projects can provide living laboratories for administering large-scale experiments under natural conditions. In marine systems, previous studies have concluded that free-living taxa readily recruit to restored oyster reef habitat 1-2 years post-construction (Humphries et al. 2011; Moore et al. 2020), but longer-term successional trajectories remain poorly characterized due to major differences in the location or design of reefs (La Peyre et al. 2019). For example, Brown et al. (2014) surveyed faunal assemblages at a series of natural,

recently created (<5 years), and older (>6 years) reefs, quantifying substrate type as a predictor of restoration success but not time-since restoration. In other systems, such as coral reef communities, studies have demonstrated that restored marine habitats increasingly resemble their natural counterparts through time post-restoration. Burt et al. (2011) compared patterns of community development in a time-series of breakwater structures (1-31 years old), finding that artificial reef communities resembled, but did not replicate, those in natural coral reefs. These results were confirmed by Hill et al. (2021), who used a centuries-old breakwater reef for comparison. Similarly, a recent synthesis of seagrass restoration projects (3-32 years old) found distinct differences in community composition between restored and reference plots even after >30 years (Rezek et al. 2019). In our system, we used a space-for-time approach to analyze long-term successional patterns in oyster reef communities. We predicted that the diversity of parasites and hosts would increase post-restoration, with older reefs approaching the diversity of natural reefs. Below, we discuss our results and how they advance the theoretical and applied framework for using multi-host parasites as surrogate species.

Parasites Indicate Successional Changes in Oyster Reef Communities

We found that parasites were better indicators of successional changes in oyster reefs compared to free-living taxa. While there was variability in the parasite data, mean estimates of richness and diversity of crustacean and fish parasites increased with elapsed time (Fig. 1, C-D; Fig. S2, C-D). This trend was not apparent for free-living taxa (Fig. 1, A-B; Fig. S2, A-B), whose relative abundance varied from month-to-month (Fig. S4, A-B). These results suggest that conventional methods of sampling for oyster reef fauna (e.g., minnow traps, settlement trays/habitat collectors) using free-living organisms may be less effective at capturing successional changes compared to sampling for trophically transmitted parasites. We also

observed important differences between free-living and parasite taxa when comparing effect sizes, which provide an estimate of the practical differences between groups (Ho et al. 2019). When comparing data from each restored reef to the Natural reefs in a stepwise fashion, we would expect the magnitude of difference to be larger in comparison to more recently restored reefs (e.g., 5-Natural) as opposed to older reefs (e.g., 24-Natural) that have had more time to accrue trophic complexity. This trend was only apparent for the parasite data, wherein the difference across all comparisons decreased with elapsed time (Fig. 1, E-F). In addition, the overall magnitude of the effect was larger for the parasite data than the free-living data (Fig. S3; Table S2), which indicates that there is a stronger relationship between the parasite data and reef age. While we sampled more free-living taxa than parasite taxa, it should be noted that effect size measurements are independent of sample size (Ho et al. 2019).

In some cases, habitat structure can serve as a predictor of parasite transmission pathways (Rossiter and Sukhdeo 2014). However, our results cannot solely be attributed to differences in the structural complexity of reef habitat, at least not for the parameters we measured. For example, the average number of live oysters was not different among reefs, nor was height different between the Natural and New (<10 years) reefs (Table S7). On the other hand, the Natural reefs were larger than the restored reefs (Figure S5; Table S7). The relationship between size/area and species richness is well known (i.e., Connor and McCoy 1979), and it is possible that the greater size of these reefs may contribute to greater species diversity. Nevertheless, the Old reefs are much closer in size to the New reefs than they are to the Natural reefs (Figure S5; Table S7). If reef area were a better predictor of diversity, then the parasite diversity of the Old reefs should more closely resemble the New reefs. Instead, parasite diversity in the Old reefs was more similar to diversity of the Natural reefs (Fig. 1, C-D; Fig S2, C-D).

While the parasite data were more informative overall, we identified important differences between parasites of crustaceans and parasites of fish, which has implications for using parasites as surrogates of overall taxonomic diversity (Fig. 2). Although there was no host-parasite relationship in the New (<10 years) reefs for the crustacean hosts, there was a significant positive relationship in the Old (>20 years) and Natural reefs (Fig. 2, A-C), demonstrating that the close connection between host and parasite diversity developed with time. In contrast, we did not document this same relationship in fish (Fig. 2, D-F), likely due to the greater mobility and functional diversity (e.g., life history, habitat preferences) of species sampled compared to crustaceans (Table S6). In general, crustacean host-parasite diversity may be a more accurate indicator of site-level effects because of the limited mobility of xanthid crabs (Toscano et al. 2014). While there was no relationship between overall fish taxa richness and parasite richness (Fig. 2, D-F), this does not preclude single species of site-resident fish from functioning as sentinels of parasite diversity, a phenomenon we documented in oyster toadfish (Tables S8-9).

Toadfish are a Key Host Species in Oyster Reef Food Webs

Parasitism in food webs is a non-random process (Chen et al. 2008). Hosts with high parasite diversity tend to consume a wide variety of prey items and occupy network positions close to many types of prey (Marcogliese 2002). While trophically transmitted parasites typically require multiple hosts (Huspeni and Lafferty 2004), parasite persistence may be driven by a single host species (Fenton et al. 2015), increasing the probability of successful transmission over evolutionary time if that host is central to the food web (Anderson and Sukhdeo 2011). It is thus possible to map parasite transmission onto food webs since trophically transmitted parasites move along pathways in which key hosts act as trophic links (Thompson et al. 2005; Poulin and Leung 2011).

Past research has suggested that toadfish can mediate trophic cascades in oyster reef communities via predation (Grabowski and Kimbro 2005; Grabowski et al. 2008). Species like toadfish that occupy a prominent place in the middle of food webs often harbor higher numbers of endoparasites because they feed on both invertebrates and small fish (Marcogliese 2002; Poulin and Leung 2011). For example, in a study of three intertidal food webs, Chen et al. (2008) reported that species serving as intermediate hosts had more predators (mostly birds) and were incorporated into more food chains than those not serving as intermediate hosts. Indeed, parasites using intermediate hosts rely on predation to facilitate "trophic ascent" (i.e., Parker et al. 2015) to larger hosts at higher trophic levels where reproduction and dispersal occur (Esch and Fernandez 1993). Toadfish can therefore serve as a model organism for mapping parasite transmission within oyster reefs. By comparing parasite diversity in toadfish from New (<10 years), Old (>20 years), and Natural reefs, we can depict the trophic connections represented by each toadfish-parasite interaction and infer the composition of the overall host community.

Moreover, toadfish undergo ontogenetic shifts in diet (Wilson et al. 1982), which has implications for the upward incorporation of parasites in toadfish-driven food webs (Fig. 3). For example, juvenile toadfish are primarily infected with larval cestodes, while larger adults have higher parasite diversity because of the wider range of prey items they consume (Table S9). Multiple cestode taxa (e.g., *Diphyllobothrium*, *Triaenophorus*) use copepods as first-intermediate hosts followed by small fish (Pasternak et al. 1995). Smaller fish like juvenile toadfish are more likely to consume an infected copepod or other microcrustacean serving as a first intermediate host for cestode parasites (Poulin and Leung 2011). While we documented larval cestode infections in adult toadfish from multiple reefs (Table S9), these individuals were likely infected as juveniles because microcrustaceans would not be an important prey item of adult toadfish

(Pasternak et al. 1995). Importantly, we did not document any mature cestode infections in toadfish, which implies that toadfish are not the final hosts for these parasites. It is also interesting that nearly all infected juvenile toadfish were sampled from the Natural reefs (Table S9). Indeed, the discrepancy in the abundance of larval cestode-infected fish was mostly responsible for driving differences in fish parasite abundance as a function of reef age (Tables S4-5). The preponderance of cestode-infected toadfish in the Natural reefs may imply that the process of trophic ascent for cestode parasites from first to final host is more efficient. Without an abundance of juvenile toadfish, cestodes may encounter a trophic "vacuum" (Parker et al. 2015) that would inhibit life cycle completion.

Larger toadfish are capable of consuming larger and more diverse prey such as small fishes, shrimps, and mudcrabs (Linton 1905; Wilson et al. 1982). While we identified multiple parasite taxa in adult toadfish (Table S9), the diversity of parasites and life history strategies differed among reefs (Fig. 3, Table S9). For the most part, parasites like nematodes, trematodes, and acanthocephalans were absent from the New reefs. The lack of parasite diversity in fishes sampled from New reefs may suggest that the corresponding reef community is host-poor. However, low sample size may also account for these results, as we sampled fewer toadfish overall from the New reefs (n=4) compared to the Old (n=20) and Natural reefs (n=32) (Table S8). In both the Old and Natural reefs, multiple parasite taxa require toadfish as intermediate or paratenic hosts to fill the trophic transmission vacuum between first and final hosts (Benesh et al. 2014). Adult toadfish in these reefs were also parasitized by mature nematodes, trematodes, and acanthocephalans consistent with the prediction that the diversity of adult parasites in a specific host increases with increasing trophic level (Poulin and Leung 2011). It is noteworthy that adult trematodes were absent from the Old reefs, while toadfish from the Natural reefs were infected

with both intermediate and adult-stage trematodes. Trematode life cycle completion requires trophic transmission, an outcome that is more favorable when there is a high abundance of intermediate hosts with overlapping niches (Choisy 2003). This may suggest that the overall host community is not as diverse in the Old reefs compared to the Natural reefs, which also had an adult acanthocephalan infection. However, it is likely that we under-sampled parasites like trematodes and acanthocephalans because our collection method was size-limited and biased towards smaller adult toadfish. Ultimately, our discussion of toadfish as key hosts in parasite food webs is meant to be illustrative rather than comprehensive.

Conclusions

We demonstrate that parasites can be a valuable tool for monitoring community diversity and trophic complexity in restored oyster reefs. Indeed, our results show that parasites are better indicators of community succession in restored reef habitat than free-living taxa. Compared to pre-existing natural reefs, parasite-driven differences in trophic complexity were most apparent 10 years post-restoration – roughly similar to studies of benthic community development in artificial and natural coral reefs (Burt et al. 2011; Hill et al. 2021). While most studies monitor for a maximum of 1-3 years post-restoration (Baggett et al. 2015), our results suggest that complexity emerges over longer time intervals. Finally, given that toadfish and their parasites appear to act as sentinels of community diversity, we suggest including these key host species (and their parasite infracommunities) in future studies. Collections should target larger toadfish in natural reefs to obtain a more complete picture of toadfish-parasite diversity in reference ecosystems.

Acknowledgments

Funding for this work was provided to C. Moore by the Garden Club of America's 2019 Fellowship in Ecological Restoration; support and equipment from East Carolina University to A. Blakeslee and R. Gittman; and grants to R. Gittman from the North Carolina Department of Environmental Quality (#NA15NOS4190091), NOAA (#A19-0052-001), U.S. Fish and Wildlife Service (#F19AP 00230), and the North Carolina Policy Collaboratory (#20170 12593 221010 KOAST). We gratefully acknowledge the following individuals for their assistance: T. Boyd, C. Deaton, S. Donaher, M. Plafcan, S. Sutcliffe, S. Trackenburg, and E. Wellman.

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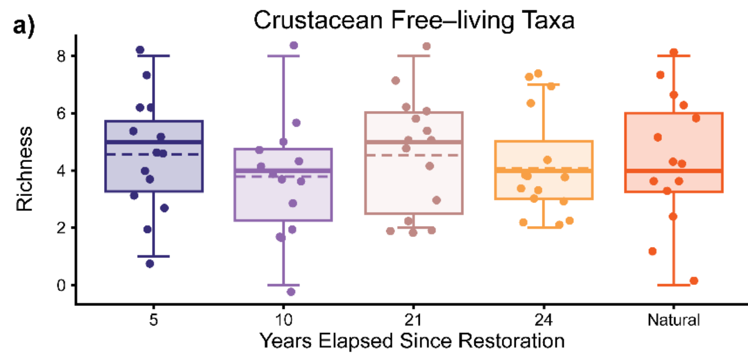
a)
Crustacean Free–living Taxa
Richness
Years Elapsed Since Restoration
5
10
21
24
Natural

b)
Fish Free–living Taxa
Richness
Years Elapsed Since Restoration
5
10
21
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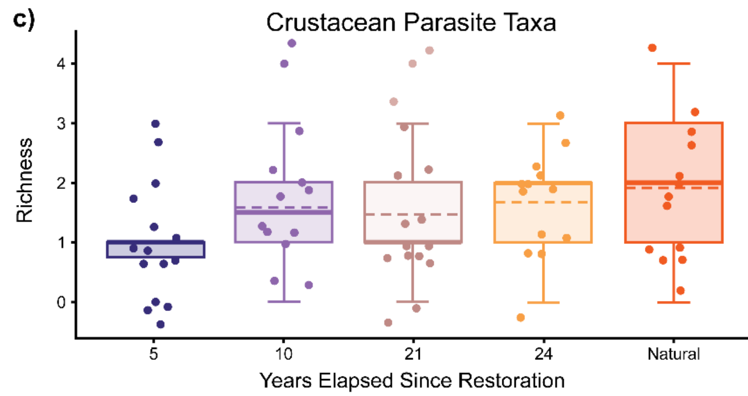
Natural


c)
Crustacean Parasite Taxa
Richness
Years Elapsed Since Restoration
5
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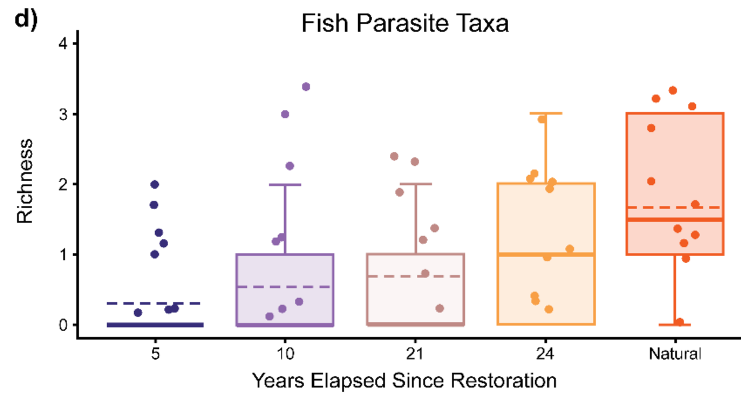
d)
Fish Parasite Taxa
Richness
Years Elapsed Since Restoration
5
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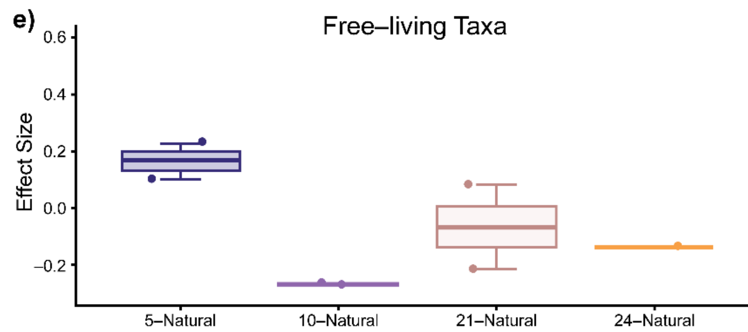
Natural


e)
Free–living Taxa
Effect Size
5–Natural
10–Natural
21–Natural
24–Natural

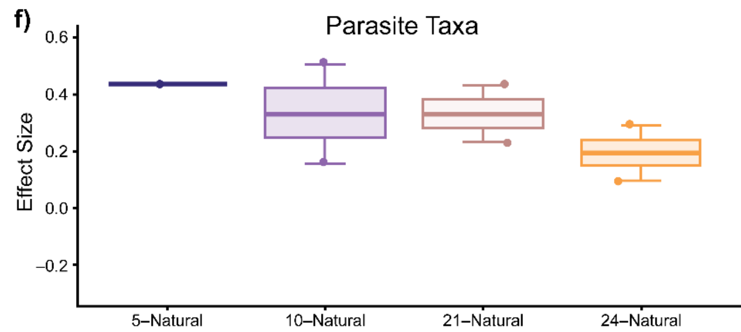
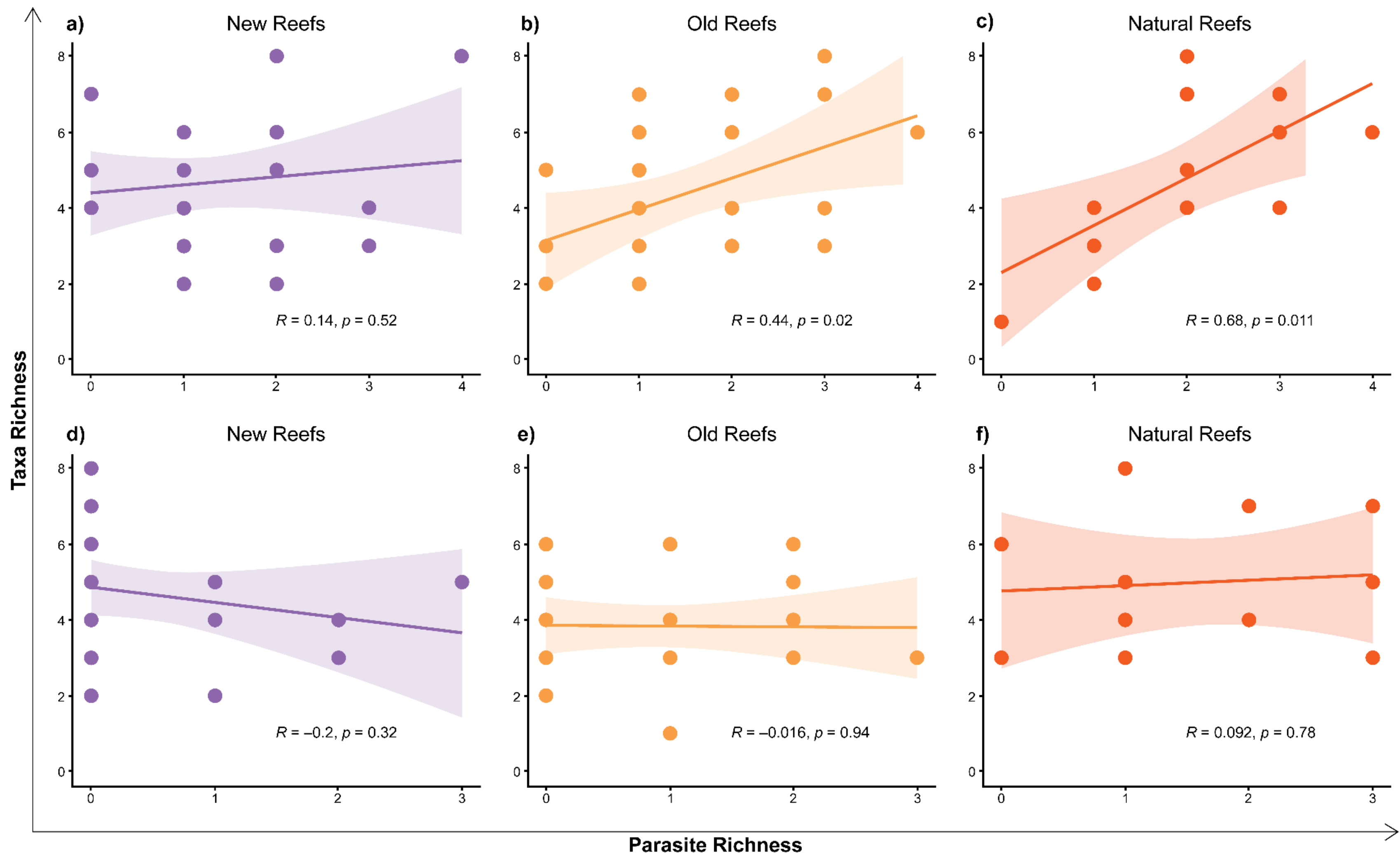
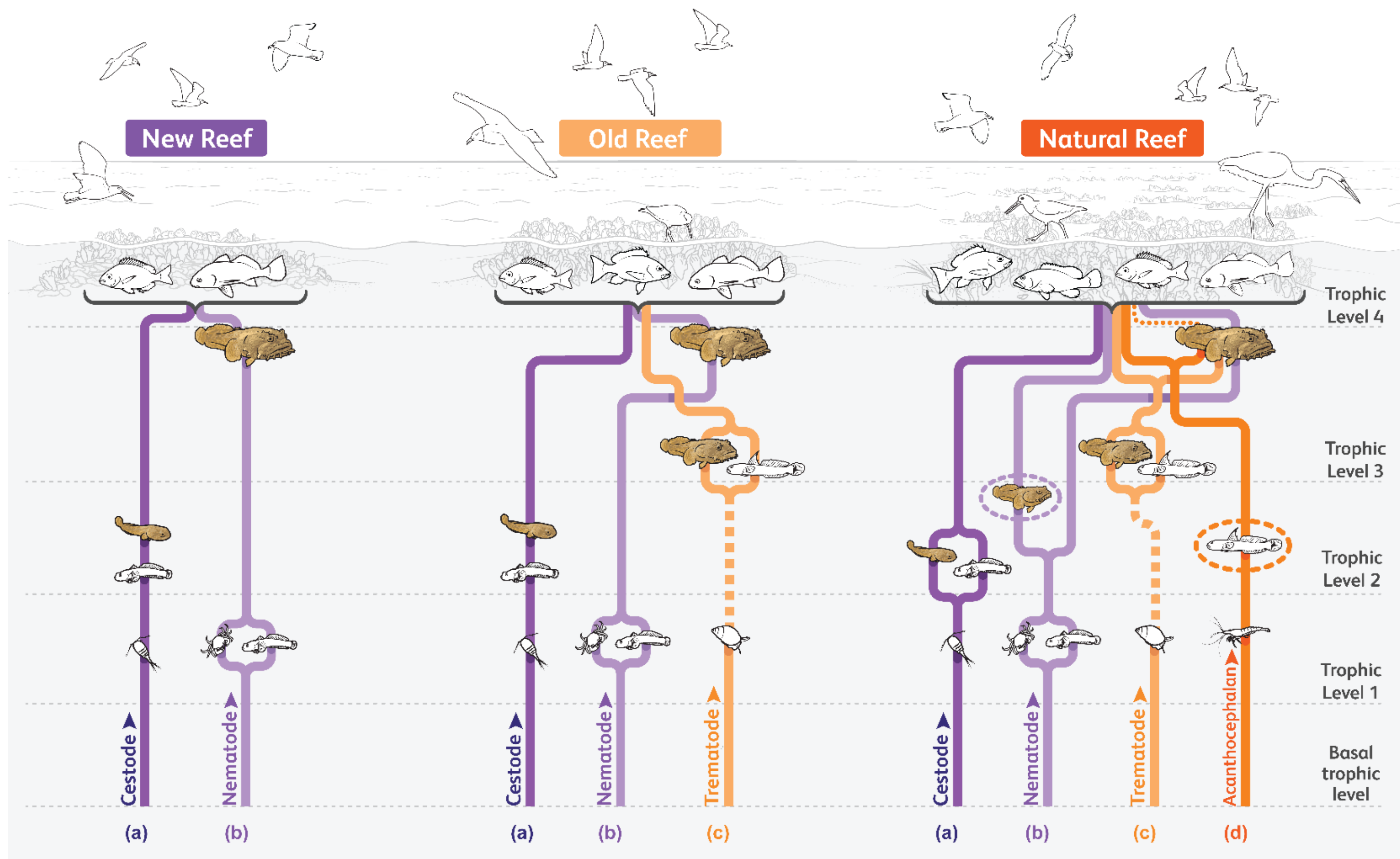

f)
Parasite Taxa
Effect Size
5–Natural
10–Natural
21–Natural
24–Natural

Fig. 1. Free-living crustacean and fish taxa richness (a-b); crustacean and fish parasite taxa richness (c-d); and effect size measurements between free-living taxa (e) and parasite taxa (f). Solid lines represent the median of each treatment, and dashed lines represent mean estimates. Mean estimates of free-living taxa richness were highly variable as a function of elapsed time since restoration (a-b), while mean estimates of parasite taxa richness increased through time (c-d). In plot D, fish parasite richness was greater in the Natural reefs compared to reefs restored in 2016 (X^2=12.5, df=4, p=0.02). For the free-living taxa, there was no trend when comparing effect size measurements between restored and natural reefs (e); however, the magnitude of effect steadily decreased through time for the parasite taxa when comparing the data from reefs restored 5, 10, 21, and 24 years ago to data from the natural reefs (f).



a)
New Reefs
R = 0.14, p = 0.52
b)
Old Reefs
R = 0.44, p = 0.02
c)
Natural Reefs
R = 0.68, p = 0.011
d)
New Reefs
R = –0.2, p = 0.32
e)
Old Reefs
R = –0.016, p = 0.94
f)
Natural Reefs
R = 0.092, p = 0.78
Taxa Richness
Parasite Richness

Fig. 2. Correlation between free-living and parasite taxa richness in crustaceans (a−c) and fish (d−f) across New, Old, and Natural Reefs. Shaded areas represent the 95% confidence interval for each fitted curve. For the crustacean data (a-c), the correlation between host and parasite taxa richness strengthened from New (<10 years) [R=0.14, t=0.66, df=22, p=0.52], to Old (>20 years) [R=0.44, t=2.49, df=26, p=0.02], to Natural reefs [R=0.68, t=3.07, df=11, p=0.01]. In contrast, there was no relationship between host and parasite fish taxa richness (d-f).



(a) Cestode- Juvenile toadfish are infected with larval cestodes via trophic transmission. As intermediate hosts, toadfish remain infected until they are consumed by a suitable final host.

(b) Nematode- Toadfish may serve as intermediate, final, or paratenic hosts. Nematodes infect larger juvenile toadfish and adult toadfish via direct penetration or trophic transmission.

(c) Trematode- Toadfish serve as intermediate hosts in Old and Natural reefs when infected by larval cercariae shed from snails. Toadfish serve as final hosts in Natural reefs.

(d) Acanthocephalan- Toadfish serve as final hosts for acanthocephalans, which can undergo post-cyclic transmission if consumed by an additional final host. Other taxa may act as paratenic hosts.

Trophic transmission =
Environmental transmission =
Potential post-cyclic transmission =
Paratenic host stage =

Fig. 3. Food webs illustrating how parasite diversity in toadfish differs in New (<10 years), Old (>20 years), and Natural reefs. Oyster toadfish are the only species depicted in solid form, as they are the focal organism in this figure, while other host taxa are depicted as silhouettes.

Supplementary Figures

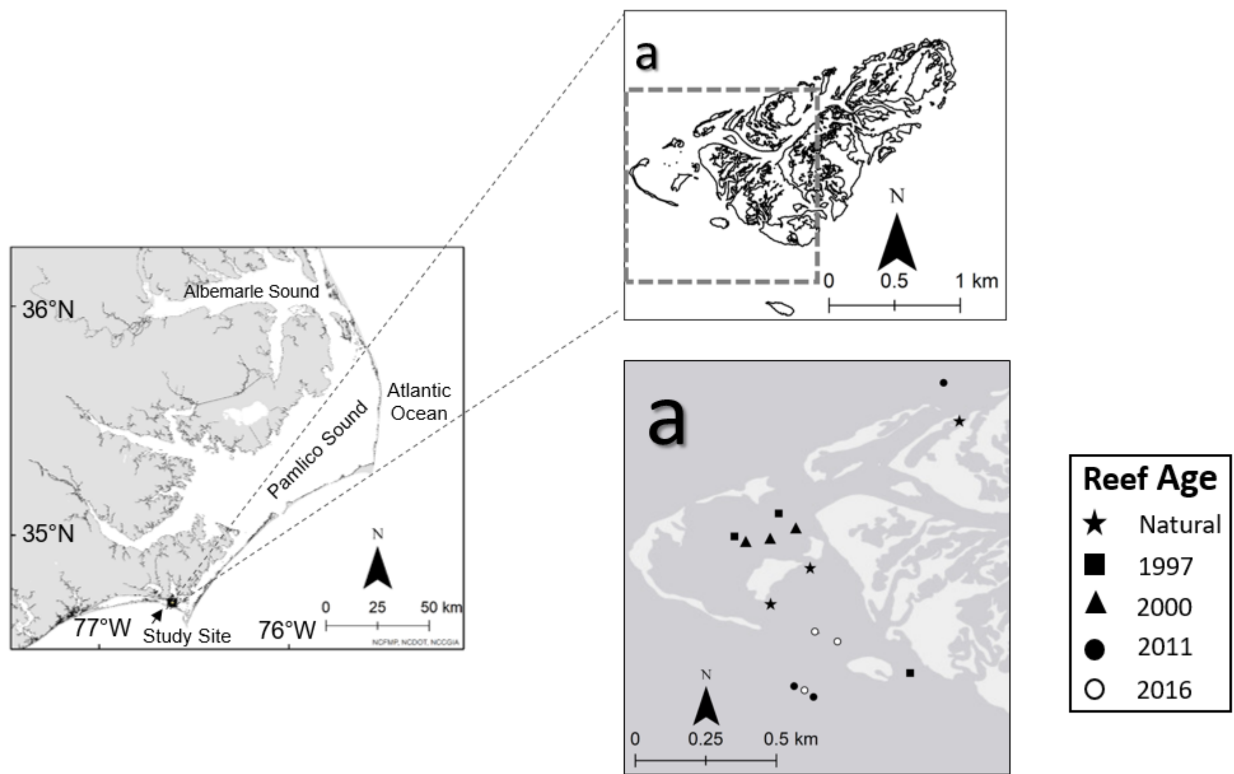


Figure S1. Map showing study site location along the central North Carolina coast and enhanced view of Middle Marsh and project area (a).

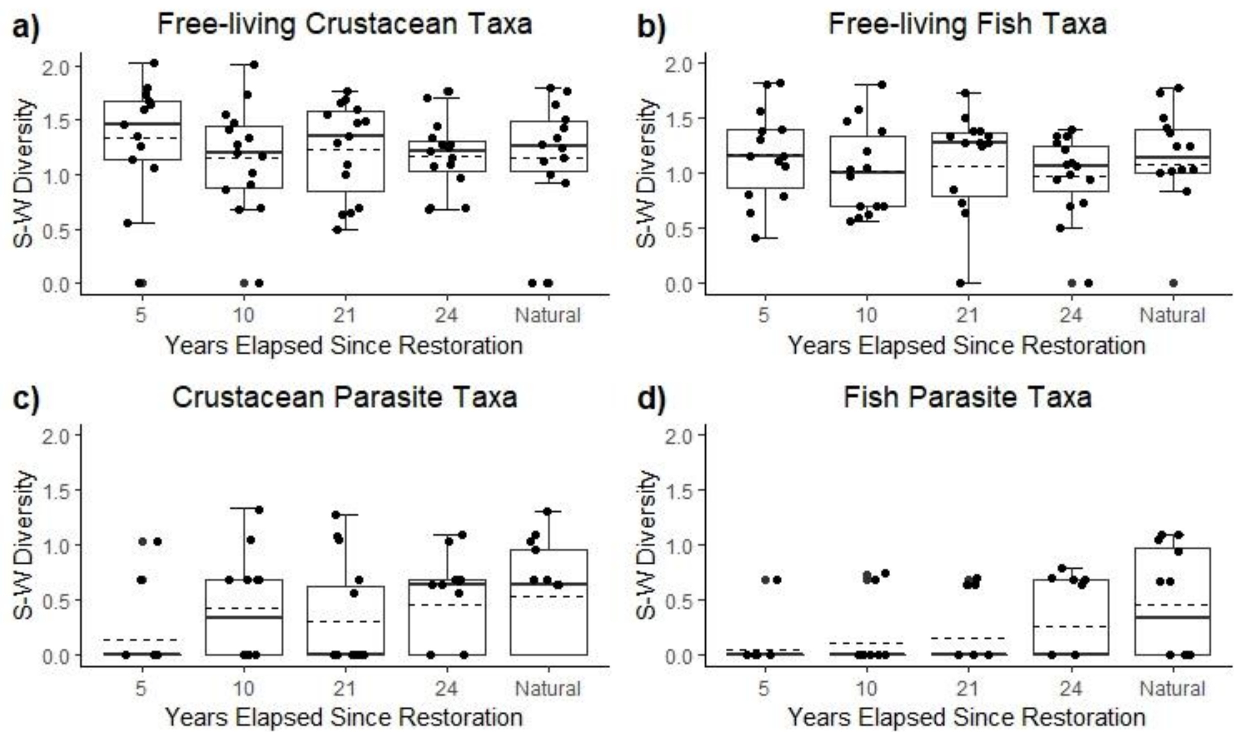


Figure S2. Shannon-Wiener diversity of free-living crustacean and fish taxa (a-b) and crustacean and fish parasite taxa (c-d) as a function of time elapsed since restoration. Solid lines represent the median of each treatment, and dashed lines represent mean estimates. In plot D, fish parasite diversity increased over time (X^2=8.6, df=4, p=0.07); however, post-hoc testing revealed no significant differences between treatments.

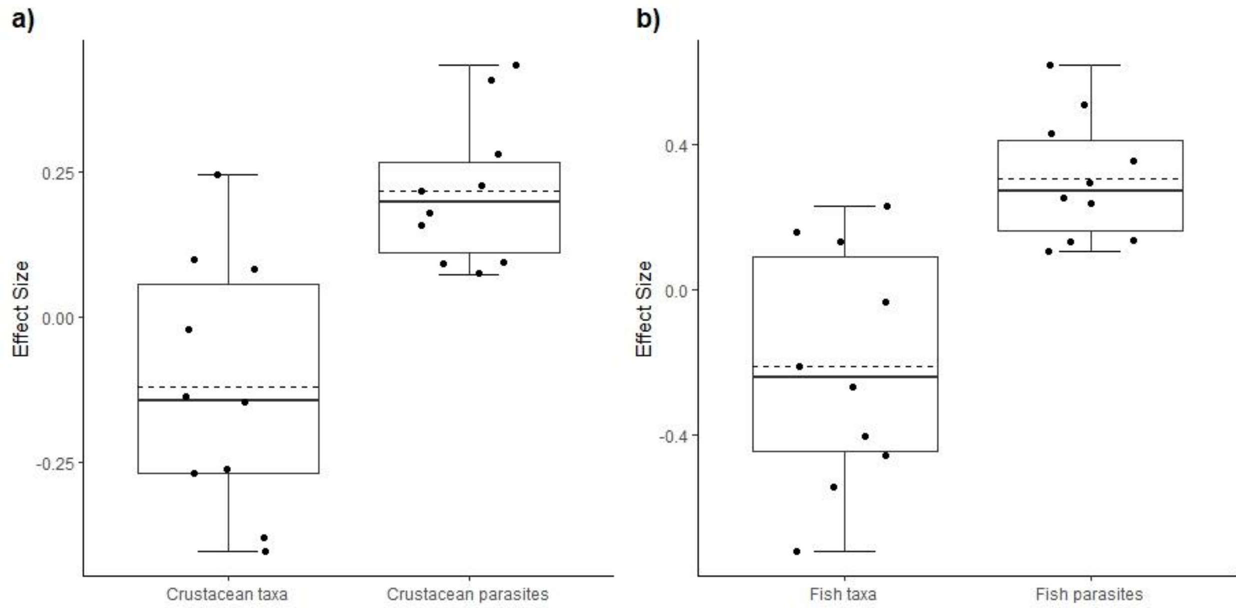


Figure S3. Comparison of effect sizes between free-living crustacean taxa and crustacean parasites (a) and free-living fish taxa and fish parasites (b). Solid lines represent the median of each treatment, and dashed lines represent mean estimates. The magnitude of effect was greater for crustacean parasites compared to free-living crustacean taxa (t=-4.2, df=14.5, p=0.0008) and for fish parasites compared to free-living fish taxa (t=-4.5, df=13.7, p=0.0006).

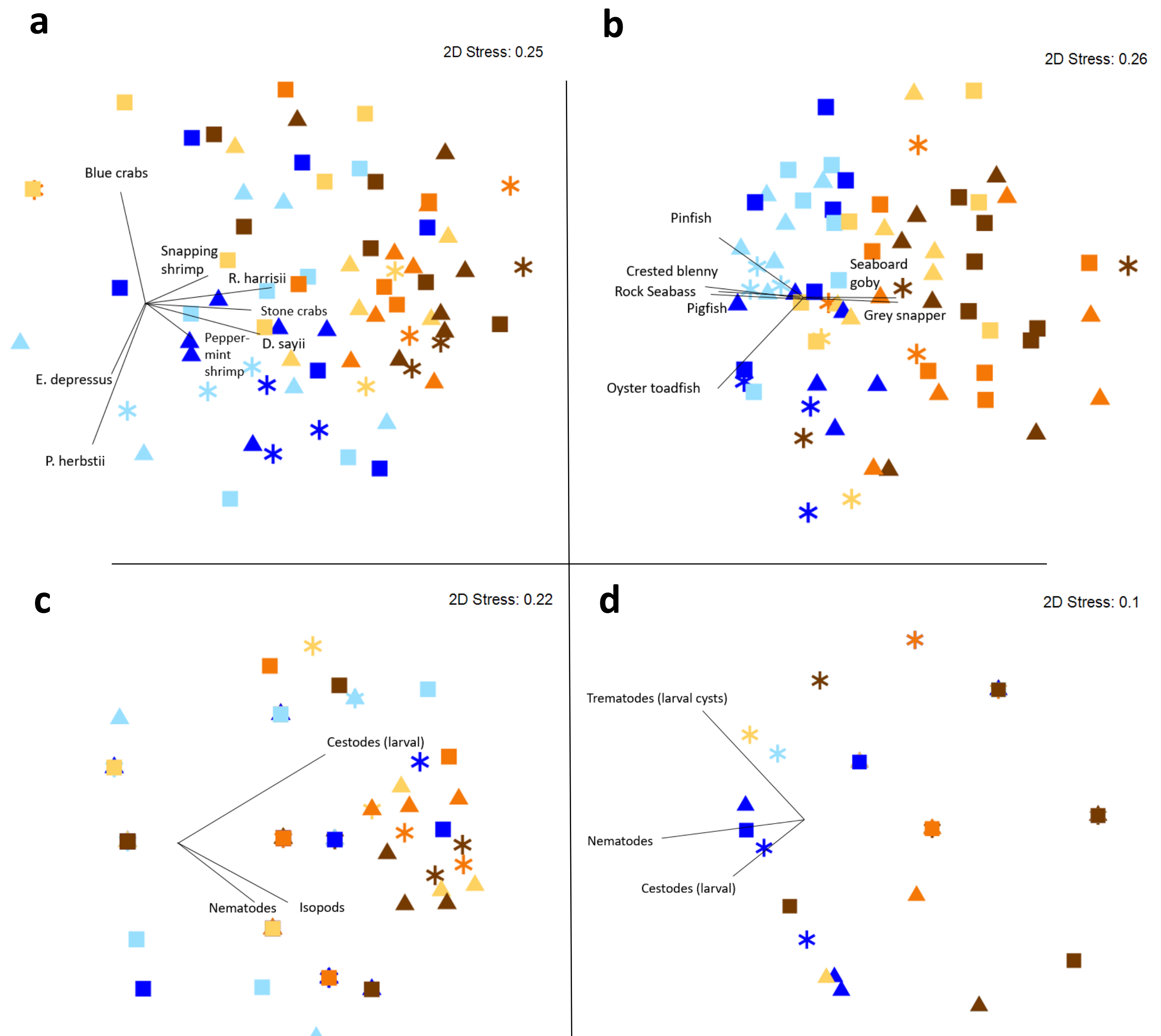


Figure S4. nMDS plots of host-parasite abundance in New (< 10 years), Old (> 20 years), and Natural reefs showing community change over time. Clockwise: plots of crustacean taxa abundance (a), fish taxa abundance (b), crustacean parasite abundance (c), and fish parasite abundance (d). Taxa overlays represent Pearson correlation coefficients ≥ 0.35.

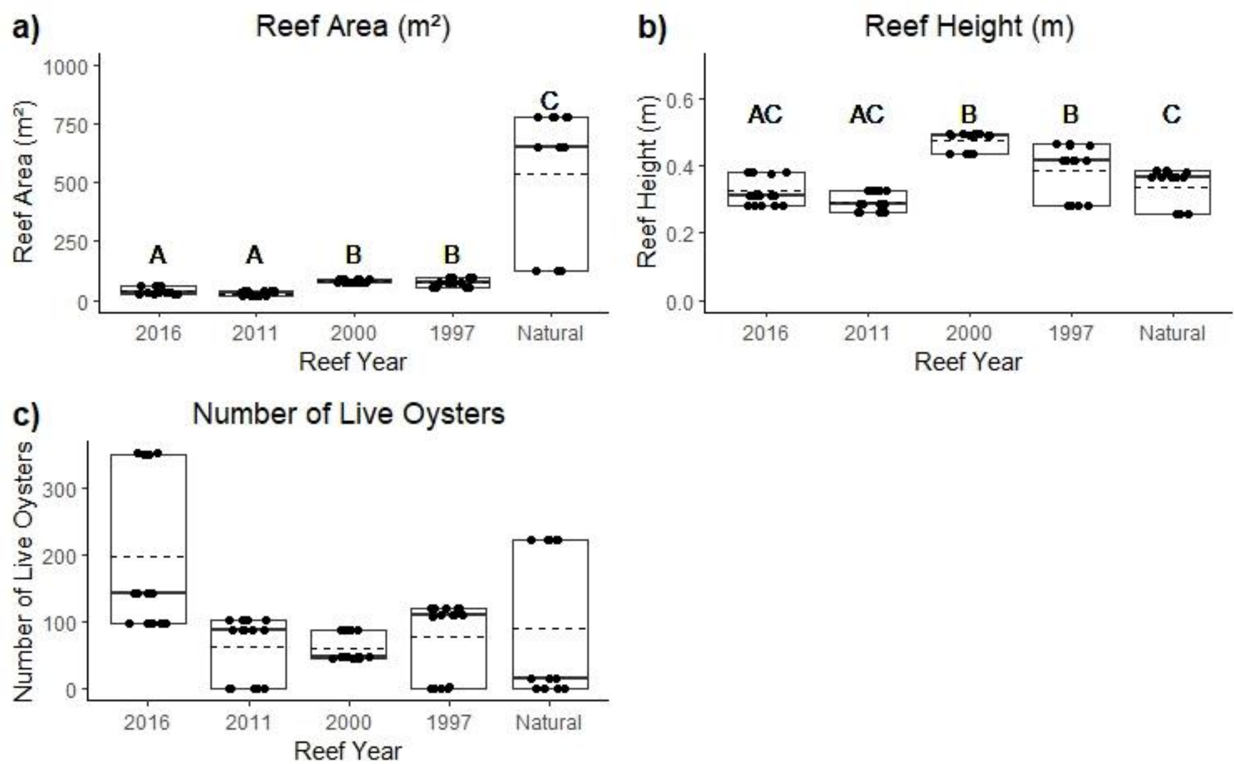


Figure S5. Box plots showing differences in the following habitat parameters: reef area (m^2) (a), reef height (m) (b), and the number of live oysters (c). Solid lines represent the median of each treatment, and dashed lines represent mean estimates. Clockwise: the area of 2016/2011=New reefs (A) was less than the area of 2000/1997=Old reefs (B) (X^2=58.6, df=2, p=9.1x10^{-10}); however, Natural reefs were larger in size than both Old and New reefs (C) (X^2=58.6, df=2, p=2.3x10^{-7}). In terms of height, 2016/2011=New reefs (AC) were smaller than 2000/1997=Old reefs (B) (X^2=29, df=2, p=4.4x10^{-6}), which were taller than Natural reefs ((X^2=29, df=2, p=0.0001) (C). There was no difference in height between New and Natural reefs. There were no differences in the number of live oysters between age groupings.

Supplementary Tables

Table S1. Abiotic measurements collected during each sampling event.

Salinity (ppt)	
Mean (SD)	34.4 (3.01)
Median [Min, Max]	35.5 [30.2, 37.4]
Temperature (°C)	
Mean (SD)	27.2 (3.46)
Median [Min, Max]	27.2 [23.5, 32.0]
Dissolved Oxygen (mg/L)	
Mean (SD)	6.46 (0.901)
Median [Min, Max]	6.43 [5.37, 7.85]

Table S2. Results from Cohen's D (crustacean and fish taxa) and Wilcoxon effect size tests (crustacean and fish parasites). Free-living taxa data are depicted next to the corresponding parasite data to facilitate comparison.

Free-living Crustaceans							Crustacean Parasites						
Group Comparison		p-value	Effect Size	95% CI (low)	95% CI (high)	Magnitude	Group Comparison		p-value	Effect Size	95% CI (low)	95% CI (high)	Magnitude
5	Natural	1	0.1	-0.68	0.8	negligible	5	Natural	0.22	0.44	0.1	0.73	moderate
10	Natural	0.94	-0.26	-1.18	0.51	small	10	Natural	0.64	0.16	0.01	0.49	small
21	Natural	1	0.08	-0.72	0.82	negligible	21	Natural	0.54	0.23	0.01	0.58	small
24	Natural	1	-0.14	-1	0.59	negligible	24	Natural	0.72	0.1	0.0055	0.46	small
10	5	0.84	-0.41	-1.39	0.34	small	10	5	0.54	0.28	0.01	0.64	small
10	21	0.85	-0.38	-1.26	0.32	small	10	21	0.72	0.07	0.0052	0.45	small
10	24	1	-0.15	-0.9	0.7	negligible	10	24	0.72	0.09	0.0059	0.49	small
21	5	1	-0.02	-0.84	0.71	negligible	21	5	0.54	0.21	0.01	0.57	small
21	24	0.97	0.25	-0.43	1.09	small	21	24	0.59	0.18	0.0097	0.54	small
24	5	0.96	-0.27	-1.13	0.45	small	24	5	0.22	0.41	0.07	0.73	moderate

Free-living Fish							Fish Parasites						
Group Comparison		p-value	Effect Size	95% CI (low)	95% CI (high)	Magnitude	Group Comparison		p-value	Effect Size	95% CI (low)	95% CI (high)	Magnitude
5	Natural	0.97	0.23	-0.53	1.11	small	5	Natural	0.02	0.62	0.34	0.86	large
10	Natural	0.94	-0.27	-1.1	0.55	small	10	Natural	0.06	0.51	0.16	0.8	large
21	Natural	0.96	-0.21	-1.13	0.53	small	21	Natural	0.11	0.43	0.08	0.72	moderate
24	Natural	0.83	-0.4	-1.27	0.34	small	24	Natural	0.3	0.29	0.02	0.63	small
10	5	0.61	-0.54	-1.56	0.19	moderate	10	5	0.62	0.1	0.0068	0.47	small
10	21	1	-0.04	-0.82	0.69	negligible	10	21	0.57	0.13	0.0064	0.54	small
10	24	1	0.13	-0.63	0.88	negligible	10	24	0.34	0.24	0.01	0.59	small
21	5	0.67	-0.46	-1.47	0.23	small	21	5	0.34	0.25	0.01	0.6	small
21	24	1	0.15	-0.63	0.91	negligible	21	24	0.57	0.14	0.0065	0.5	small
24	5	0.42	-0.72	-1.77	0.04	moderate	24	5	0.19	0.35	0.03	0.65	moderate

Table S3. Pearson correlation coefficients associated with free-living and parasite communities. Correlations are shown for each nMDS axis and coefficients with an absolute value of **0.35** or higher are shown on nMDS plots (Fig. S4).

Free-living crustaceans

nMDS axis	Atlantic mud crab (*P. herbstii*)	White fingered mud crab (*R. harrissi*)	Flat back mud crab (*E. depressus*)	Say's mud crab (*D. sayii*)	Unknown mud crabs (juv.)	blue crab	stone crab	grass shrimp	snapping shrimp	peppermint shrimp	spider crab	porcelain crab	blueleg pistol shrimp
MDS1	-0.2157343	**0.595458**	-0.1459065	**0.5541956**	**0.488265**	**-0.044**	**0.45469**	0.02907	**0.365877**	0.3049326	-0.0517	0.192578	0.2035472
MDS2	**-0.7418309**	0.06052	**-0.351327**	-0.1796125	-0.0459688	**0.5527**	-0.043	0.06756	0.113187	-0.27763	0.0006	-0.03962	0.127569

Free-living fish

nMDS axis	striped blenny	oyster toadfish	skilletfish	darter goby	rock seabass	pinfish	feather blenny	grey snapper	lane snapper	slippery dick	crested blenny	pigfish	seaboard goby
MDS1	-0.0302814	**-0.47459**	0.1509272	-0.2019809	**-0.3518794**	-0.404	-0.0075	**0.46674**	0.319243	0.2034314	**-0.4605**	**-0.40778**	**0.4492658**
MDS2	-0.180126	**-0.37339**	0.3077703	0.0498562	0.0581532	0.3304	0.24692	-0.0644	0.044808	-0.1465135	0.0833	0.04863	-0.0218777
nMDS axis	seaweed blenny	Nassau grouper	bank butterflyfish	fringed filefish	yellowedge grouper	red grouper	black sea bass	sand perch	spottail pinfish	spot	planehead filefish	mottled mojara	sergeant major
MDS1	-0.2473329	0.107453	0.0482428	0.0399056	-0.1069361	-0.111	0.09815	-0.095	-0.240383	-0.133304	-0.1734	0.129556	0.1358829
MDS2	0.1260796	0.318303	-0.0836354	-0.0862978	-0.1798112	-0.082	-0.23	-0.1407	0.145228	0.2240831	-0.099	-0.06292	0.2390153
nMDS axis	black grouper	striped killifish	jack crevalle	dusky damselfish									
MDS1	-0.1729158	-0.11029	-0.1102859	0.1737883									
MDS2	-0.0017676	-0.26998	-0.2699777	-0.1972268									

Crustacean parasites

nMDS axis	Trematodes (larval cysts)	Cestodes (larval)	Nematodes	Isopods (entoniscid)	*Loxothylacus panopaei*
MDS1	0.1184922	**0.701035**	**0.3974782**	**0.6861579**	0.0564004
MDS2	0.1204186	**0.428862**	-0.2568348	-0.3258426	-0.0539354

Fish Parasites

nMDS axis	Trematodes (larval cysts)	Cestodes (larval)	Trematodes (adult)	Nematodes
MDS1	**-0.4998021**	**-0.62038**	-0.3151432	**-0.8410983**
MDS2	-0.2911265	**0.678579**	-0.0998042	-0.0654139

Table S4. Results from nested PERMANOVA design on Bray-Curtis similarity matrices demonstrating that fish parasite abundance differed as a function of reef age [New (< 10 years), Old (> 20 years), Natural reefs]. Age was a fixed factor and sampling event was included as a random factor within age to avoid pseudo-replication.

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Age	2	6442.5	3221.3	7.5172	0.0103	9939
Sampling event (Age)	12	5121.1	426.75	0.92788	0.5367	9931
Residuals	59	27136	459.92			
Total	73	38981				

PAIR-WISE TESTS: Term: 'Age'

Groups	t	P(perm)	Unique perms
Natural, Old	2.3268	0.0528	1259
Natural, New	4.7571	0.0045	1258
Old, New	1.852	0.1546	126

Table S5. Similarity percentage (SIMPER) results depicting contributions of fish parasite abundance to dissimilarity between age groups.

Group Natural					
Average similarity: 40.69					
Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Cestodes (larval)	0.85	30.63	0.97	75.28	75.28

Group Old					
Average similarity: 15.21					
Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Nematodes	0.35	7.13	0.4	46.84	46.84
Cestodes (larval)	0.27	4.73	0.29	31.08	77.92

Group New					
Average similarity: 5.56					
Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Cestodes (larval)	0.19	4.33	0.25	77.97	77.97

Groups Natural & Old						
Average dissimilarity = 78.00						
	Group: Natural	Group: Old				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Cestodes (larval)	0.85	0.27	38.22	1.09	49	49
Nematodes	0.57	0.35	22.07	1.06	28.3	77.29

Groups Natural & New						
Average dissimilarity = 86.72						
	Group: Natural	Group: New				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Cestodes (larval)	0.85	0.19	48.03	1.27	55.39	55.39
Nematodes	0.57	0.12	22.61	1.11	26.07	81.46

Groups Old & New						
Average dissimilarity = 91.55						
	Group: Old	Group: New				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Cestodes (larval)	0.27	0.19	33.85	0.92	36.97	36.97
Nematodes	0.35	0.12	28.59	1.06	31.23	68.2
Trematodes (larval cysts)	0.26	0.06	22.58	0.71	24.66	92.86

Table S6. Mean abundances (±SD) of site-resident (R) and transient (T) free-living crustaceans and fish grouped by reef age and year.

	1997	**2000**	**2011**	**2016**	**Natural**	**Overall**		
	Old	**Old**	**New**	**New**	**Natural**	**New**	**Old**	**Natural**
	(N=15)	**(N=15)**	**(N=15)**	**(N=15)**	**(N=14)**	**(N=30)**	**(N=30)**	**(N=14)**
Say's mud crab (*D. sayii*) (R)	4.87 (5.18)	4.53 (4.94)	2.64 (4.31)	2.93 (3.20)	5.14 (5.29)	2.79 (3.73)	4.70 (4.98)	5.14 (5.29)
Atlantic mud crabs (*P. herbstii*) (R)	1.47 (1.85)	2.00 (1.93)	1.93 (2.70)	1.00 (1.18)	2.64 (2.10)	1.46 (2.10)	1.73 (1.87)	2.64 (2.10)
White-fingered mud crab (*R. harrissi*) (R)	1.20 (1.66)	1.40 (2.16)	0.286 (0.611)	0.857 (1.70)	1.50 (1.91)	0.571 (1.29)	1.30 (1.90)	1.50 (1.91)
Flat-backed mud crab (*E. depressus*) (R)	0.400 (1.30)	0.533 (1.13)	0.714 (1.07)	0.214 (0.579)	0.429 (0.756)	0.464 (0.881)	0.467 (1.20)	0.429 (0.756)
Unknown mud crabs (juv. *Panopeidae*) (R)	1.67 (2.19)	1.60 (1.84)	0.786 (2.42)	1.00 (1.18)	2.43 (4.18)	0.893 (1.87)	1.63 (1.99)	2.43 (4.18)
Stone crabs (R)	0.600 (1.30)	0.467 (0.640)	0.571 (0.756)	0.429 (0.646)	0.500 (0.855)	0.500 (0.694)	0.533 (1.01)	0.500 (0.855)
Porcelain Crabs (R)	0 (0)	0 (0)	0.143 (0.535)	0 (0)	0 (0)	0.0714 (0.378)	0 (0)	0 (0)
Snapping Shrimp (R)	2.60 (1.72)	1.87 (1.68)	1.57 (1.74)	2.50 (1.95)	1.79 (1.85)	2.04 (1.88)	2.23 (1.72)	1.79 (1.85)
Blue Crabs (T)	0.533 (1.13)	0.0667 (0.258)	1.21 (1.31)	1.50 (1.40)	0.214 (0.426)	1.36 (1.34)	0.300 (0.837)	0.214 (0.426)
Spider Crabs (T)	0 (0)	0 (0)	0 (0)	0.0714 (0.267)	0 (0)	0.0357 (0.189)	0 (0)	0 (0)
Grass Shrimp (T)	0.200 (0.561)	0.400 (1.06)	0.643 (1.60)	0.286 (0.825)	0.143 (0.363)	0.464 (1.26)	0.300 (0.837)	0.143 (0.363)
Peppermint Shrimp (T)	0.267 (1.03)	0.467 (0.834)	0.214 (0.579)	0.357 (0.633)	1.00 (1.47)	0.286 (0.600)	0.367 (0.928)	1.00 (1.47)
Blueleg Pistol Shrimp (T)	0 (0)	0 (0)	0 (0)	0 (0)	0.214 (0.802)	0 (0)	0 (0)	0.214 (0.802)
Striped Blennies (R)	0.0667 (0.258)	0.0667 (0.258)	0 (0)	0.0714 (0.267)	0.0714 (0.267)	0.0357 (0.189)	0.0667 (0.254)	0.0714 (0.267)
Oyster Toadfish (R)	1.00 (1.00)	1.07 (1.39)	0.143 (0.363)	0.714 (0.994)	2.43 (2.10)	0.429 (0.790)	1.03 (1.19)	2.43 (2.10)

	1997	2000	2011	2016	Natural	Overall		
	Old	Old	New	New	Natural	New	Old	Natural
	(N=15)	(N=15)	(N=15)	(N=15)	(N=14)	(N=30)	(N=30)	(N=14)
Skilletfish (R)	0 (0)	0.0667 (0.258)	0.214 (0.579)	0.143 (0.363)	0 (0)	0.179 (0.476)	0.0333 (0.183)	0 (0)
Darter Gobies (R)	0.133 (0.516)	0.200 (0.561)	0.0714 (0.267)	0.0714 (0.267)	0 (0)	0.0714 (0.262)	0.167 (0.531)	0 (0)
Feather Blennnies (R)	0 (0)	0.133 (0.352)	0.643 (1.34)	0.571 (1.87)	0.357 (0.633)	0.607 (1.59)	0.0667 (0.254)	0.357 (0.633)
Crested Blenny (R)	4.53 (5.59)	2.20 (2.73)	4.79 (7.97)	5.57 (8.49)	3.71 (5.98)	5.18 (8.09)	3.37 (4.48)	3.71 (5.98)
Seaboard Gobies (R)	0.667 (0.900)	0.800 (1.08)	1.00 (1.66)	1.57 (2.24)	0.143 (0.535)	1.29 (1.96)	0.733 (0.980)	0.143 (0.535)
Rock Seabass (T)	0.0667 (0.258)	0.0667 (0.258)	0.0714 (0.267)	0.0714 (0.267)	0.0714 (0.267)	0.0714 (0.262)	0.0667 (0.254)	0.0714 (0.267)
Pinfish (T)	4.53 (10.4)	4.47 (5.24)	2.00 (2.73)	4.13 (4.50)	5.07 (9.08)	3.07 (3.81)	4.50 (8.06)	5.07 (9.08)
Grey Snapper (T)	0.133 (0.352)	0.0667 (0.258)	0.214 (0.579)	0.571 (1.02)	0.0714 (0.267)	0.393 (0.832)	0.100 (0.305)	0.0714 (0.267)
Lane Snapper (T)	0.267 (0.799)	0 (0)	0.333 (1.05)	0.200 (0.414)	0.714 (1.44)	0.267 (0.785)	0.133 (0.571)	0.714 (1.44)
Slippery Dick (T)	1.13 (3.04)	0.733 (1.22)	0.267 (0.458)	1.80 (3.26)	1.00 (3.46)	1.03 (2.41)	0.933 (2.29)	1.00 (3.46)
Pigfish (T)	0.400 (0.910)	0.267 (0.594)	0.333 (1.05)	0.200 (0.414)	0.286 (0.825)	0.267 (0.785)	0.333 (0.758)	0.286 (0.825)
Seaweed Blennies (T)	0 (0)	0.200 (0.561)	0 (0)	0.0714 (0.267)	0.214 (0.802)	0.0357 (0.189)	0.100 (0.403)	0.214 (0.802)
Nassau Grouper (T)	0.0667 (0.258)	0.133 (0.352)	0.0714 (0.267)	0.143 (0.363)	0.0714 (0.267)	0.107 (0.315)	0.100 (0.305)	0.0714 (0.267)
Bank Butterflyfish (T)	0 (0)	0.0667 (0.258)	0.0714 (0.267)	0 (0)	0.0714 (0.267)	0.0357 (0.189)	0.0333 (0.183)	0.0714 (0.267)
Fringed Filefish (T)	0 (0)	0 (0)	0 (0)	0 (0)	0.0714 (0.267)	0 (0)	0 (0)	0.0714 (0.267)
Yellowedge Grouper (T)	0 (0)	0 (0)	0 (0)	0 (0)	0.0714 (0.267)	0 (0)	0 (0)	0.0714 (0.267)

	1997	2000	2011	2016	Natural	Overall		
	Old	**Old**	**New**	**New**	**Natural**	**New**	**Old**	**Natural**
	(N=15)	**(N=15)**	**(N=15)**	**(N=15)**	**(N=14)**	**(N=30)**	**(N=30)**	**(N=14)**
Red Grouper (T)	0 (0)	0 (0)	0 (0)	0.0714 (0.267)	0.0714 (0.267)	0.0357 (0.189)	0 (0)	0.0714 (0.267)
Black Seabass (T)	0.733 (1.79)	0.133 (0.352)	0.200 (0.414)	0.0667 (0.258)	0 (0)	0.133 (0.346)	0.433 (1.30)	0 (0)
Sand Perch (T)	0 (0)	0 (0)	0 (0)	0 (0)	0.0714 (0.267)	0 (0)	0 (0)	0.0714 (0.267)
Spottail Pinfish (T)	0.133 (0.516)	0.0667 (0.258)	0.133 (0.352)	0.133 (0.352)	0.143 (0.363)	0.133 (0.346)	0.100 (0.403)	0.143 (0.363)
Spot (T)	0 (0)	0.133 (0.516)	0.0667 (0.258)	0 (0)	0 (0)	0.0333 (0.183)	0.0667 (0.365)	0 (0)
Planehead Filefish (T)	0 (0)	0 (0)	0 (0)	0 (0)	0.0714 (0.267)	0 (0)	0 (0)	0.0714 (0.267)
Mottled Mojara (T)	0 (0)	0 (0)	0.0667 (0.258)	0 (0)	0 (0)	0.0333 (0.183)	0 (0)	0 (0)
Sergeant Major (T)	0 (0)	0 (0)	0.267 (1.03)	0 (0)	0 (0)	0.133 (0.730)	0 (0)	0 (0)
Black Grouper (T)	0 (0)	0.0667 (0.258)	0 (0)	0 (0)	0 (0)	0 (0)	0.0333 (0.183)	0 (0)
Striped Killifish (T)	0 (0)	0 (0)	0 (0)	0 (0)	0.0714 (0.267)	0 (0)	0 (0)	0.0714 (0.267)
Jack Crevalle (T)	0 (0)	0 (0)	0 (0)	0 (0)	0.0714 (0.267)	0 (0)	0 (0)	0.0714 (0.267)
Dusky Damselfish (T)	0.0667 (0.258)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.0333 (0.183)	0 (0)

Table S7. Summary statistics for measurements of reef habitat complexity grouped by New (< 10 years), Old (> 20 years), and Natural reefs and associated statistical test results.

	New	**Old**	**Natural**
Reef Area (m^2)			
Mean (SD)	34.3 (13.9)	78.2 (12.7)	538 (293)
Median [Min, Max]	30.6 [16.1, 60.0]	77.6 [56.2, 97.5]	649 [124, 781]
Reef Height (m)			
Mean (SD)	0.308 (0.0387)	0.431 (0.0740)	0.338 (0.0563)
Median [Min, Max]	0.298 [0.261, 0.379]	0.450 [0.281, 0.497]	0.365 [0.258, 0.384]
Live Oyster Count (n)			
Mean (SD)	130 (110)	68.7 (42.1)	90.0 (109)
Median [Min, Max]	100 [0, 351]	67.5 [1.00, 121]	15.0 [0, 222]

Kruskall-Wallis				Wilcoxon post-hoc	
Variable	**Chi-sq**	**df**	**p-value**	**Comparison**	**p-value**
Reef area (m^2)	58.6	2	1.862e-13	Old and New	9.1e-10
				Natural and New	2.3e-07
				Natural and Old	2.3e-07
Reef height (m)	29.0	2	5.162e-07	Old and New	4.4e-06
				Natural and New	0.1878
				Natural and Old	0.0001
Live oyster count (n)	3.49	2	0.1739	Old and New	0.19
				Natural and New	0.44
				Natural and Old	0.51

Table S8. All parasitized fish species and parasite taxa sampled from New (< 10 years), Old (> 20 years), and Natural reefs. Fish parasite numbers refer to the number of infected hosts.

	New (N=16)	Old (N=30)	Natural (N=32)	Overall (N=78)
Fish Species				
Oyster toadfish	4 (25.0%)	20 (66.7%)	32 (100%)	56 (71.8%)
Crested blenny	9 (56.3%)	8 (26.7%)	0 (0%)	17 (21.8%)
Seaboard goby	3 (18.8%)	1 (3.33%)	0 (0%)	4 (5.13%)
Striped blenny	0 (0%)	1 (3.33%)	0 (0%)	1 (1.28%)
Fish Parasites				
Adult trematodes	2 (12.5%)	0 (0%)	2 (6.25%)	4 (5.13%)
Larval cestodes	5 (31.3%)	8 (26.7%)	16 (50.0%)	29 (37.2%)
Larval trematodes	6 (37.5%)	12 (40.0%)	2 (6.25%)	20 (25.6%)
Nematodes	3 (18.8%)	10 (33.3%)	11 (34.4%)	24 (30.8%)
Acanthocephalans	0 (0%)	0 (0%)	1 (3.13%)	1 (1.28%)
Site				
1S5 2011	12 (75.0%)	0 (0%)	0 (0%)	12 (15.4%)
2S5 2011	2 (12.5%)	0 (0%)	0 (0%)	2 (2.56%)
CCA2 2016	1 (6.25%)	0 (0%)	0 (0%)	1 (1.28%)
CCA3 2016	1 (6.25%)	0 (0%)	0 (0%)	1 (1.28%)
Mudflat Reef 1 1997	0 (0%)	9 (30.0%)	0 (0%)	9 (11.5%)
Mudflat Reef 1 2000	0 (0%)	5 (16.7%)	0 (0%)	5 (6.41%)
Mudflat Reef 2 1997	0 (0%)	5 (16.7%)	0 (0%)	5 (6.41%)
Mudflat Reef 2 2000	0 (0%)	3 (10.0%)	0 (0%)	3 (3.85%)
Mudflat Reef 3 1997	0 (0%)	4 (13.3%)	0 (0%)	4 (5.13%)
Mudflat Reef 3 2000	0 (0%)	4 (13.3%)	0 (0%)	4 (5.13%)
Natural Reef 1	0 (0%)	0 (0%)	7 (21.9%)	7 (8.97%)
Natural Reef 2	0 (0%)	0 (0%)	5 (15.6%)	5 (6.41%)
Natural Reef 3	0 (0%)	0 (0%)	20 (62.5%)	20 (25.6%)

Table S9. Toadfish parasites grouped by sexual maturity of toadfish hosts (top) and reef age (bottom).

	Toadfish Sexual Maturity		
	Adult (N=45)	**Juvenile (N=11)**	**Overall (N=56)**
# Toadfish Infected			
Acanthocephalans	1 (2.22%)	0 (0%)	1 (1.79%)
Adult trematodes	2 (4.44%)	0 (0%)	2 (3.57%)
Larval cestodes	18 (40.0%)	8 (72.7%)	26 (46.4%)
Larval trematodes	5 (11.1%)	0 (0%)	5 (8.93%)
Nematodes	19 (42.2%)	3 (27.3%)	22 (39.3%)

	New (N=4)	**Old (N=20)**	**Natural (N=32)**	**Overall (N=56)**
# Toadfish Infected				
Larval cestodes	3 (75.0%)	7 (35.0%)	16 (50.0%)	26 (46.4%)
Nematodes	1 (25.0%)	10 (50.0%)	11 (34.4%)	22 (39.3%)
Larval trematodes	0 (0%)	3 (15.0%)	2 (6.25%)	5 (8.93%)
Adult trematodes	0 (0%)	0 (0%)	2 (6.25%)	2 (3.57%)
Acanthocephalans	0 (0%)	0 (0%)	1 (3.13%)	1 (1.79%)
Toadfish Sexual Maturity				
Adult	3 (100%)	10 (90.9%)	12 (60.0%)	25 (73.5%)
Juvenile	0 (0%)	1 (9.09%)	8 (40.0%)	9 (26.5%)
Toadfish length (TL, mm)				
Mean (SD)	126 (6.00)	129 (23.4)	107 (40.6)	116 (35.1)
Median [Min, Max]	126 [120, 132]	135 [69.0, 156]	128 [29.0, 154]	130 [29.0, 156]
Site				
1S5 2011	3 (75.0%)	0 (0%)	0 (0%)	3 (5.36%)

Site	New (N=4)	Old (N=20)	Natural (N=32)	Overall (N=56)
CCA2 2016	1 (25.0%)	0 (0%)	0 (0%)	1 (1.79%)
Mudflat Reef 1 1997	0 (0%)	3 (15.0%)	0 (0%)	3 (5.36%)
Mudflat Reef 1 2000	0 (0%)	5 (25.0%)	0 (0%)	5 (8.93%)
Mudflat Reef 2 1997	0 (0%)	4 (20.0%)	0 (0%)	4 (7.14%)
Mudflat Reef 2 2000	0 (0%)	2 (10.0%)	0 (0%)	2 (3.57%)
Mudflat Reef 3 1997	0 (0%)	4 (20.0%)	0 (0%)	4 (7.14%)
Mudflat Reef 3 2000	0 (0%)	2 (10.0%)	0 (0%)	2 (3.57%)
Natural Reef 1	0 (0%)	0 (0%)	7 (21.9%)	7 (12.5%)
Natural Reef 2	0 (0%)	0 (0%)	5 (15.6%)	5 (8.93%)
Natural Reef 3	0 (0%)	0 (0%)	20 (62.5%)	20 (35.7%)

Appendix A: IACUC Approval Letter

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

February 3, 2017

April Blakeslee, Ph.D.
Department of Biology
Howell Science Building
East Carolina University

Dear Dr. Blakeslee:

Your Animal Use Protocol entitled, "The Ecological Role of Naked Goby *Gobiosoma bosc* Fish in Host-Parasite Networks in the Pamlico-Neuse River Estuaries" (AUP #D346) was reviewed by this institution's Animal Care and Use Committee on February 3, 2017. The following action was taken by the Committee:

"Approved as submitted"

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

Enclosure

EAST CAROLINA UNIVERSITY
ANIMAL USE PROTOCOL (AUP) FORM
LATEST REVISION NOVEMBER, 2013

Project Title:

The ecological role of naked goby *Gobiosoma bosc* fish in host-parasite networks in the Pamlico-Neuse river estuaries

	Principal Investigator	Secondary Contact
Name	Dr. April Blakeslee	Christopher Moore
Dept.	Biology	Biology
Office Ph #	252-737-5255	252-737-5255
Cell Ph #	443-366-4555	336-577-6204
Pager #	Click here to enter text.	Click here to enter text.
Home Ph #	Click here to enter text.	Click here to enter text.
Email	Blakesleeap14@ecu.edu	Moorech16@students.ecu.edu

For IACUC Use Only

AUP # D346			
New/Renewal New 2/3/17			
Full Review/Date	DR/Date		
Approval Date 2/3/17			
Study Type diversity- habitat			
Pain/Distress Category C			
Surgery	Survival	Multiple	
Prolonged Restraint			
Food/Fluid Regulation			
Other			
Hazard Approval/Dates	Rad	IBC	EHS
OHP Enrollment			clove oil
Mandatory Training			
Amendments Approved			

Satellite animal facility - 5103 Howell

Appendix B: Scientific and/or Collection Permits

The following scientific and/or collection permits were issued for the research in this dissertation:

- Field collections were authorized by the North Carolina Division of Marine Fisheries (Scientific or Educational Permit #706671). The attached copy of this permit is from 2016, the first year of the author's research. DMF reauthorized this permit every year through the conclusion of the author's work in 2022.

- The author supervised the thesis research of a student in the Honor's College at ECU who studied the effects of elastomer tagging on naked goby fish. The North Carolina Department of Natural and Cultural Resources authorized this research at Goose Creek State Park (permit # 2018_0236).

- The author conducted research in the Carrot Island portion of the Rachel Carson Estuarine Reserve. This work was funded jointly by North Carolina Sea Grant and the North Carolina Coastal Reserve. This work was permitted by Dr. Brandon Puckett, Research Coordinator for the Reserve (permit # 1-2018).

- The author conducted research in the Middle Marsh portion of the Rachel Carson Estuarine Reserve. This work was funded by the Garden Club of North America. This work was permitted by Dr. Brandon Puckett, Research Coordinator for the Reserve (permit # 13-2019).

North Carolina Division of Marine Fisheries

Proof of Purchase

UPDATE : Scientific or Educational Collection Permit : Permit Number 706671

Permit Number : 706671 **NC Residency :** **Sales Outlet :** DMF Wilmington Office
Permit Year : 2016 **Qualifying Product :** **Terminal Number :** MJCHUFFO

Effective Date/Time : 02/19/2016 08:44 **Fee :** 0.00
Expiration Date/Time : 12/31/2016 23:59 **Status :** Active
Issue Date/Time : 02/19/2016 08:44 **Status Date :** 02/19/2016

Permit Holder : 660357 EAST CAROLINA UNIVERSITY (HARGITTAI) **Business Type:**

Physical Address : DEPT. OF BIOLOGY MAIL STOP 551, HOWELL SCIENCE COMPLEX, GREENVILLE, NC, 27858-4353, United States
County : Pitt

Mailing Address : DEPT. OF BIOLOGY MAIL STOP 551, HOWELL SCIENCE COMPLEX, GREENVILLE, NC, 27858-4353, United States
County : Pitt

Race : **Eyes :** **Weight :**
Gender : **Date of Birth :** **Hair :** **Height :** ft. Inches

Home Phone : **Primary Residence :** NC **Prior Names :**
Business Phone : (252) 328-6355 **Secondary Residence :**
Fax : (252) 328-4178
Ids : **E-Mail :**

Business Agent : 1066638 HARGITTAI, PAL TIBOR

Physical Address : 213 GLORIA STREET, GREENVILLE, NC, 27858, United States
County : Pitt

Mailing Address :
County :

Race : Other **Eyes :** Brown **Weight :** 160
Gender : Male **Date of Birth** 05/13/1951 **Hair :** Brown **Height :** 5 ft. 10 Inches

Home Phone : (252) 756-7691 **Primary Residence :** **Prior Names :**
Business Phone : (252) 328-6355 **Secondary Residence :**
Fax : (252) 328-4178
Ids : **E-Mail :** HARGITTAIP@ECU.EDU

Contact Information

Contact Person	Contact Person DOB	Contact Person Telephone #
PAL TIBOR HARGITTAI	05/13/1951	(252) 756-7691

Purpose of Collection

- [X] Research
- [X] Teaching Specimens
- [X] Educational Display (Aquariums)
- [ ] Other (specify)

Collectors

North Carolina Division of Marine Fisheries

Proof of Purchase

UPDATE : Scientific or Educational Collection Permit : Permit Number 706671

Permit Number : 706671 **NC Residency :** **Sales Outlet :** DMF Wilmington Office
Permit Year : 2016 **Qualifying Product :** **Terminal Number :** MJCHUFFO

Effective Date/Time : 02/19/2016 08:44 **Fee :** 0.00
Expiration Date/Time : 12/31/2016 23:59 **Status :** Active
Issue Date/Time : 02/19/2016 08:44 **Status Date :** 02/19/2016

JILLIAN OSBORN	**12/12/1988**	**(704) 232-5172**
APRIL BLAKESLEE	**04/16/1976**	**(443) 366-4555**
CHRISTOPHER MOORE	**10/05/1982**	**(336) 577-6204**
REBECCA BARNARD	**08/17/1991**	**(508) 523-9978**

Restriction Information

Restriction Type : **RESTRICT TO**
Gear Code : Gear Quantity : Gear Units :
Species Group : Species Quantity : Species Size : Species Units :
Water Body :
Time :
Start Time: End Time:
Comments: **GEARS OR METHODS: GILL NET; TRAWL; BEACH SEINE; DREDGE; CAST NET; POUND NET; ROTENONE (CONTAINED); FYKE NET; HOOK AND LINE**

Restriction Type : **RESTRICT TO**
Gear Code : Gear Quantity : Gear Units :
Species Group : Species Quantity : Species Size : Species Units :
Water Body :
Time :
Start Time: End Time:
Comments: **WATERBODIES: ALL COASTAL WATERS IN NORTH CAROLINA**

Restriction Type : **RESTRICT FROM**
Gear Code : Gear Quantity : Gear Units :
Species Group : Species Quantity : Species Size : Species Units :
Water Body :
Time :
Start Time: End Time:
Comments: **NO NIGHT OR POLLUTED AREA SHELLFISHING.**

Request To Sell? ☐ **Authorized to Sell?** ☐

Issued By: [signature] **Issued Date:** 5/5/16

North Carolina Department of Natural and Cultural Resources
Office of the Secretary

Governor Roy Cooper | Secretary Susi H. Hamilton

Monday, May 7th, 2018

Dr. April Blakeslee
East Carolina University
E 10th Street, Howell Science Bldg.
Biology Department
Greenville, NC 27858

Dear Dr. Blakeslee:

Your project entitled "Assessing Movement Patterns of Naked Goby Fish in Mallard Creek" at Goose Creek State Park has been approved. Your permit is enclosed (2018_0236). Please be sure to consult with park staff before making site visits, and to carry a copy of your permit (including project description) with you during your research activities.

Thank you for your interest in conducting your research in North Carolina State Parks. If you have any questions regarding your permit, please contact Ed Corey of our Natural Resources Program, at 919-841-4037, or Ed.Corey@ncparks.gov.

Sincerely,

Jerry D. Lequire

JL/jl

Attachment

cc: Ed Corey

MAILING ADDRESS:
4601 Mail Service Center
Raleigh, NC 27699-4600

Telephone: (919) 807-7250
Fax: (919) 733-1564

LOCATION:
109 East Jones Street
Raleigh, NC 27601

Research Permit

N.C. Coastal Reserve & National Estuarine Research Reserve

Brandon Puckett, Research Coordinator
101 Pivers Island Road
Beaufort, NC 28516
Phone: 252-838-0851
Email: brandon.puckett@ncdenr.gov
Fax: 252-838-0890

Date: Month/Day/Year 12/7/2017

Principle Investigator name and title: Dr. April Blakeslee

Address: Biology Department, ECU; E 10th St., Howell Science Bldg.

City: Greenville State: NC Zip Code: 27858

Email: blakesleeap14@ecu.edu

Phone: 252-737-5255 Fax:

Designated contact person and address (if different from above):
Christopher Moore; 2611 Tryon Dr. Greenville, NC 27858

Students, please provide the following:

Major advisor: Dr. April Blakeslee

School: East Carolina University

Degree sought: PhD

Expected number of participants for field work at Reserve: 7-8

Project Title: Parasites as Novel Indicators of Biodiversity in Restored Coastal Habitats

Funding sources: 2018 NC Sea Grant Coastal Research Fellowship (in review); additional material support provided by the Blakeslee lab

Funded amount/year: $10,000 (fellowship proposal pending)

Project Duration: 1 year fellowship period with 3 additional years of sampling planned using future funding sources

---

Project Description (please fill out A-D or attach a concise 1-2 page project summary):

A. Abstract

In order to evaluate the success of coastal habitat restoration, we will use the diversity of trophically transmitted parasites (those requiring multiple hosts, e.g. trematodes, acanthocephalans, cestodes) in common intermediate hosts as proxies for overall community biodiversity. There is mounting evidence of the important keystone role that parasites play in marine food webs, and research suggests that they can greatly enhance ecosystem connectedness and stability because of the links they form with other host taxa.[1] Moreover, trophically transmitted parasites can serve as sentinels of community biodiversity in marine ecosystems and have proven effective for monitoring and evaluating the recovery of salt marsh and other habitats following significant human-mediated disturbance.[2] Likewise, the abundance of trophically transmitted parasites in intermediate hosts has been found to reflect patterns of land use and overall animal biodiversity[3] in organisms as diverse as shorebirds, diamondback terrapins,[4] and small fishes and benthic invertebrates.5 From both a management and ecological standpoint, it is thus more efficient to survey parasites in abundant upstream hosts (e.g., mollusks, crustaceans, benthic fish) as opposed to surveying intensively for an elusive final host. We will therefore use parasites as proxies for community biodiversity to assess restoration success at Carrot Island in the Rachel Carson Reserve.

B. Sampling locations (list both Reserve and non-Reserve sampling locations)

• Reserve: Most of the sampling for this project will be conducted on Carrot Island within the Rachel Carson Reserve. Five shoreline treatments will be sampled on Carrot Island, and each treatment will have three replicates. These five treatments are: 1) Natural "living shoreline; 2) fringing marsh without reef; 3) restored creek with loose cultch; 4) shoreline stabilized with oyster catcher reef material; 5) shoreline stabilized with oyster bags.

• Non-reserve: The mainland side of Taylor's Creek will be sampled in the vicinity of Fisherman's Park. Specific locations along the north side of Taylor's Creek will be identified at a later date.

C. Project Objectives

Collectively, these data will be used to determine the efficacy of specific materials (e.g. oyster reef/bags, oyster sills, burlap, native marsh plantings) in restoring or enhancing biodiversity. This project will also provide initial data on post-restoration biodiversity. Monitoring will continue over the next 3 years as part of the applicant's dissertation research.

D. Methods

Trophically transmitted parasites utilize a wide array of marine taxa to further their life cycles including invertebrates like mollusks, crustaceans, and worms, and vertebrates like fish, birds, and reptiles.[1] In order to assess community diversity using parasite diversity as a proxy, select organisms common to the region and known to serve as intermediate hosts for trophically transmitted parasites (e.g. mud snails, Panopeid mud crabs, gobioid fishes) will be collected at sample locations using transect-quadrat sampling for snails (see below) and passive collectors for crabs and fish. Collectors are small plastic milk crates (19 x 22 x 16 cm) filled with autoclaved oyster shell, and are modeled on the successful methodology employed by the Smithsonian Environmental Research Center.[2] Although organisms can freely move inside and outside the crates, they are attracted to the complex three-dimensional habitat that the shell provides (Moore & Blakeslee, unpub). Each crate will be zip-tied to 0.75m wooden stakes secured in the shallow subtidal zone. Mud crabs and small benthic fish will be collected by sorting the contents of each crate using a large sieve (56 x 56 x 13 cm) with 2 mm mesh. An additional common intermediate host, the eastern mudsnail Ilyanassa obsoleta, will also be collected at each site in the lower intertidal zone within the vicinity of each crate. We will collect ~100 snails per site using a randomized quadrat sampling design (with 5 total quadrats) along a 30-m transect running parallel to the shoreline at low tide. As seabird diversity has been found to correlate with parasite diversity in first-intermediate hosts,[3] we will also obtain some understanding of final host abundance and diversity at a site by identifying and counting birds during the first and last 10 minutes of sampling. We will also take abiotic measurements at each site, including water temperature, salinity, dissolved oxygen, pH, and wave exposure. Following field sampling, organisms will be transported to the Blakeslee Lab at East Carolina University (ECU), where they will be assessed for parasite diversity using standard protocols and keys.[4] When necessary, we will use standard barcoding markers to identify some parasite taxa.[5]

[1] Huspeni TC et al...In: Bortone SA, ed. Estuarine Indicators (2005); [2] Roche DG et al...Aquat Inv (2007); 3 Byers JE et al....Ecology (2008); [4] Blakeslee AMH et al... J of Biogeography (2012); [5] Phelan K et al...Parasitol Res. (2016).

Additional Questions:

How were you informed of the requirement to apply for a research permit? X Reserve staff ☐ Reserve website

☐ Colleagues ☐ Other [please specify] ____________________

Are you aware of the Reserve's long-term water quality and meteorological monitoring data? No, not aware

If so, what data, if any, do you plan to use for your research (e.g., meteorological data from Masonboro Island)?

____

If not, please contact us or visit http://cdmo.baruch.sc.edu/ for more details.

☒ By checking this box, the applicant agrees to (1) adhere to the research permit terms and conditions listed in the permit guidelines, including the submission of a Final Report at the conclusion of the project and (2) keep Reserve staff appraised of permit deviations.

Please mail or email this application to Brandon Puckett at the contact information above.

Applicant Signature: April Blakeslee & Christopher Moore Date: December 7, 2017
Note: Typed name will serve as an official electronic signature.

Please include any attachments with your Research Permit Application.

Permit # (to be filled in by Reserve Staff):	#1-2018

Reserve Research Staff Approval: ______________ Date: 1/2/2018

Permit Expiration Date (maximum duration = 3 years, may be renewed): 3 years; 1/1/2021

Research Permit

N.C. Coastal Reserve & National Estuarine Research Reserve
deq.nc.gov/coastalreserve

Submit this form to research coordinator Brandon Puckett (brandon.puckett@ncdenr.gov).

Name: Christopher Moore

☑ Check if you are a student Advisor: ________ Degree: Ph.D. (in progress)

Institution: East Carolina University

Email: moorech16@students.ecu.edu Phone: 336-577-6204

Address 2611 Tryon Drive, Greenville, NC 27858

Project Title: Parasites as Indicators of Biodiversity in Restored Oyster Reefs

Duration of field work: Summer 2019

Project completed (final report submitted): No

Funding (source & annual amount): Garden Club Fellowship in Ecological Restoration ($8,000)

Project Contact (if different from above): ________

Email: ________

Expected number of participants for field work at Reserve site: 4-5

How were you informed of the requirement to apply for a research permit?

☑ Reserve staff ☐ Reserve website ☐ Colleagues ☐ Other: ________

Are you aware of the Reserve's long-term water quality and meteorological monitoring data? ☑ yes ☐ no

If so, what data, if any, do you plan to use for your research (e.g., meteorological data from Masonboro Island)? No plan at present to incorporate these data

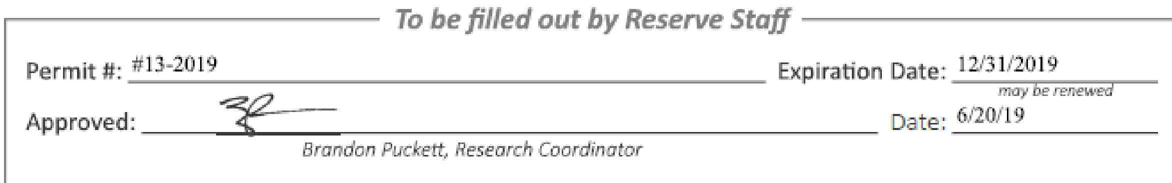
If not, please contact us or visit the NERRS Centralized Data Management Office.

☑ I have attached a 1-2 page project description that includes project objectives, sampling locations (GPS locations of sites within Reserve and list of sites outside of Reserve sites), and research methods.

☑ I agree to (1) adhere to the research permit terms and conditions listed in the permit guidelines, including submitting Final Report at the end of the project, and (2) notify Reserve staff of any permit deviations.

Signature: Christopher Moore Date: 6.7.19

Typed name will serve as an official electronic signature.



To be filled out by Reserve Staff

Permit #: #13-2019 Expiration Date: 12/31/2019 *may be renewed*

Approved: [signature] Date: 6/20/19

Brandon Puckett, Research Coordinator