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THEME SECTION

Invasion of Atlantic coastal ecosystems by Pacific lionfish

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This Theme Section focuses on understanding the ecological effects of the devastating marine invasion of Atlantic coastal ecosystems by Pacific lionfish (*Pterois volitans/miles*), reasons why lionfish are such successful invaders, and practical means of managing the invasion.

Photo: Jens Petersen

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Contribution to the Theme Section 'Invasion of Atlantic coastal ecosystems by Pacific lionfish'

INTRODUCTION

Lionfish: a major marine invasion

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ABSTRACT: Indo-Pacific lionfish *Pterois volitans/miles* were likely introduced to Florida coastal waters via the aquarium trade and have spread rapidly along the southeastern coast of the United States and throughout the greater Caribbean region, including Bermuda and the Gulf of Mexico. This mesopredator has strong consumptive effects on native demersal fishes, especially on coral reefs but also including a variety of other nearshore habitats. The invader may also have substantial indirect effects on reef ecosystems by overconsuming ecologically important species. Given growing concern over what is likely the most damaging marine fish invasion to date globally, this Theme Section presents findings reported during a lionfish symposium at the Gulf and Caribbean Fisheries Institute annual conference convened in Panama City, Panama, in November 2015. New findings include mechanisms that enhance the success of the invader, the extremely broad and variable diet of invasive lionfish, the ecological effects of the invader on native fish populations in various environmental contexts, and non-consumptive interactions between invasive lionfish and native predators.

KEY WORDS: Lionfish · Invasion · Predation · Reef fishes

The invasion of tropical and subtropical Western Atlantic coastal ecosystems by Indo-Pacific lionfish *Pterois volitans/miles* (Fig. 1) first became apparent in the early 2000s when multiple individuals were documented off the coast of North Carolina (Whitfield et al. 2002). Lionfish were likely introduced through aquarium releases (Semmens et al. 2004), and not by Hurricane Andrew (www.sciencemag.org/news/2010/04/mystery-lionfish-dont-blame-hurricane-andrew). Their geographic spread has been rapid and broad: up the eastern seaboard of the United States as far north as Rhode Island (summer recruits dying back to Cape Hatteras during the winter), across to Bermuda, throughout the Bahamas and the greater Caribbean region to Brazil, and into the Gulf of Mexico (Schofield 2010). Invaded habitats include coral reefs, other hard seafloors, seagrass beds, mangroves, river estuaries, and various artificial structures, and individuals have been sighted from manned submersibles as deep as 300 m (reviews by Morris 2012, Albins & Hixon 2013, Côté et al. 2013a). This unprecedented invasion has been facilitated by rapid growth rates of individual lionfish (Pusack et al. 2016), exponentially increasing local population sizes (Green et al. 2012, Albins & Hixon 2013), and resulting high densities (Green & Côté 2009, Kulbicki et al. 2012, Dahl & Patterson 2014), indicating that these largely piscivorous invaders are quickly converting native prey into lionfish biomass.

The success of lionfish has likely been enhanced by the lack of substantial biotic resistance by invaded communities. Native predators are apparently de-

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Fig. 1. Invasive red lionfish *Pterois volitans* in the Bahamas (photo by Rich Carey)

terred by the unusual shape, cryptic coloration, and venomous spines of lionfish, although there is debate regarding whether large native groupers are a major threat to the invader (Mumby et al. 2011, Hackerott et al. 2013, Valdivia et al. 2014, Bruno et al. doi: 10.7287/peerj.preprints.139v1, Mumby et al. doi: 10.7287/peerj.preprints.45v1). Few parasites attack invasive lionfish (Sikkel et al. 2014), native mesopredators appear to be ineffective competitors (Albins 2013), and even highly territorial damselfish do not chase them (Kindinger 2015). Additionally, lionfish use a variety of feeding behaviors, including ambushing and corralling prey (Morris & Akins 2009), as well as blowing jets of water at prey (Albins & Lyons 2012). Combined with cryptic coloration and unusual appearance, such flexible feeding behavior apparently allows high consumption rates of native prey (Albins & Hixon 2008, Côté & Maljković 2010, Green et al. 2011, Cure et al. 2012). To date, the only local controls of the invasion have been targeted fisheries and removals by divers, especially lionfish derbies (Barbour et al. 2011, Frazer et al. 2012, Morris 2012). In the absence of such local controls, lionfish eventually reach densities where they may become self-limiting (Benkwitt 2013).

Lionfish are rapidly depleting local abundances of native reef fishes, as shown by both controlled field experiments (Albins & Hixon 2008, Albins 2013, 2015, Green et al. 2014, Benkwitt 2015) and observational studies (Green et al. 2012, Benkwitt 2016a). Lionfish diets comprise a broad variety of native fishes and invertebrates (Morris & Akins 2009, Muñoz et al. 2011, Valdez-Moreno et al. 2012, Côté et al. 2013b, Dahl & Patterson 2014), including not only small species but also the juveniles of larger species. Native prey consumed as juveniles include commercially and recreationally important groupers and snappers, as well as ecologically important grazers such as surgeonfishes and parrotfishes, which keep reef surfaces clean so that corals can flourish (review by Hixon 2015). Invasive lionfish also have non-consumptive effects, in that their mere presence inhibits grazing activity by these reef fishes (Kindinger & Albins 2016). The list of native species consumed by lionfish will undoubtedly continue to grow with additional diet studies from across the invaded region. However, studies of lionfish prey selection suggest that solitary, narrow-bodied fish that reside near the seafloor are most vulnerable (Green & Côté 2014). Importantly, extirpation of native fishes is evident (Albins 2015). Consequently, the lionfish invasion has been identified as one of the greatest emerging threats to global biodiversity (Sutherland et al. 2010).

Given this unprecedented invasion, a symposium on invasive lionfish (the 8th thus far) was hosted by the 68th Gulf and Caribbean Fisheries Institute annual conference in Panama City, Panama, on November 11, 2015, funded by the Florida Sea Grant Program, the Florida Fish and Wildlife Conservation

Commission, the National Oceanic and Atmospheric Administration, and the Reef Environmental Education Foundation. With over 80 submissions from across the invaded region, the symposium featured 24 talks and as many posters, presenting a broad range of information related to lionfish biology, the ecological and economic effects of the invasion, and efforts to manage the invasion. The 9 articles in this Theme Section reflect much of the diversity of topics covered during the symposium:

Several papers focus on the success of lionfish as invasive species. Stevens et al. (2016, this Theme Section [TS]) found that the skin bacteria of lionfish show antibacterial activity against known fish pathogens, indicating that resistance to disease may be added to the list of mechanisms that enhance the success of the invader. One established measure of success is that invasive lionfish are generalist mesopredators. Harms-Tuohy et al. (2016, this TS) used DNA metabarcoding to characterize the complete gut contents of lionfish in Puerto Rico, finding that the diets are even broader than detectable by traditional visual identification: 63 lionfish had consumed 39 native fish species representing 16 families. Eddy et al. (2016, this TS) further documented the broad diet of the invader. Lionfish in Bermuda, near the northern limit of the invasive range, consume more crustaceans and fewer fishes than further south.

Most papers examine the ecological effects of the invasion. Dahl et al. (2016, this TS) compared fish communities on artificial reefs in the northern Gulf of Mexico before and after the invasion, and conducted a field experiment comparing no versus single versus repeated removals of lionfish. Lionfish rapidly recolonized removal reefs. Although the experiment was confounded by possible effects of the Deepwater Horizon oil spill, there was a clear indication that smaller resident fishes were less abundant following the invasion. Working in Panama, Palmer et al. (2016, this TS) conducted a shorter-term lionfish removal experiment that also indicated a negative effect on the abundance of several native fishes, yet no effect on the genetic diversity of the bicolor damselfish *Stegastes partitus*.

A field experiment by Ingeman (2016, this TS) in the Bahamas demonstrated that invasive lionfish add substantial density-independent mortality of the native fairy basslet *Gramma loreto* to the density dependence caused by native predators, pushing some local populations toward extirpation. Aquarium experiments by Kindinger & Anderson (2016, this TS) showed that lionfish prefer fairy basslet over blackcap basslet *G. melacara*, whereas a native mesopredator, graysby grouper *Cephalopholis cruentata*, prefers blackcaps. Thus, interactions among native prey, native predators, and invasive lionfish may have unforeseen indirect effects on community interactions. Benkwitt (2016b, this TS) documented movements and behaviors of lionfish on patch reefs in the Bahamas, showing that at higher densities, lionfish forage over surrounding seagrass meadows at night. These findings indicate that localized culling of lionfish may limit the area over which the invaders affect native fish populations.

The ecological effects of lionfish may be moderated by large native fishes having negative effects on the invader. Red grouper *Epinephelus morio* defend karst solution holes in Florida Bay. Ellis & Faletti (2016, this TS) experimentally demonstrated that, relative to holes where both predators were excluded, juvenile reef fish abundance was much higher where only grouper were present, much lower where only lionfish were present, and not different if both predators were present. This result indicates that grouper somehow inhibit piscivory by lionfish, which switch to invertebrate prey in the presence of the larger native predator.

In summary, the lionfish invasion is unprecedented and potentially catastrophic from multiple perspectives. Given that eradication is currently not possible with the tools and resources available, the possibility remains that this major marine fish invasion will permanently alter the ecology of coastal ecosystems of the greater Caribbean region and southeastern United States, especially coral-reef systems already degraded by various human activities (Albins & Hixon 2013).

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Bacteria associated with lionfish (*Pterois volitans/miles* complex) exhibit antibacterial activity against known fish pathogens

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ABSTRACT: Fish support microbial communities that serve a variety of functions, including disease resistance. In addition to fish microbiota acting as a defense against disease, fish mucus often contains antimicrobial compounds. This study investigated the antibacterial activity of bacteria isolated from external surfaces of native (e.g. Indo-Pacific) and invasive (e.g. Western Atlantic, Caribbean) lionfish (*Pterois volitans/miles* complex) and native Caribbean squirrelfish *Holocentrus adscensionis* against 6 known fish pathogens (*Vibrio* spp., *Photobacterium damselae*), and evaluated the antibacterial activity of lionfish mucus against these pathogens and lionfish- and squirrelfish-associated bacteria. The 16S rRNA gene was sequenced for bacteria exhibiting pathogen inhibition, providing information on their taxonomic affiliations. Antibacterial metabolites were produced by 36.2% (54 of 149) of lionfish-derived bacterial cultures, with similar percentages of producing organisms recovered from the native and invaded ranges. Only 1 of 13 squirrelfish isolates inhibited pathogens. Interestingly, similar genera exhibiting antibacterial activity were detected in both ranges (e.g. *Alteromonas*, *Pseudoalteromonas*, *Photobacterium*), even though previous work suggested that external bacterial communities were not vertically transmitted. Antibacterial activity was detected after 24 h of growth, and the amount of inhibition did not increase over a 14 d incubation period. Conversely, organic and aqueous mucus extracts from lionfish were not active against the 6 pathogens or against bacteria isolated from lionfish and squirrelfish. These findings indicate that the external bacterial communities of lionfish may provide disease resistance to their hosts, a trait that would enhance the ability of lionfish to successfully establish as an invasive species.

KEY WORDS: Lionfish · *Pterois volitans/miles* complex · Invasive · Disease resistance · Fish pathogens · Bacterial isolates

INTRODUCTION

In the marine environment, the surfaces of eukaryotic organisms serve as substrates for the growth of microorganisms and have been shown to support different bacterial communities than what is found in the surrounding seawater (e.g. Taylor et al. 2005, Penesyan et al. 2010, Burke et al. 2011, Stevens & Olson 2013), suggesting that the bacteria may be specifically adapted to the microenvironment of their host (Holmström & Kjelleberg 1999, Harder et al. 2003). Some fish are known to produce antimicrobial compounds as a protective mechanism against pathogens (Hellio et al. 2002, Bragadeeswaran et al. 2011), but apparently healthy fish support microbial communities, indicating that the presence of these microorganisms is not necessarily detrimental but may instead benefit the host (Cahill 1990, Austin 2002). For example, bacteria associated with fish have been shown to aid in disease resistance (Olsson

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et al. 1992, Sugita et al. 2002, Chabrillón et al. 2005, O'Brien & Wright 2011), drag reduction (Bernadsky & Rosenberg 1992), and food digestion (Ganguly & Prasad 2012, Ray et al. 2012). In return, fish are thought to provide a nutrient-rich surface for bacterial colonization in an otherwise oligotrophic marine environment (Sar & Rosenberg 1987, Penesyan et al. 2010).

Disease resistance facilitated by microorganisms occurs through both direct and indirect mechanisms. Fish-associated bacteria can directly inhibit the growth of pathogens on the mucosal lining by competing for space (Chabrillón et al. 2005), while indirect antagonism occurs through the production and release of compounds inhibitory to potential pathogens (Chabrillón et al. 2005, O'Brien & Wright 2011). The host fish will be protected from disease caused by organisms sensitive to these inhibitory compounds (Penesyan et al. 2010). To optimize both direct and indirect disease resistance mechanisms, the host is thought to support a dense, diverse, and non-pathogenic resident microbiota (Verschuere et al. 2000, Chabrillón et al. 2005).

In addition to the protective metabolites produced by their associated microorganisms, some fish also release antimicrobial compounds in their external mucus (reviewed in Ellis 2001). However, studies of fish mucus have primarily focused on freshwater and temperate marine species (Hellio et al. 2002, Fernandes et al. 2004, Bergsson et al. 2005, Subramanian et al. 2008, Bragadeeswaran et al. 2011), thus little is known about antibacterial metabolites in the mucus of fish species with a broader distribution range. One example in the tropical environment showed that the mucus cocoons of the queen parrotfish inhibited the growth of several bacterial fish pathogens (Videler et al. 1999). However, the ability of parrotfish surface-associated bacteria to produce the inhibition detected in the mucus was not assessed.

The present study examined potential antibacterial defenses of lionfish (*Pterois volitans/miles* complex) from both the native Indo-Pacific and the invaded western Atlantic Ocean, and Caribbean squirrelfish (*Holocentrus adscensionis*). Previous work demonstrated that the bacterial communities associated with invasive lionfish were more diverse than those associated with native squirrelfish, and included no known fish pathogens (Stevens & Olson 2013). Additionally, lionfish retained a core surface-associated bacterial community in both the native and invaded ranges, suggesting that these associations are species specific and may play an ecological role (Stevens & Olson 2015). Therefore, the present study investigated the capacity of lionfish- and squirrelfish-associated bacteria and lionfish mucus to function in pathogen resistance. Additionally, potential mechanisms for pathogen inhibition were examined by investigating the presence of biosynthetic genes for known bioactive molecules, including polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS), and the 16S rRNA gene from bacteria demonstrating pathogen inhibition was sequenced.

MATERIALS AND METHODS

Field collection

Lionfish were collected from locations within the invaded (Honduras [n = 5], Key Largo Florida [n = 11], Belize [n = 7], and Bahamas [n = 8]) and native (Taiwan [n = 9], Philippines [n = 4], and Indonesia [n = 3]) ranges. In the invaded range, fish were collected by SCUBA divers with pole spears, pithed, and placed into individual Whirl-pak bags (UA IACUC protocol no. 11-358-2). In the native range, fish were collected by SCUBA divers with nets, placed into individual Whirl-pak bags, and released following sample collection. The surface of the skin of each fish was swabbed with a sterile cotton swab, which was placed into 500 µl of filter-sterilized (0.22 µm) artificial seawater (ASW: 1.9 l DI H_2O, 40.6 g NaCl, 1.16 g KCl, 18.39 g $MgCl_2{\cdot}6H_2O$, 2.58 g $CaCl_2{\cdot}2H_2O$, 6.58 g Na_2SO_4, 0.32 g $NaHCO_3$, 0.00027 g $Na_2H_2PO_4$) for ≤20 min. After vigorously mixing the swab in the ASW, the swab was removed and 100 µl of the ASW solution was plated onto a Marine Agar 2216 (MA, Difco) plate. A 1:100 dilution of the inoculated ASW solution was made and 100 µl was plated onto another MA plate. The plates were sealed with Parafilm, maintained at room temperature, and transported to the laboratory. To provide a comparison with native Caribbean fish, bacteria from the mucus of squirrelfish were also isolated as described above. Individual colonies were streaked for isolation onto fresh MA plates and grown for 24 to 48 h at room temperature (~24°C). Following incubation, isolated strains were transferred to MA slants.

Mucus was collected from lionfish in Belize (n = 12) and the Bahamas (n = 12) by adding 10 ml of a 100 mM NaCl solution to a Whirl-pak containing only the pithed fish. The fish was carefully moved back and forth in the solution for ~1 min to slough off the mucus (Subramanian et al. 2008). The resulting mucus–salt solution was collected in 15 ml sterile centrifuge tubes and immediately frozen at −20°C until use.

Culture screening for potential antibacterial activity

Known fish pathogens *Vibrio vulnificus* (2 strains: 1-FT-1, 76-FC-1), *V. parahaemolyticus* (2 strains: DI-ST-7, LA-4T-1), and *V. harveyi* (strain VH536ED) were generously provided by C. Arias (Auburn University). *Photobacterium damselae* ssp. *piscicida* (strain DSMZ 22834) was purchased from the German Collection of Microorganisms and Cell Cultures. All pathogens were grown in marine broth (MB; Difco) overnight at 30°C with shaking (220 rpm). Using a hemocytometer, cell concentrations were standardized to approximately 1×10^7 cells ml^{-1}. Seeded plates were made by adding 1 ml of the standardized cell solution to 100 ml of molten MA, mixing thoroughly, and placing 10 ml aliquots of the inoculated medium into square Petri dishes (100 × 100 mm; Fisher Scientific). All seeded plates had a final concentration of approximately 1×10^5 cells ml^{-1} and were stored at 4°C and used within 48 h.

All lionfish- and squirrelfish-associated isolates were grown in individual culture tubes containing 10 ml of SYZ–ASW broth (soluble starch 15 g, yeast extract 2 g, NZ-amine 4 g, dextrose 2 g, ASW 750 ml, DI H_2O 250 ml) for 14 d at 30°C with shaking (220 rpm) in a New Brunswick Scientific C25 incubator shaker. After growth, 1 ml portions of the bacterial liquid cultures were transferred to 1.5 ml sterile microcentrifuge tubes and centrifuged for 3 min at 16 200 × *g* in an Eppendorf 5415D tabletop centrifuge to pellet cells. Filter paper disks (n = 6) were infused with 20 µl of the cell-free supernatant (CFS) from each isolate and placed onto plates seeded with each fish pathogen. For a positive control, a disk infused with 10 µg gentamicin (BD BBL Sensi-Disc) was placed onto the agar surface of each plate. Plates were incubated for 24 h at 37°C (*V. vulnificus* strains, *V. parahaemolyticus* strains, and *V. harveyi*) or 28°C (*P. damselae* ssp. *piscicida*). Following incubation, plates were examined for the presence of zones of growth inhibition around the disks. When present, zones of inhibition were measured to the nearest 0.5 mm.

Growth inhibition assays

Cultures that were active against more than one of the pathogens (n = 23) were regrown for 14 d and CFSs prepared as above. Microtitre plates were prepared by making dilutions of the CFS in MB. For each culture, triplicate wells containing CFS concentrations of 100% (undiluted), 50% (1:1 dilution), 25% (1:3 dilution), and 12.5% (1:7 dilution) were used in the assay. To the 50 µl volumes of CFS (undiluted and diluted), 10 µl of an overnight culture of *V. parahaemolyticus* DI-ST-7 standardized to 2×10^4 cells ml^{-1} in MB was added to each well. Triplicate control wells were inoculated with 60 µl MB (negative control), and 10 µl pathogen and 50 µl MB (positive control). The final volume per well was 60 µl. Plates were incubated overnight at 37°C with shaking and the absorbance in each well was measured at 600 nm on a µQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments).

Absorbance measurements were converted to percent pathogen inhibition using the absorbance values from the control wells. Once converted, means and standard deviations for the percent of pathogen inhibition were calculated and linear regression was conducted to test for overall effects of dilution on inhibition. One-way ANOVA was used to test for the effect of dilution on inhibition for each strain individually.

Initiation of secondary metabolite production

To assess when antibacterial metabolite production began in lionfish-associated bacteria, 6 isolates (3 per range, native and invaded) were grown to exponential phase before 500 µl volumes were preserved in 15% glycerol and frozen. Ten ml SYZ-ASW tubes were inoculated daily for 14 d with the preserved cells at a 1:200 dilution so that each tube received the same inoculum. After the 14 d, CFSs were prepared for the 14 cultures for each isolate. Microtitre plates were prepared and incubated as above with 2×10^4 cells ml^{-1} liquid culture of *V. parahaemolyticus* DI-ST-7 to test pathogen growth inhibition. Linear regression was used to test for the effects of dilution and time on pathogen inhibition.

Taxonomic affiliations of bacteria that produced antibacterial metabolites

DNA was extracted from the isolates that demonstrated antibacterial activity by placing cells into 100 µl of a sterile 5% Chelex 100 resin (Bio-Rad Laboratories) in water solution in a sterile 1.5 ml microcentrifuge tube. Each tube was vortexed for 30 s, incubated at 70°C for 15 min, vortexed again for 30 s, and placed on ice for 30 min. This cycle was repeated 3 times before microcentrifuge tubes were centrifuged for 1 min at 16 200 × *g* to pellet Chelex beads and cellular debris. The supernatants were used for subsequent amplification reactions.

PCR was performed using universal prokaryotic primers 8F (5′-AGAGTTTGATCMTGGCTCAG-3′; Edwards et al. 1989) and 1392R (5′-ACGGGCGGTGTGTACA-3′; Lane 1991) to amplify an approximately 1385 bp region of the 16S rRNA gene. Each reaction consisted of 1.25 U PerfectTaq (5-Prime), 1X PerfectTaq buffer, 1.25 mM $Mg(OAc)_2$, 0.06 mM deoxynucleoside triphosphates, 25 pmol of each primer, 4 µl of DNA and sterile DI water to a final volume of 25 µl. Reaction conditions were 85°C for 5 min, followed by 25 cycles of 94°C for 45 s, 62°C for 90 s, and 72°C for 90 s, with a final 10 min extension at 72°C with ramp speeds at 1°C s^{-1} (Stevens et al. 2013). Amplification products were visualized by electrophoresis on 1.5% agarose gels containing GelRed (Biotium) for 70 min at 70 V, and visualized under UV transillumination with a gel imaging system (Fotodyne). Negative reagent controls without template were run with each reaction.

PCR products (20 µl) were digested with *Hae*III restriction endonuclease (New England BioLabs) for 8 h at 37°C prior to inactivating the enzyme at 80°C for 30 min. Digestion products were visualized on 1.5% agarose gels as described above and banding patterns were compared using Bionumerics v6.6 software (Applied Maths). A representative PCR product for each banding pattern was randomly selected for sequencing, cleaned using the EZNA Cycle Pure Kit (Omega Bio-Tek), and bi-directionally sequenced by Eurofins MWG Operon. Sequences were compared with the NCBI database using the MEGABLAST algorithm. Closely related sequences were included in an RDP alignment to generate a maximum likelihood tree in Geneious v6.1.7 using the PhyML algorithm with 100 bootstraps and the HKY85 model of nucleotide substitution (Fig. 1).

Screening for secondary metabolite biosynthetic genes

PCR was used to evaluate the presence of genes found within biosynthetic pathways associated with antimicrobial metabolite production. DNA from each active lionfish-associated isolate was screened with primers specific to non-ribosomal peptide synthetase (NRPS; A3F and A7R; Ayuso-Sacido & Genilloud 2005) and polyketide synthetase (PKS I; K1F and M6R; Ayuso-Sacido & Genilloud 2005; and PKS II; KSα and ACP; Seow et al. 1997) genes. Each reaction consisted of 2.5 U PerfectTaq (5-Prime), 1X PerfectTaq buffer, 1.25 mM $Mg(OAc)_2$, 0.06 mM deoxynucleoside triphosphates, 25 pmol of each primer, 5% dimethyl sulfoxide, 2 µl of DNA and sterile DI water to a final volume of 50 µl. Reaction conditions were 85°C for 5 min, followed by 30 cycles of 94°C for 1 min, 56°C for the PKS primers or 60°C for the NRPS primers for 90 s, and 72°C for 2 min, with a final 10 min extension at 72°C. Amplification products were visualized by electrophoresis on 1.0% agarose gels containing GelRed (Biotium) for 70 min at 70 V, and visualized under UV transillumination with a gel imaging system (Fotodyne). DNA from *Streptomyces scopuliridis*, a species with known NRPS and PKS type I and II genes, was used as a positive control. Both positive and reagent negative controls were run with each reaction.

Preparation of mucus extracts

Mucus samples were lyophilized by freezing at −80°C immediately followed by freeze drying in a FreeZone 2.5 benchtop freeze dry system (Labconco) and stored at −20°C. Extracts were prepared following Hellio et al. (2002) and Subramanian et al. (2008) with modifications. To prepare organic extracts (polar and nonpolar phases), lyophilized mucus was suspended in 95% ethanol at a concentration of 1 mg ml^{-1} and centrifuged in a Sorvall RC6+ centrifuge (Thermo Scientific) for 30 min at 4°C and 11 000 × *g*. The supernatant was transferred to a sterile 50 ml conical tube and the pellet was resuspended 2 additional times in the same volume of 95% ethanol. The 3 supernatants were combined and evaporated under vacuum at 40°C using a Rotavapor Collegiate (Buchi). To separate the nonpolar and polar phases of the organic extract, the dried extract was resuspended in 10 ml distilled water and partitioned 3 times with 5 ml (3 × 5 ml) dichloromethane (DCM). The DCM nonpolar phases were combined and evaporated under a nitrogen stream and the polar phases were combined and lyophilized.

The aqueous extract of mucus was prepared by resuspending lyophilized mucus in sterile distilled water at a concentration of 1 mg ml^{-1}. After a 2-h incubation at 4°C, the samples were centrifuged at 9500 × *g* for 10 min at 4°C in a Sorvall RC6+ centrifuge and the supernatant was decanted and lyophilized. Mucus processing resulted in isolation of aqueous and organic extracts, with the organic extract further separated into nonpolar and polar phases.

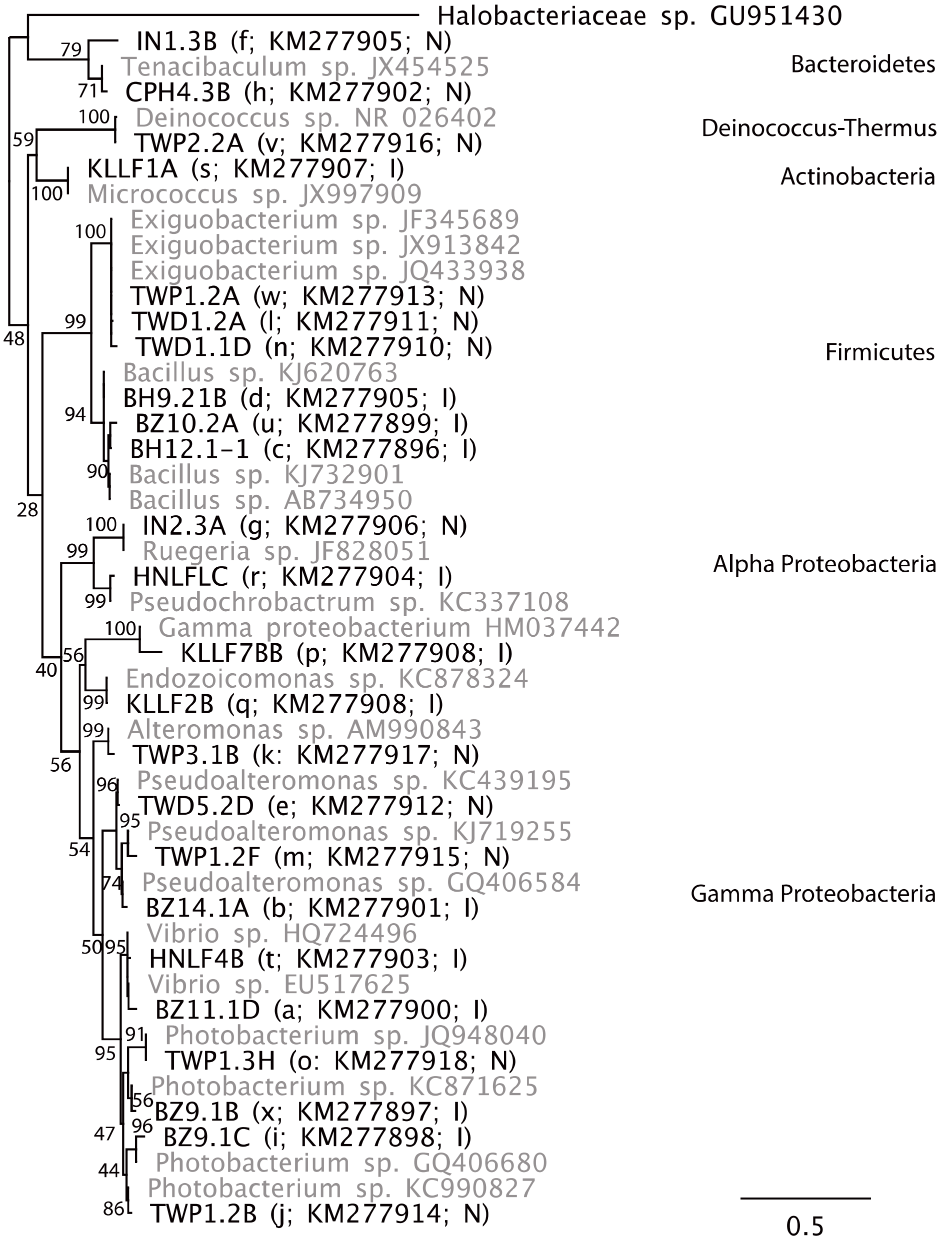

Fig. 1. Maximum likelihood tree showing the phylogenetic relationships of the lionfish-associated bacterial isolates and related reference sequences (in grey) based on ~1385 bp of 16S rRNA genes. *Halobacteriaceae archaeon* was used as an outgroup for this tree. Numbers at each node indicate the bootstrap support from 100 iterations, and the scale bar indicates the nucleotide change between organisms. GenBank accession numbers are provided after each sequence, and isolates from this study also have the letter of the corresponding banding pattern and are designated as being recovered from either the native (N) or invaded (I) range

Mucus screening

The aqueous extract and polar organic phase were resuspended in 1 ml sterile distilled water and the nonpolar organic phase was resuspended in 1 ml of a 5% DMSO solution (Bergsson et al. 2005). Filter paper disks were infused with 20 µl of extract and placed onto seeded plates of the 6 pathogens, 7 lionfish isolates, and 7 squirrelfish isolates. After an overnight incubation at 37°C for the *Vibrio* spp. and 24°C for *Photobacterium damselae* ssp. *piscicida* and fish-associated isolates, the plates were visually inspected for zones of inhibition.

RESULTS

Taxonomic affiliations of bacteria that produced antibacterial metabolites

A total of 149 bacterial isolates were cultivated from surface swabs of 47 lionfish collected in the native (n = 16) and invaded (n = 31) ranges. After completion of disk diffusion assays on lawns of 6 strains of known fish pathogens, 54 isolates representing 13 bacterial genera showed activity against at least one of the pathogens (Table 1, Fig. 1). Twenty-one of the active isolates were from samples

Table 1. Diameter of zones of inhibition (mm) exhibited by lionfish-associated bacteria against the 6 known fish pathogens and the putative taxonomic affiliations of the sequenced isolates. The reported diameter excludes the 6.35 mm diameter filter disk. The range of the fish (native or invaded) is indicated by N or I before the isolate designation, and GenBank accession numbers are included in parentheses for those that were sequenced. Taxonomic affiliations and percent identity of the BLAST match are provided for these isolates as well as the banding pattern that each represents (designated by a lowercase letter in parentheses). For isolates that shared banding patterns and were thus not sequenced, the letter of the appropriate banding pattern and the isolate designation for the sequenced organism are shown. The first 2 letters of the isolate designation indicate the collection location of the source fish. BH = Bahamas, BZ = Belize, CP = Philippines, HN = Honduras, IN = Indonesia, KL = Key Largo Florida, TW = Taiwan. The presence/absence (+/−) of potential antibacterial genes (NRPS: non-ribosomal peptide synthetase; PKS: polyketide synthase types I and II) was assessed via PCR

Isolate (accession number)	*Vibrio parahaemolyticus* LA-4T-1	*Vibrio parahaemolyticus* DI-ST-7	*Vibrio vulnificus* 1-FT-1	*Vibrio vulnificus* 76-FC-1	*Vibrio harveyi* VH536ED	*Photobacterium damselae* ssp. *piscicida*	Taxonomic affiliation and percent identity	Presence of potential antibacterial genes (NRPS/PKS I/PKS II)
I-BZ11.1D (KM277900)	0	0	0	0	0	2.0	*Vibrio* sp. EU517625 (a) 99 %	– / – / –
I-BZ14.1A (KM277901)	2.0	2.0	0	0	0	10.0	*Pseudoalteromonas* sp. GQ406584 (b) 99 %	– / – / –
I-BH12.1-1 (KM277896)	0	0	7.0	0	0	0	*Bacillus* sp. AB734950 (c) 100 %	+ / – / –
I-BH9.2-1B (KM277895)	0	0	10.0	0	0	0	*Bacillus pumilus* KJ620763 (d) 100 %	– / – / –
I-BH6.2-2B	0	0	8.0	0	0	0	BH9.2-1B (d)	– / – / –
I-BZ10.1B	0	0	0	0	0	12.0	BH9.2-1B (d)	– / – / –
I-BZ9.2A	0	0	2.0	0	0	0	BH9.2-1B (d)	– / – / –
I-BZ9.1A	3.0	0	0	0	0	0	BH9.2-1B (d)	– / – / –
N-TWD5.2D (KM277912)	8.0	10.0	0	0	0	0	*Pseudoalteromonas* sp. KC439195 (e) 99 %	– / – / –
I-HNLF4A	0	0	5.0	4.0	0	0	TWD5.2D (e)	+ / – / +
N-IN1.3B (KM277905)	10.0	7.0	0	8.0	0	0	Uncultured bacterium JX939763 (f) 99 %	– / – / –
N-IN2.3A (KM277906)	2.0	3.0	0	0	0	0	*Ruegeria* sp. JF828051 (g) 99 %	– / – / –
N-IN1.3C	2.0	3.0	0	0	0	0	IN2.3A (g)	– / + / –
N-CPH4.2C	0	0	0	0	6.0	0	IN2.3A (g)	– / – / –
N-CPH2.3B	2.0	2.0	0	0	0	0	IN2.3A (g)	– / – / –
N-CPH4.3B (KM277902)	2.0	0	0	0	0	0	*Tenacibaculum* sp. JX454525 (h) 97 %	– / – / –
I-BZ9.1C (KM277898)	0	0	2.0	0	0	0	*Photobacterium* sp. GQ406680 (i) 97 %	– / – / –
I-BZ9.1D	0	0	4.0	0	0	0	BZ9.1C (i)	+ / – / –
N-TWP1.2B (KM277914)	2.0	0	0	0	0	0	*Photobacterium* sp. KC990827 (j) 99 %	– / – / –
I-KLLF11B	0	0	2.0	0	0	0	TWP1.2B (j)	– / – / –
N-TWD1.1E	0	4.0	0	0	0	0	TWP1.2B (j)	– / – / –
N-TWP3.1B (KM277917)	0	0	0	8.0	0	0	*Alteromonas* sp. AM990843 (k) 98 %	– / – / –
N-TWP2.1E	6.0	0	0	0	0	0	TWP3.1B (k)	– / + / –
N-TWP2.1H	0	0	6.0	0	0	5.0	TWP3.1B (k)	– / – / –
I-BZ10.1C	6.0	0	0	0	0	0	TWP3.1B (k)	– / – / –
I-BZ10.1D	3.0	0	0	0	0	0	TWP3.1B (k)	– / – / –
I-HNLF1A	2.0	0	0	0	0	0	TWP3.1B (k)	– / – / –
I-HNLF9A	6.0	4.0	0	0	0	0	TWP3.1B (k)	– / – / –
I-HNLF12	4.0	0	0	0	0	0	TWP3.1B (k)	– / – / –
I-KLLF11A	3.0	2.0	0	6.0	0	0	TWP3.1B (k)	– / – / –
I-KLLF12B	0	4.0	0	0	0	0	TWP3.1B (k)	– / – / –
N-TWP2.1A	0	0	2.0	0	0	0	TWP3.1B (k)	– / – / –
N-TWD5.2B	6.0	6.0	0	0	0	0	TWP3.1B (k)	– / – / –
I-KLLF6AD	0	4.0	0	0	0	0	TWP3.1B (k)	– / – / –
I-KLLF6BA	0	3.0	0	0	0	0	TWP3.1B (k)	– / – / –
N-TWP4.3A	0	2.0	0	4.0	0	0	TWP3.1B (k)	– / – / –

(Table 1 continued on next page)

Table 1 (continued)

Isolate (accession number)	*Vibrio parahaemolyticus* LA-4T-1	*Vibrio parahaemolyticus* DI-ST-7	*Vibrio vulnificus* 1-FT-1	*Vibrio vulnificus* 76-FC-1	*Vibrio harveyi* VH536ED	*Photobacterium damselae* ssp. *piscicida*	Taxonomic affiliation and percent identity	Presence of potential antibacterial genes (NRPS/PKS I/PKS II)
N-TWD1.2A (KM277911)	0	2.0	0	3.0	0	0	*Exiguobacterium* sp. JX913842 (l) 100%	–/–/–
N-TWP1.2F (KM277915)	6.0	10.0	0	0	0	0	*Pseudoalteromonas* sp. KJ719255 (m) 96%	–/–/–
N-TWD1.1D (KM277910)	0	3.0	0	0	0	0	*Exiguobacterium* sp. JF345689 (n) 98%	–/–/–
N-TWP1.3H (KM277918)	7.0	0	0	0	8.0	0	*Photobacterium* sp. JQ948040 (o) 100%	–/–/+
I-KLLF7BB (KM277909)	0	0	0	4.0	0	0	γ Proteobacterium HM037442 (p) 93%	–/–/–
I-HNLFLB	2.0	0	0	0	0	4.0	KLLF7BB (p)	–/+/–
I-KLLF2B (KM277908)	4.0	2.0	0	4.0	0	0	*Endozoicomonas* sp. KC878324 (q) 100%	–/+/–
I-HNLFLC (KM277904)	4.0	0	0	0	0	0	*Pseudochrobactrum* sp. KC337108 (r) 98%	–/–/–
I-HNLF7A	2.0	0	0	0	0	0	HNLFLC (r)	–/+/–
I-KLLF1A (KM277907)	5.0	0	0	4.0	0	0	*Micrococcus* sp. JX997909 (s) 99%	–/–/–
I-BZ10.1E	6.0	0	7.0	0	0	0	KLLF1A (s)	–/+/–
I-HNLF4B (KM277903)	1.0	2.0	0	0	0	0	*Vibrio* sp. HQ724496 (t) 96%	–/–/–
I-HNLFLA	4.0	4.0	0	0	0	0	HNLF4B (t)	–/–/–
I-BZ10.2A (KM277899)	0	0	0	0	0	17.0	*Bacillus* sp. KJ732901 (u) 98%	–/–/–
I-BZ11.1C	0	0	0	0	0	7.0	BZ10.2A (u)	–/–/–
N-TWP2.2A (KM277916)	0	1.0	0	5.0	0	0	*Deinococcus* sp. NR_026402 (v) 99%	+/–/–
N-TWP1.2A (KM277913)	4.0	2.0	0	0	0	0	*Exiguobacterium* sp. JQ433938 (w) 100%	–/–/–
I-BZ9.1B (KM277897)	0	0	3.0	0	0	0	*Photobacterium rosenbergii* KC871625 (x) 98%	–/–/–

obtained in the native range; the other 33 were obtained from the invaded range. Following gel electrophoresis of *Hae*III digested PCR products, the 54 isolates were separated into 24 broad taxonomic groups representing distinct banding patterns; the 16S rRNA gene from a single representative of each group was sequenced and the sequences were submitted to GenBank under accession numbers KM277895–KM277918 (Table 1, Fig. 1). Using similar banding patterns as a proxy for taxonomy, 15 of the 54 isolates were most closely related to *Alteromonas* spp. (6 from the native range, 9 from the invaded), with another 4 most closely related to *Pseudoalteromonas* spp. (2 each from the native and invaded ranges). *Bacillus* spp. and *Photobacterium* spp. were the closest relatives of 8 (all invaded) and 7 (3 native, 4 invaded) additional isolates, respectively. A single *Ruegeria* sp. was the closest relative of 4 isolates (all native), while members of the genera *Vibrio* and *Exiguobacterium* were each most closely related to 3 isolates (all invaded for *Vibrio*, all native for *Exiguobacterium*). Two isolates were each most related to members of the Gammaproteobacteria, *Micrococcus*, and *Pseudochrobactrum* (all invaded). Single isolates exhibiting unique banding patterns were most closely related to *Bacteroidetes* (native), *Endozoicomonas* (invaded), *Deinococcus* (native), and *Tenacibaculum* spp. (native).

Antibacterial activity of lionfish bacterial isolates

From the native range, 47.7% (21 of 44) isolates showed activity compared with 31.4% (33 of 105) of isolates from the invaded range. The 2 strains of *Vibrio parahaemolyticus* were the fish pathogens most commonly inhibited by lionfish-associated bacterial isolates (Table 1); the growth of *V. parahaemolyticus* strain LA-4T-1 was inhibited by 28 isolates whereas strain DI-ST-7 was inhibited by 22 isolates. Twelve and 10 isolates inhibited the growth of *V. vulnificus* strains 1-FT-1 and 76-FC-1, respectively, whereas *Photobacterium damselae* ssp. *piscicida* was inhibited by 7 isolates and *V. harveyi* was inhibited by 2 isolates. However, only 10 of the 54 active isolates tested positive for the presence of a gene within characterized NRPS and/or PKS pathways (Table 1).

Within the native Indo-Pacific, lionfish bacterial isolates from 7 genera (*Alteromonas*, *Deinococcus*, *Exiguobacterium*, *Photobacterium*, *Pseudoalteromonas*, *Ruegeria*, and *Tenacibaculum*) and 1 class (*Bacteroidetes*) demonstrated activity against the pathogens, while isolates from 8 genera (*Alteromonas*, *Bacillus,* *Endozoicomonas*, *Micrococcus*, *Photobacterium*, *Pseudoalteromonas*, *Pseudochrobactrum*, and *Vibrio*) cultivated from lionfish in the invaded Western Atlantic were active. Isolates from 3 genera (*Alteromonas*, *Photobacterium*, and *Pseudoalteromonas*) were recovered from lionfish in both ranges, while some of the other genera identified from different ranges belong to the same families (e.g. *Bacillus* and *Exiguobacterium* are members of the *Bacillales*; *Vibrio* and *Photobacterium* are members of the *Vibrionaceae*). The presence of similar genera of skin-associated bacteria that were able to inhibit the growth of known pathogens suggested that the core bacterial community may function in pathogen inhibition.

Interestingly, isolates sharing the same banding pattern from digestion of the amplified 16S rRNA gene did not always exhibit similar antibacterial activity against the 6 tested pathogens. For example, putative species of *Alteromonas* (banding pattern k; n = 15) showed considerable variability in antibacterial activity, with at least one isolate active against all of the tested pathogens except *V. harveyi* (Table 1). While differences were noted in the extent of growth inhibition, only isolates within banding patterns g (3 of 4 isolates), i, r, t, and u displayed activity against the same pathogens.

One of 13 isolates recovered from squirrelfish swabs (n = 2) also showed activity against *V. parahaemolyticus* DI-ST-7, indicating that antibacterial activity of fish-associated bacteria is likely common. This isolate did not inhibit the growth of any of the other fish pathogens. Because of the disparate number of lionfish and squirrelfish isolates tested, direct comparisons cannot be made.

Growth inhibition assays

Serial dilution of the CFSs significantly impacted the amount of pathogen inhibition for the lionfish-associated bacteria (linear regression, $p < 0.0001$), with 21 of the 23 isolates showing a significant reduction in antibacterial activity with increasing dilution (Fig. 2). One of the exceptions, an isolate obtained from a lionfish in Belize (BZ14.1A), maintained greater than 67% growth inhibition of *V. parahaemolyticus* strain DI-ST-7 in all dilutions of CFS tested (12.5 to 100%). Only the 100% and 12.5% CFS concentrations were significantly different (ANOVA, $p = 0.03$) for this isolate. The second exception, an isolate from a fish in the native range (IN1.3C), showed greater than 30% growth inhibition in all dilutions tested. Overall, the interaction between isolate and CFS dilution had a significant effect on percent pathogen inhibition (ANOVA, $p < 0.0001$).

When isolates were grouped by the range of their lionfish host (native or invaded), there were no differences in the amount of pathogen inhibition (ANOVA, $p > 0.05$). The mean (±SD) percent pathogen inhibition of isolates was 47.81 ± 22.57% and 51.37 ± 26.76% for lionfish caught in the native and invaded ranges, respectively.

Metabolite production initiation assays

To evaluate whether antibacterial activity was being appropriately captured in our disk diffusion and growth inhibition assays, which used 2-wk-old cultures, broth cultures were inoculated daily for 14 d with aliquots of cryopreserved cells from 6 isolates (n = 3 native range, n = 3 invaded range) and the antibacterial activity was tested using the growth inhibition assay. This approach indicated that production of antibacterial metabolites began after 24 h of growth for the 6 bacterial isolates tested (Fig. 3). Overall, the length of incubation of the cultures did not have a significant effect on the inhibition of *V. parahaemolyticus* DI-ST-7 (regression, $p > 0.05$). However, overall, significant effects were seen in the concentration of CFS that was inhibitory (regression, $p < 0.0001$).

The inhibitory activity of isolate BZ14.1A was not significantly reduced by serial dilution of cultures incubated for >5 d (ANOVA, $p > 0.05$; Fig. 3A). In the 25% CFS dilution, antibacterial metabolite production was variable across days, but the other concentrations of CFS (100%, 50%, and 12.5%) remained relatively stable throughout the 14 d. The remaining 5 isolates exhibited between-day variability in the strength of pathogen inhibition for all concentrations.

Examination of mucus extracts for antibacterial activity

The volume of mucus collected from each fish was variable (15 to 25 ml), so it was not possible to determine what volume of mucus was appropriate for testing antibacterial activity. Instead, the concentration of freeze-dried samples was standardized for all chemical extractions at 1 mg ml^{-1} solvent. Using disk diffusion assays to evaluate the presence of antibacterial metabolites, the lionfish mucus extracts did not exhibit any activity on plates seeded individually with the 6 bacterial fish pathogens, 7 lionfish bacterial isolates, or 7 squirrelfish bacterial isolates.

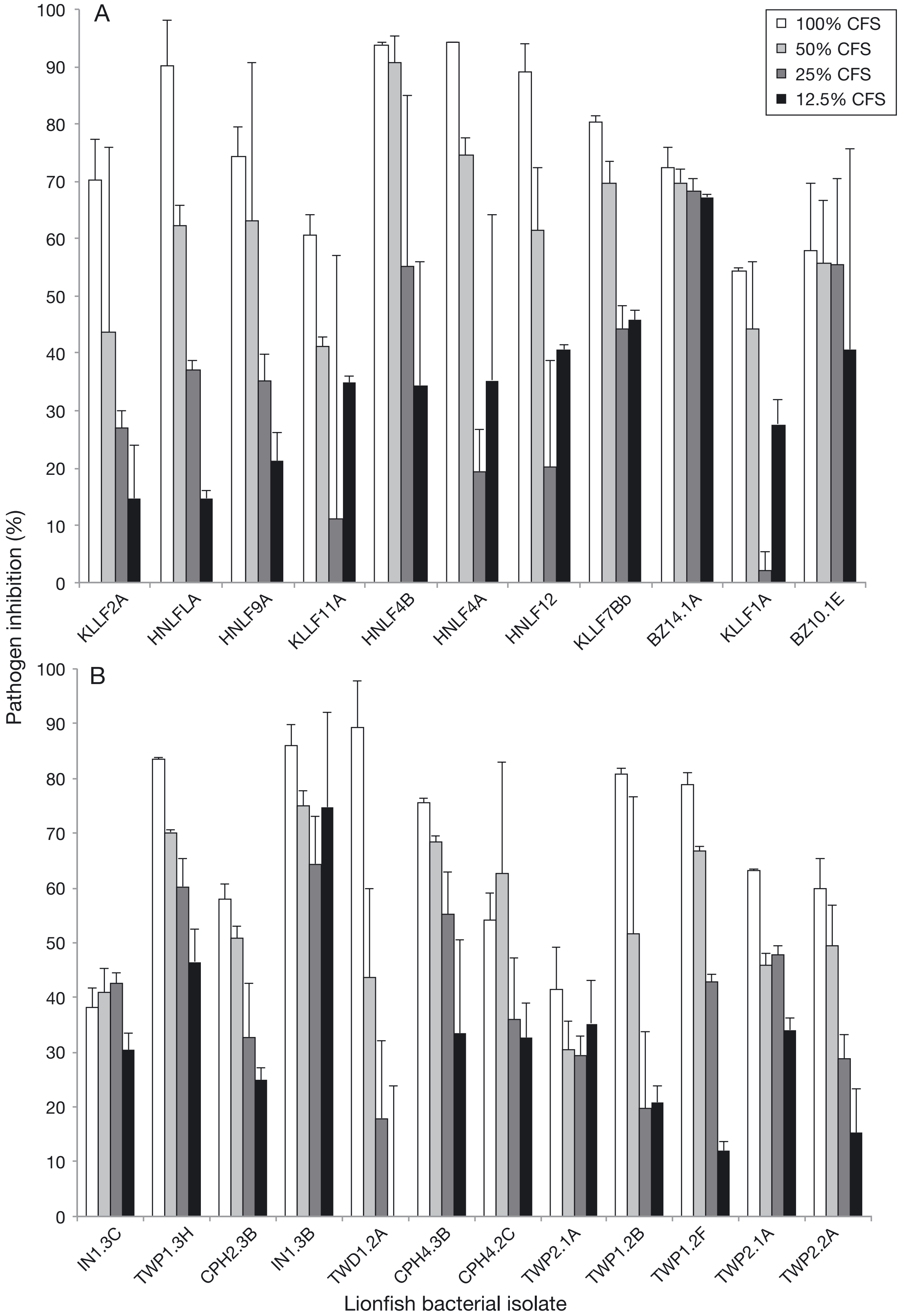

Fig. 2. *Vibrio parahaemolyticus* strain DI-ST-7 growth inhibition in growth inhibition assays using 100%, 50%, 25%, and 12.5% cell-free supernatant for the 23 isolates that showed activity against 2 or more of the 6 pathogens tested. Error bars (SD) represent triplicate wells per concentration of cell-free supernatant. Isolates from lionfish surfaces in the (A) invaded and (B) native range are shown

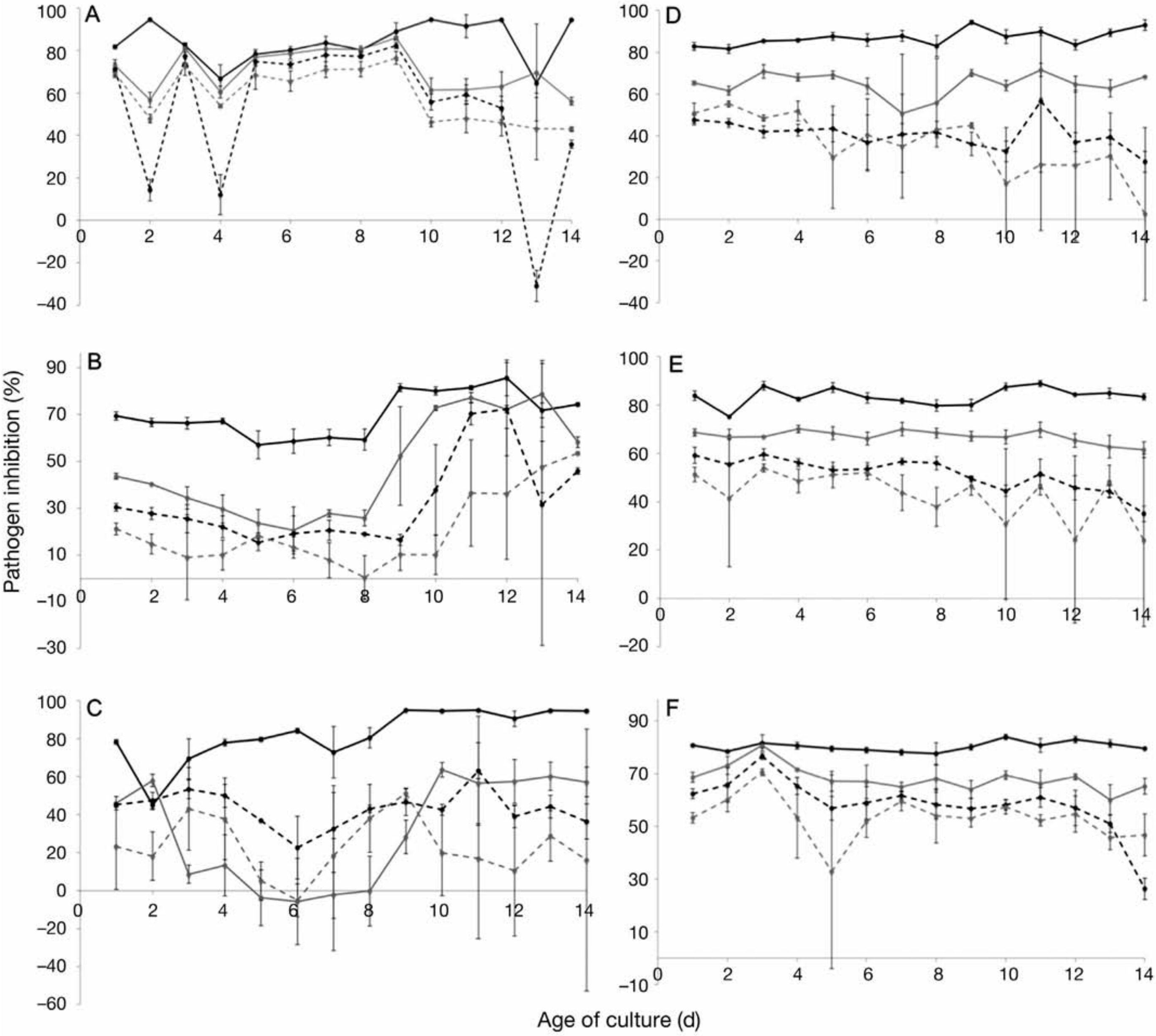

Fig. 3. Pathogen inhibition was detected after 24 h of incubation for each of the 6 tested isolates: 100% cell-free supernatant (CFS; black lines), 50% CFS (gray lines), 25% CFS (black dashed lines), and 12.5% CFS (gray dashed lines). (A) Invaded 1 BZ14.1A; (B) invaded 2 HNLFLA; (C) invaded 3 HNLF9A; (D) native 1 TWD1.2A; (E) native 2 TWP1.3H; and (F) native 3 CPH4.3B

DISCUSSION

Lionfish skin supports a diverse group of bacteria capable of producing antibacterial metabolites, with activity against one or more fish pathogens observed in more than a third of lionfish-associated bacterial isolates. Similar or higher levels of pathogen inhibition by associated bacterial communities were also found on healthy adult humans (Toshima et al. 2007) and brittlestars (Strahl et al. 2002). However, other studies in the marine environment reported that less than 20% of host-associated bacteria were able to inhibit pathogen growth (Dobretsov & Qian 2002, Zhang et al. 2009, Leyton & Riquelme 2010). This variability in pathogen inhibition by associated bacterial communities may explain differences in disease resistance between species or even individuals. Interestingly, only 18.5% (10 of 54) of the bacteria that demonstrated antibacterial activity yielded an amplification product for the presence of genes involved in the NRPS or PKS pathways, suggesting that alternative metabolites were used for their antibacterial activity.

Host species have been shown to use the chemical metabolites produced by their associated microorganisms to avoid infection by pathogens (Harder et al. 2003, O'Brien & Wright 2011). For example, microorganisms isolated from the mucus of sole prevented the adhesion of *Photobacterium damselae* ssp. *piscicida* (Chabrillón et al. 2005). Similarly, rainbow trout harbored pseudomonads on their skin that inhibited the proliferation of *Vibrio anguillarum* (Spanggaard et al. 2001). However, the capacity for mucus-associated bacteria to inhibit or outcompete pathogens is highly variable (Lee et al. 2003), so harboring multiple strains of bacteria with the ability to produce antibacterial metabolites could serve as a bet-hedging mechanism for pathogen resistance. In fact, diverse fish-associated bacterial communities have been linked to greater resilience and disease resistance (Verschuere et al. 2000, Chabrillón et al. 2005). Invasive lionfish, which have a more diverse bacterial community than some native Bahamian fishes (Stevens & Olson 2013), may use their bacterial community as a mechanism to aid in pathogen resistance, which would likely influence their ability to successfully establish in the invaded range.

Previous work demonstrated that invasive lionfish did not appear to harbor any known pathogenic or opportunistic bacteria on their skin (Stevens & Olson 2013). However, several fish pathogens, including one of the pathogens used in the present study, *P. damselae* ssp. *piscicida*, were previously detected on the skin of Caribbean squirrelfish (Stevens & Olson 2013). The antibacterial activity exhibited by lionfish-associated bacteria against known fish pathogens may explain the absence of these pathogens in the previous study. However, the present study assayed pathogen inhibition rather than investigated the mechanism(s) of activity, so we cannot comment on whether the pathogens were killed. The presence of biosynthetic genes for the production of potentially bioactive PKS and NRPS metabolites was evaluated but no other potential mechanisms of inhibition were assessed.

The diversity of lionfish-associated bacteria capable of inhibiting the fish pathogens was evident in the phylogenetic assessment. The taxonomic affiliations of these bacteria included several genera that are known to inhibit pathogen growth in other marine hosts and that were previously detected in lionfish-associated bacterial communities (Stevens & Olson 2013). Although the present study used cultivation-dependent approaches that are known to limit bacterial diversity, comparisons of the taxonomic identifications of our isolates to the lionfish bacterial clone libraries published previously (Stevens & Olson 2013) showed considerable overlap. Members of the genera *Alteromonas*, *Pseudoalteromonas*, *Tenacibaculum*, and *Vibrio* and the phylum *Bacteroidetes* were recovered in both studies, indicating that these bacteria may be important components of the lionfish surface-associated bacterial community. For example, *Vibrio* spp. isolated from sole were shown to be active against *P. damselae* ssp. *piscicida* (Chabrillón et al. 2005). *Bacillus pumilus*, an isolate recovered in the present study but not found in the previous culture-independent study, inhibited *V. parahaemolyticus* and *V. harveyi* infections in shrimp (Hill et al. 2009). *Vibrio* spp. and *Bacillus* spp. present in the intestines and on the skin of flounder and *Bacillus* spp. associated with brittlestars were active against a suite of known fish pathogens (Strahl et al. 2002, Sugita et al. 2002). The present study isolated a *Micrococcus* sp., a member of the phylum *Actinobacteria*, with antibacterial activity while, interestingly, Chabrillón et al. (2005) failed to detect pathogen inhibition by multiple species of *Micrococcus*. However, each study utilized different suites of test pathogens, reinforcing the concept that antibacterial activity is likely limited to specific pathogens. Isolates most closely related to members of the *Alteromonas* and *Pseudoalteromonas* were commonly cultivated from both the native and invaded ranges. Members of these genera (which were split by Gauthier et al. 1995) are known to produce antibacterial metabolites active against both human and fish pathogens (Dopazo et al. 1988, Barja et al. 1989). The variability in pathogen inhibition by the isolates recovered in the present study and other studies further supports the role of diverse microbial communities in promoting disease resistance of their hosts.

The fish pathogens used in the present study are ubiquitous in tropical waters worldwide and cause fish disease in both the Indo-Pacific and Atlantic Oceans (Linkous & Oliver 1999, Austin & Zhang 2006, Farmer & Hickman-Brenner 2006). As a result, differences in the activity of lionfish-associated bacteria against pathogens specific to the native or invaded range could not be assessed. Because of the lack of endemicity of fish pathogens to either the Indo-Pacific or Atlantic, it was not possible to fully explore whether lionfish, like other invasive organisms, escaped from pathogens through their establishment in the invaded range (sensu Vermeij 2005). If fish pathogens specific to particular ranges can be identified, it would be an intriguing question for future studies. However, 2 ubiquitous fish pathogens, *V. vulnificus* and *V. parahaemolyticus*, are also considered pathogens of concern for humans (Linkous & Oliver 1999, Farmer & Hickman-Brenner 2006), making their control in the marine environment an interesting and timely issue.

The epidermal mucus of some fish contributes to innate immunity and responds to environmental shifts and pathogen exposure by altering the composition and/or rate of excretion of mucus (Ellis 1974, Subramanian et al. 2008). However, antimicrobial activity testing of mucus extracts currently remains limited and results vary by species of fish (Hellio et al. 2002, Subramanian et al. 2008). In the present study, although ~1/3 of the lionfish-associated bacteria obtained from lionfish mucus were active against one or more of the 6 bacterial fish pathogens, no antibacterial activity of lionfish mucus extracts was detected against any of the fish pathogens or fish-associated (lionfish and squirrelfish) bacteria. There are several possible reasons for this disparity in activity. First, a previous study found no known fish pathogens associated with lionfish mucus (Stevens & Olson 2013), suggesting that skin conditions may not have required production of defensive molecules at detectable concentrations. Second, the concentration of freeze-dried mucus was standardized across samples prior to chemical extractions following Hellio et al. (2002), but did not take into account the size of the fish collected. Thus, it remains unknown whether the concentration tested was ecologically relevant. Chemical analyses of the activity of fish mucus have not examined the effect of correcting sample concentration for differences in fish size (Hellio et al. 2002, Bergsson et al. 2005, Subramanian et al. 2008, Bragadeeswaran et al. 2011). Thus, studies are needed to provide information regarding the antibacterial activity of fish mucus at ecologically relevant concentrations.

Previous work conducted in our laboratory indicated that lionfish retained a core bacterial community in both the native and invaded ranges, but that differences were apparent when the bacterial communities were examined by individual collection locations (Stevens & Olson 2015). As no bacteria were found associated with lionfish eggs, vertical transmission of these communities was not expected (Stevens & Olson 2013). In the present study, nearly half of the presumably environmentally acquired isolates from fish caught in the native range inhibited pathogen growth while nearly one-third of the isolates from the invaded range were active. Similar isolate diversity was found in both the native and invaded ranges and the percent of pathogen inhibition was not different between ranges, suggesting that the retained organisms may provide a beneficial function for the host through the inhibition of pathogen growth.

The continual exposure to potential pathogens requires that marine organisms are able to prevent the growth and proliferation of pathogens. The methods used in the present study did not consider the ecological relevance of antibacterial metabolite production as all experiments were conducted *in vitro* with pure cultures, but provided the framework for further analyses to determine whether laboratory results reflect the actual relationship between lionfish and their associated bacteria. Although lionfish mucus does not appear to prevent the growth of potential pathogens, the ability of their skin-associated bacteria to inhibit pathogen growth may be effective in preventing disease. By maintaining a high diversity of bacteria with the ability to inhibit pathogen growth throughout both the native and invaded ranges of the lionfish, it is likely this bacterial community plays a role in innate immune function and ultimately contributes to the invasive success of lionfish.

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Use of DNA metabarcoding for stomach content analysis in the invasive lionfish *Pterois volitans* in Puerto Rico

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ABSTRACT: Studies of lionfish feeding ecology seek to document the ecological impact of this invasive predatory species and determine which native prey species are at greatest risk. There are 2 common approaches to feeding ecology through gut content analysis: morphological identification to the lowest possible taxonomic rank and/or DNA barcoding of individual prey components in the stomach. The major disadvantage of both techniques is their inability to use advanced digested material. This study introduces next-generation sequencing to lionfish feeding ecology, employing DNA metabarcoding to analyze all components of the gut contents, including the previously unidentifiable portion. Sixty-three lionfish were caught from the inshore and offshore reefs of La Parguera, Puerto Rico. Stomach contents were separated into 2 sample components — a liquid (i.e. digested) and undigested tissue. A 313 bp region of the cytochrome oxidase subunit I (COI) gene was amplified from extracted DNA using specific primers for Caribbean reef fish. Samples were sequenced with an Illumina MiSeq platform, and the resulting 950+ sequences were compared against GenBank and the Barcode of Life Database to identify specimens at the lowest taxonomic level. Thirty-nine fish species from 16 families were identified (35 each in the digested and tissue fractions), including members of Pomacentridae, Acanthuridae, Gobiidae, Apogonidae, and Scaridae. Using the digested liquiform material proved efficient in detecting prey species, especially those that would have been missed with traditional methods.

KEY WORDS: Reef fish · Feeding ecology · Invasive species · Caribbean · Cytochrome oxidase subunit I · COI · Next-generation sequencing · NGS

INTRODUCTION

Invasive species are capable of altering ecosystems, evolving with their new environment (Mooney & Cleland 2001) and driving native species extinctions (Pimm 1987, Fritts & Rodda 1998). In response, management of invasive species attempts to mitigate their ecological and economic impacts (Buckley 2008). However, marine invasive species present a difficult management scenario where vectors promoting their spread and establishment may be known (i.e. ballast transport, aquarium trade) but cannot be easily regulated or avoided without strict enforcement (Bax et al. 2003). Marine invaders, once established, often become integrated into the ecosystem, whereby complete eradication is unfeasible (Thresher & Kuris 2004). This scenario is exacerbated when their presence extends to areas that remain inaccessible to management, such as mesophotic depths, or in cases where the spread of the invasive species is driven by larval dispersal. Aside from investigating management strategies, invasion ecologists must simultaneously seek to identify which native communities may be at greatest risk, either ecologically or economically.

Invasive species alter ecosystems through competition, niche displacement, hybridization, and predation, among other processes (Mooney & Cleland 2001). In particular, predation in the marine environment is a driving force structuring the fish communi-

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ties on coral reefs (Hixon 1991). Aside from observing this predator–prey interaction *in situ*, predation can also be documented using visual inspection or, more recently, DNA barcoding to assess biodiversity in diet from gut contents or feces (Sheppard & Harwood 2005). Over a decade has passed since DNA barcoding first proved useful in biodiversity applications (Hebert et al. 2003), and has recently been promoted as an ecological tool for addressing issues including a species' invasion potential, trophic interactions, and food webs (Joly et al. 2014). With the advancement and lower cost of DNA sequencing and massive growth of reference databases, a metabarcoding approach using next-generation sequencing (NGS) has quickly emerged as a promising method for higher-resolution diet analysis (Pompanon et al. 2012, Taberlet et al. 2012, de Barba et al. 2014, Deagle et al. 2013). Metabarcoding is the combination of DNA-based identification and high-throughput DNA sequencing that reduces sampling effort and maximizes species-level identification of tissue remnants that were previously undetected or underused by traditional methods.

There are known constraints on metabarcoding, including the inability to quantify the species information obtained (Deagle et al. 2010, 2013, Bowles et al. 2011, Murray et al. 2011). Results are limited or biased to the frequency of occurrence, which still provides useful information when seeking to understand localized effects of an invasive predator. However, the underlying variability in DNA quality, differential breakdown of that DNA during digestion, and differences in digestion stages (Deagle & Tollit 2007, Troedsson et al. 2009, Valentini et al. 2009b), as well as the objective of identifying several different organisms within the same sample (i.e. the gut) (Valentini et al. 2009a), still hinder the quantification aspect in metabarcoding of gut contents. Despite these disadvantages, metabarcoding is quickly gaining popularity as a tool for assessing biodiversity in animal diets (Leray et al. 2013, de Barba et al. 2014). NGS allows for the highest degree of confidence in gut content analysis (Pompanon et al. 2012) with significantly reduced sampling effort (Taberlet et al. 2012), but has only recently been applied to fish feeding ecology (Leray et al. 2013, 2015).

Understanding the extent and possible ecological impact of the lionfish *Pterois volitans* invasion of the Western Atlantic, Gulf of Mexico and Caribbean is an issue that employs all facets of lionfish biology and ecology. Of particular interest is how this Indo-Pacific fish will affect native coral reef fauna, especially commercially and ecologically important reef fishes. Researchers have sought to address what lionfish consume, in terms of species and size classes, in an effort to document which species may suffer the greatest level of mortality. Feeding ecology has been a key component in many lionfish studies, resulting in our current understanding of site specificity in dietary preferences (Côté & Maljković 2010, Muñoz et al. 2011, Layman & Allgeier 2012) and overall diversity of diet (Albins & Hixon 2008, Morris & Akins 2009, Green et al. 2011).

There are 2 common approaches to lionfish feeding ecology through gut content analysis: morphological identification to the lowest possible taxon (i.e. using morphological characters to identify whole or only partially digested specimens) or a DNA barcoding approach, which involves sequencing of the mitochondrial 16S rRNA or cytochrome oxidase subunit I (COI) genes from all distinct prey components of the stomach. Morphological identification relies heavily on the ability to identify digested organisms to the species level, which is not possible in many cases (Baker et al. 2014). This technique discards useful information that could be obtained in the digested portion of the stomach contents (the liquids or digested pulp). However, the traditional morphological method is widely applied (Albins & Hixon 2008, Morris & Akins 2009, Alexander & Haynes 2011, Jud et al. 2011, Muñoz et al. 2011, Green et al. 2012, Frazer et al. 2012, Layman & Allgeier 2012, Green & Côté 2014), while the more accurate DNA barcoding approach has been less frequently used (Barbour et al. 2010, Valdez-Moreno et al. 2012, Côté et al. 2013). Despite the higher resolution attained with this approach, traditional DNA barcoding also has disadvantages. This technique does not reduce sampling effort (Coissac et al. 2012) and can be applied only to items in the stomach contents for which barcode information is available either in databases or can be generated during concomitant sequencing of possible prey from the area. However, as opposed to morphological identification, analyzed items can include unrecognizable specimens, liquids, or pulp (Saitoh et al. 2003), but this approach requires molecular cloning and is therefore labor intensive and costly. These digested products may contain underrepresented prey items, or prey items that have yet to be acknowledged within the diet.

In this study, we used metabarcoding analysis of all lionfish stomach contents, regardless of their digestive stage, to provide a more accurate profile of the lionfish prey in Puerto Rico while demonstrating that the methodological approach is applicable to all other regions of the invasion. Metabarcoding resolution of lionfish stomach contents is supported by the *a priori* knowledge, albeit site specific, of the lionfish diet (Côté & Maljković 2010, Muñoz et al. 2011, Layman & Allgeier 2012), whereas the use of COI as a marker often allows for identification to the species-level in online reference databases. The specific objectives were (1) to identify the prey of Puerto Rican lionfish in stomach contents through the use of NGS, (2) to compare inshore and offshore diets of lionfish in La Parguera, Puerto Rico, and (3) to assess the general suitability of the NGS metabarcoding approach compared to published studies using other gut content analysis methods.

MATERIALS AND METHODS

Collection and locations

Sixty-three lionfish were used for metabarcoding of entire stomach contents. Approximately half of the lionfish came from inshore reefs of La Parguera (17° 58′ 12.33″ N, 67° 2′ 45.83″ W) while half were collected from offshore shelf-edge reefs in the same region from June 2013 to January 2014 (Fig. 1). La Parguera is a natural reserve on the southwest coast of Puerto Rico that is heavily affected by environmental and anthropogenic stressors resulting in low coral cover, high macroalgal abundance, and diminished populations of large-bodied fish species, resulting in the system being dominated by small-bodied planktivores and piscivores (Pittman et al. 2010). The inshore reefs are subjected to high particle suspension and lower water quality (García-Sais et al. 2005, 2008) and are connected through a series of shallow patch and linear reefs, mangroves, and seagrasses critical for ontogenetic migrations (Aguilar-Perera & Appeldoorn 2007, 2008). The offshore shelf-edge reefs are characterized by spur and groove formations and better water quality, with exposure to stronger currents (Pittman et al. 2010). The inshore and offshore reefs harbor dissimilar fish richness and biomass (Pittman et al. 2010), where inner reefs are comparatively lower in species richness than shelf-edge reefs (Nemeth 2013), thus providing a potential spatial comparison of lionfish diets. Lionfish were collected by pole spear and SCUBA at depths ≤30 m. On the boat, the venomous spines were immediately removed and specimens were placed on ice to slow digestive processes and preserve DNA (Baker et al. 2014). All metrics pertaining to lionfish size, sex, reproductive state, and weight were recorded (see Table S1 in the Supplement at www.int-res.com/articles/suppl/m558p181_supp.pdf). The stomachs were removed <2 h after lionfish capture, and preserved whole in a −80°C freezer until further processing.

DNA extraction and COI amplification

Samples were thawed at room temperature until the liquefied digested materials could be removed. Only a few prey items could be identified with visual inspection, thus morphological identification was not coupled with this study. DNA was extracted (Qiagen DNeasy Blood & Tissue Kit) following the guidelines of the manufacturer from 2 components of the 63 whole stomach contents: (1) the tissues of the remaining partially digested organisms (as with a DNA barcoding approach) and (2) the liquids of completely digested organisms, resulting in 126 samples. Cross contamination was avoided by subjecting dissection utensils to an open flame, followed by an ethanol rinse between each sample, or in some cases new utensils were used for each stomach. The quality and quantity of extracted DNA was measured with the NanoDrop 2000 (Thermo Fisher Scientific). Samples were stored in a −20°C freezer for later analysis.

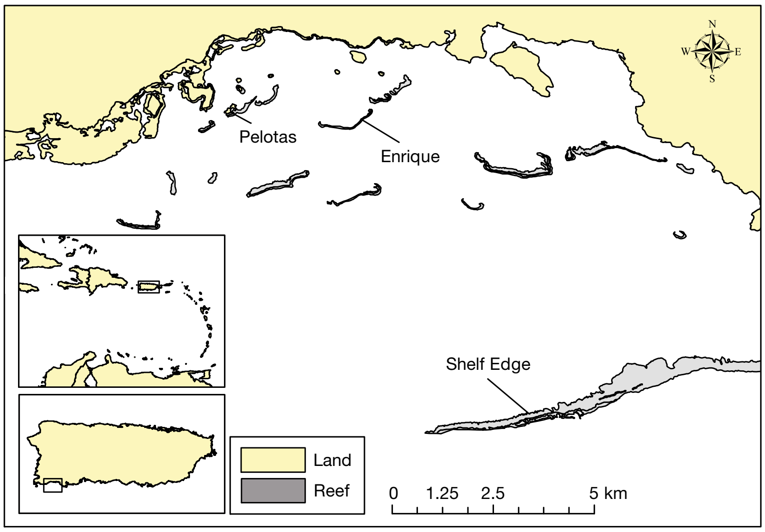

Fig. 1. La Parguera, Puerto Rico, and the insular shelf, with 3 study sites identified. Pelotas and Enrique reefs were the inshore collection sites, while the shelf edge was the offshore location. All sampling was performed up to 30 m depth

PCR amplification of a 313 bp COI fragment from prey mtDNA was performed on each of the 126 samples (tissues and liquid). This gene was chosen for its exceptional coverage of Caribbean fishes (Weigt et al. 2012) and other marine metazoan taxa (Bucklin et al. 2011). It is also the most widely accepted DNA barcode, where its rapid evolution allows for discrimination between closely related species (Hebert et al. 2003). Taxon-specific primers (for fish and invertebrates in coral reef fish guts) were utilized; the mlCOIintF forward primer (5′GGW ACW GGW TGA ACW GTW TAY CCY CC) in conjunction with the jgHCO2198 reverse primer (5′TAI ACY TCI GGR TGI CCR AAR AAY CA) (Leray et al. 2013). The specific region of COI is adequately represented in online databases for Caribbean coral reef fishes and invertebrates (Leray et al. 2013), as well as estimates of relative abundance of species in benthic samples (Leray & Knowlton 2015). The DNA amplification was completed in a total volume of 20 µl on the MyCycler (Bio-Rad Laboratories). The PCR recipe contained 0.6 µl of 10 µM of each forward and reverse primers, 10 µl of MyTaq DNA polymerase mix (Bioline), and 0.5 µl of genomic DNA. This recipe varied slightly depending on the success of the PCR, in which the concentration of DNA was increased up to 1.5 µl and all other ingredients varied accordingly to maintain a 20 µl reaction. We adopted the PCR profile from Leray et al. (2013) and conducted 16 initial cycles: denaturation for 10 s at 95°C, annealing for 30 s at 62°C, and extension for 60 s at 72°C. This initial set of cycles was followed by 25 cycles at 46°C annealing temperature with the same denaturation and extension steps, with a final extension at 72°C for 6 min. Success of PCR amplifications was validated on 1.5% agarose gels. The second step of the PCR process involved addition of the barcode identifiers. COI amplicons were ligated with a unique 3 base identifier (ATG), followed by a specific 6 base barcode added to the forward and/or reverse primer that would allow for identification of each sequence back to a particular lionfish stomach, as well as whether it was sampled from the liquid or tissue portion of the diet (see Tables S2 & S3 in the Supplement). We produced 126 unique combinations of barcodes from 16 forward primers and 7 reverse primers, including the original PCR primers.

All samples were loaded into a 2% agarose gel with TAE buffer and allowed to run for 45 min. The gel was briefly placed under a low-intensity UV light to identify the presence of the bands. Each sample was then excised from the gel using the ‘freeze–squeeze’ method (Tautz & Renz 1983), avoiding primer dimers, and was placed into individually labeled 1.5 ml centrifuge tubes. In total, 109 samples were successfully acquired. Successful samples represented 59 offshore samples and 50 inshore samples, divided into 57 tissue samples and 52 liquid samples.

Sequencing and bioinformatics

Samples were multiplexed and sequenced in 1 Illumina MiSeq lane (Scripps Research Institute, CA). Resulting reads were cleaned in the FASTQ filing and extended using FLASH pair software (Mago & Salzberg 2011). Extended fragments were converted to FASTA files. To utilize the insert in both directions, the reverse complement of the extended read (‘FASTX’) was combined with the original extended fragment. Sequences were then demultiplexed to identify reads back to their original stomach sample.

In total, 966 sequences were obtained. These were manually trimmed of the original PCR primers in Notepad++ v6.8, and each sequence was individually inspected. All sequences shorter than 200 bp length were discarded, along with duplicates and chimeric sequences. The resulting 313 bp COI fragment sequences were blasted (BLASTn) in GenBank (August 2015) to identify matches. A confident match was identified as 98% or higher for vertebrates and 80% or higher for invertebrates. The difference in acceptance of matches is based on the limited availability of invertebrate references in GenBank. Sequences were also referenced in the Barcode of Life Database (BOLD Systems v.3) using known and validated barcode identification numbers corresponding to voucher specimens (Victor et al. 2015) and accepted at a 98% match (September 2015). All cleaned reads were translated into amino acids using ExPASy Translate tool (Artimo et al. 2012) and MEGA 6 (Tamura et al. 2013) to further support accurate matches to references in both databases. Cleaned reads were separated by species and aligned in MEGA 6 to identify insertions, deletions, and frame shifts. If stop codons were present in the sequence, the sequence was rejected. An insertion of an amino acid (3 bases) was accepted, and all reads with 1 or 2 insertions and 1 deletion were accepted. A sequence was discarded if a series of ‘N’s representing unknown bases were present in the read, indicating sequencing ambiguity. All vertebrate sequences with less than 98% match were removed from subsequent analysis. Species that were represented by only 1 sequence were retained, in an effort to document rare and under-represented items from the gut contents that might previously have been unreported. All sequences obtained from this study are available in GenBank (accession numbers KX140056–KX140702) and a BOLD dataset (DS-PARG2016).

RESULTS

Lionfish diet in La Parguera, Puerto Rico, was diverse, with gut content analysis through metabarcoding revealing 2 phyla, 5 orders, 18 families, 23 genera, and 40 species. We assume that all prey DNA recovered from the gut was prey of lionfish. All fish sequences matched a reference in BOLD and GenBank. Of the 966 sequences recovered, 442 had fish species-level matches to 98% or greater and an additional 205 sequences were the lionfish itself, resulting in a 65% metabarcoding efficiency at the 98% similarity threshold for fish. Of those fish sequences, excluding lionfish, 17 had up to 2 insertions while 8 had 1 deletion and 7 sequences had an additional amino acid. Thirty-seven sequences could not be demultiplexed to the appropriate stomach and were labeled as unclassified. Eleven sequences reported discrepancies in similarities between databases, but were included in the final count if at least 1 match met the acceptance criteria. Forty-six sequences had stop codons present and were discarded, 99 were duplicated sequences from de-multiplexing errors and were discarded. Additionally, 18 chimeric or nonsensical sequences were discarded as well as 2 sequences shorter than 200 bp. Lastly, 99 sequences could not be matched at 98% or higher to either database. At a similarity match of 80 to 100% in GenBank and BOLD, 22 sequences corresponded to invertebrates, with 5 matched at the species level; however, a disagreement of identification occurred when comparing both reference databases. Thus, these species were placed in a higher taxon resulting in 18 Decapoda sequences, 1 Penaeidae, 2 Portunidae, and 1 sequence of the shrimp *Metapenaeopsis gerardoi*.

Fish contributed to the largest portion of the diet (95% of prey DNA recovered). Fish families with the greatest number of species represented in the diet included Gobiidae (6), Apogonidae and Scaridae (5), and Pomacentridae (4). By frequency of occurrence, Apogonidae made up 18%, while Gobiidae (9%) and Scaridae (10%) were less frequently found (Table 1). Pomacentridae had the greatest frequency of occurrence (35%), which was dominated by 3 species: *Chromis multilineata* (71%), *C. cyanea* (63%), and *Stegastes partitus* (58%) (Table 2).

Four species were observed only in the inshore lionfish stomach contents, while 8 species and 1 family were unique to offshore diets. Furthermore, 3 taxa were detected only in the liquid portion of the diet including the first account of the labrisomid *Starksia williamsi* in Puerto Rico (Table 3).

Table 1. Fish families represented in the diet of lionfish *Pterois volitans* at La Parguera, Puerto Rico. Number of species corresponds to those identified to species level except the Family Lutjanidae. Frequency indicates the number of stomachs in which they were found

Family	No. of species	Frequency (%)
Acanthuridae	1	2.18
Apogonidae	5	18.58
Chaenopsidae	1	3.00
Chaetodontidae	1	1.09
Gobiidae	6	9.56
Grammatidae	1	0.55
Haemulidae	1	1.09
Holocentridae	1	1.09
Labridae	3	3.00
Labrisomidae	2	1.91
Lutjanidae	1[a]	0.55
Pomacentridae	4	34.69
Priacanthidae	1	0.55
Scaridae	5	10.65
Serranidae	3	9.29
Synodontidae	1	2.18

[a] Only identified to Family level

Invertebrates represented a small portion of the diet, accounting for only 5% of the sequences obtained from gut content analysis. All cleaned sequences reported at least an 82% similarity to a reference in GenBank, which was usually complemented by a better match in BOLD. The Order Decapoda was the most abundant taxon (76%) (Table 4). Two families, Penaeidae and Portunidae, were documented only in offshore samples, and only from the tissue. The only species-level identification was the shrimp *Metapenaeopsis gerardoi*.

Lionfish DNA was present in every stomach, indicating the overwhelming abundance of predator DNA in the samples. For this reason, lionfish was not included in the prey profiling.

DISCUSSION

This study presents the first case of DNA metabarcoding for lionfish stomach contents. Overall, fish were identified as the most dominant prey component in the diet of lionfish in La Parguera. Representatives of several fish functional groups were observed within the gut, including herbivores, piscivores, and planktivores. No commercially important species of groupers or snappers were identified, which could be due to their low abundance in the study area resulting from high fishing intensity. Fishermen in the shallow water reef systems of La Parguera typically

Table 2. Number of lionfish *Pterois volitans* stomachs in which fish species were found, by location (inshore and offshore collection sites) and gut fraction. Species could occur in both liquid and tissue samples from the same stomach. Unclassified could not be de-multiplexed back to a particular stomach. Frequency is the frequency of occurrence for all species from all stomachs, including those unclassified

Prey species	Inshore	Offshore	Liquid	Tissue	Unclassified	Frequency (%)
Acanthurus tractus	3	5	4	5	0	12.31
Apogon maculatus	7	11	9	10	2	30.77
Apogon pillionatus	3	10	9	5	0	20.00
Apogon townsendi	0	4	4	1	1	7.69
Bodianus rufus	1	2	1	2	0	4.62
Chaetodon capistratus	2	2	0	4	0	6.15
Chromis cyanea	7	27	16	24	7	63.08
Chromis multilineata	15	28	7	9	3	70.77
Clepticus parrae	0	2	1	1	0	3.08
Coryphopterus glaucofraenum	9	5	8	10	1	23.08
Coryphopterus hyalinus	1	1	1	1	0	3.08
Coryphopterus lipernes	3	8	3	8	2	20.00
Coryphopterus personatus	2	0	1	2	0	3.08
Coryphopterus tortugae	2	0	1	1	0	3.08
Emblemariopsis arawak	0	2	0	2	0	3.08
Emblemariopsis spp.	4	4	5	4	1	13.85
Gnatholepsis thompsoni	0	1	1	0	0	1.54
Gramma loreto	2	0	0	2	0	3.08
Haemulon flavolineatum	2	2	2	3	0	6.15
Halichoeres garnoti	0	6	3	3	0	9.23
Heteropriacanthus cruentatus	1	1	0	2	0	3.08
Hypoplectrus spp.	6	10	8	10	1	26.15
Hypoplectrus aberrans	0	1	1	0	0	1.54
Hypoplectrus nigricans	4	3	3	5	3	15.38
Hypoplectrus puella	2	2	2	2	2	9.23
Lutjanidae sp.	0	1	1	0	0	1.54
Malacoctenus macropus	2	2	1	3	0	6.15
Phaeoptyx conklini	8	11	13	10	1	30.77
Phaeoptyx pigmentaria	2	5	3	5	3	15.38
Sargocentron coruscum	2	1	0	3	1	6.15
Scarus iseri	14	10	14	14	3	41.54
Scarus taeniopterus	0	2	1	1	0	3.08
Scarus vetula	1	1	1	1	0	3.08
Sparisoma radians	3	0	0	3	1	6.15
Sparisoma viride	1	3	2	2	0	6.15
Starksia williamsi	0	2	2	0	1	4.62
Stegastes partitus	8	26	17	15	4	58.46
Stegastes variabilis	1	1	1	1	0	3.08
Synodus intermedius	1	6	4	3	1	12.31

Table 3. Species that were observed in only 1 habitat or type of lionfish *Pterois volitans* stomach content category

Inshore	Offshore	Liquid	Tissue
Coryphopterus personatus	*Apogon townsendi*	*Gnatholepsis thompsoni*	*Emblemariopsis arawak*
Coryphopterus tortugae	*Clepticus parrae*	Lutjanidae sp.	*Chaetodon capistratus*
Gramma loreto	*Emblemariopsis arawak*	*Starksia williamsi*	*Gramma loreto*
Sparisoma radians	*Gnatholepis thompsoni*		*Heteropriacanthus cruentatus*
	Halichoeres garnoti		*Sargocentron coruscum*
	Hypoplectrus aberrans		*Sparisoma radians*
	Lutjanidae sp.		
	Scarus taeniopterus		
	Starksia williamsi		

Table 4. Number of lionfish *Pterois volitans* stomachs in which invertebrate taxa were found, by location (inshore and offshore collection sites) and gut fraction. Stomachs could have taxa represented in both liquid and tissue factions. Frequency is the frequency of occurrence for each taxa from all stomachs. The percent similarity refers to the match to a reference in the Barcode of Life Database

Taxon	Inshore	Offshore	Liquid	Tissue	Frequency	Similarity (%)
Decapoda	11	2	6	10	76.47	97.3
Penaeidae	0	1	0	1	5.88	88.7
Portunidae	0	2	0	2	11.76	100
Metapenaeopsis gerardoi	0	1	0	1	5.88	97.6

target snappers, groupers, grunts, and parrotfishes (Pittman et al. 2010), all of which are potential prey for lionfish. Ecologically important species were identified in the gut, such as *Scarus vetula, S. taeniopterus, S. iseri*, and *Sparisoma viride*, which are known to help prevent macroalgae from displacing corals (Mumby & Steneck 2008). Some of these parrotfishes have been identified to co-occur across all seascapes in La Parguera, including the offshore reefs (Pittman et al. 2010, Nemeth 2013), supporting their presence in the diet of both inshore and offshore lionfish.

Two comprehensive studies of the La Parguera fish assemblages (Pittman et al. 2010, Nemeth 2013) and one island-wide study (NCCOS 2016) provide field-occurrence data for a comparison to observed prey frequencies within the guts (Table 5). In general, lionfish diet is representative of the particular fish assemblages observed in La Parguera and Puerto Rico, which supports the emerging trend observed from other studies in the Caribbean (Côté & Maljković 2010, Muñoz et al. 2011, Layman & Allgeier 2012) that lionfish are trophic generalists and that dietary preferences are site specific and driven by the spatial and temporal dynamics of prey. However, some species are consumed in unequal proportions to what exists in nature (Table 5), represented by the absence of *Thalassoma bisfasciatum* within the guts, and the overrepresentation of both *Chromis cyanea* and *C. multilineata.* These pomacentrids may be preferentially targeted due to their morphology (i.e. small but deep-bodied) and hovering behavior, both of which have been identified as preferred traits for lionfish prey (Green & Côte 2014).

Overall dietary profiles were very similar inshore and offshore, as would be expected given the broad spatial distribution of the dominant prey species observed. Nevertheless, differences were observed between inshore and offshore diets, as revealed by species found only in one of these categories. Eight fish species were identified only in offshore diets. Of these, *Clepticus parrae, Halichoeres garnoti*, and *Scarus taeniopterus* are typically associated with shelf-edge habitats (Pittman et al. 2010, NCCOS 2016) and were not largely represented in the lionfish diet overall. In contrast, *Stegastes partitus* was among the most frequently observed species in the gut, and despite its occurrence across the insular shelf, it showed a strong association with the shelf-edge reef system, with 28 stomachs containing this species in offshore lionfish versus only 8 in the inshore system. In general, more prey species were identified from offshore samples (n = 36 versus inshore n = 31), where their total frequency of occurrence was almost

Table 5. Percent frequency of occurrence of key species within lionfish *Pterois volitans* gut contents (this study) and on the insular shelf of La Parguera (Pittman et al. 2010, Nemeth 2013) and Puerto Rico island-wide (Clark et al. 2015). Nemeth (2013) frequencies refer to abundance in terms of percent mean density of individuals per 100 m^2

Fish species	Frequency				Rank			
	Pittman et al.	NCCOS	Nemeth	Gut	Pittman et al.	NCCOS	Nemeth	Gut
Thalassoma bifasciatum	34	75	11.3	0	5	3	1	8
Chaetodon capistratus	42.2	4	1.8	6	2	7	7	7
Acanthurus tractus	41.9	76	3	12	3	2	6	6
Coryphopterus glaucofraenum	28	21	–	23	6	6	–	5
Scarus iseri	45	49	8.1	42	1	4	3	4
Stegastes partitus	38	80	10.6	58	4	1	2	3
Chromis cyanea	6.1	31	4.7	63	7	5	4	2
Chromis multilineata	3.5	–	3.9	70	8	–	5	1

twice that observed inshore (n = 208 versus inshore n = 119). The shelf edge off La Parguera has the greatest fish species richness and biomass in the region, with up to 41 species identified in a single 100 m^2 transect (Pittman et al. 2010, Nemeth 2013). However, these results may also be due to the significantly (*t*-test, $p < 0.05$) larger size of lionfish found offshore (217.8 g, 200 mm standard length [SL]) compared to inshore (147.6 g, 167.7 mm SL). The gobies *Coryphopterus personatus* and *Coryphopterus tortugae*, the parrotfish *Sparisoma radians,* and the basslet *Gramma loreto* were found only in lionfish sampled from inshore reefs. All were sampled at low frequency, but the distributions of the first 3 species are known to be inshore. Additionally, 2 frequently occurring prey with broad distributions across the shelf, the parrotfish *Scarus iseri* and the goby *Coryphopterus glaucofraenum*, were the only species found more frequently inshore. Juveniles of the former species are common in inshore nursery areas, but are infrequently seen near the shelf edge (Cerveny 2006).

These comparisons between the distribution and frequency of prey species in lionfish stomachs relative to their distribution in the field suggest that both the list of prey species and their frequency of occurrence as determined by metabarcoding can be used to compare diets among different populations or even different habitats and life history stages. In general, smaller or juvenile lionfish have been observed to consume proportionally more invertebrates than larger, adult lionfish (Morris & Akins 2009), and at least 28 % of prey by number in stomach contents represent invertebrates (Morris & Akins 2009, Valdez-Moreno et al. 2012). In particular, shrimp are the most common invertebrate observed, representing the families Palaemonidae, Penaeidae (Barbour et al. 2010, Jud et al. 2011, Layman & Allgeier 2012), and Alpheidae (Valdez-Moreno et al. 2012, Layman et al. 2014). In our study, invertebrates were equally consumed by juvenile lionfish (n = 8, 74–181 mm SL) and adult lionfish (n = 7, 190–239 mm SL), and were observed in the guts predominantly from inshore lionfish (n = 11) versus offshore (n = 5). Overall, invertebrates did not contribute to a large portion of the diet, and proved to be the most difficult to identify given the potential number and diversity of available prey species inhabiting Caribbean reefs and the current status of the reference databases. Invertebrates are lacking in species-level identification in both BOLD and GenBank, and occasionally the 2 databases did not agree on the identification based on the submitted DNA sequences. Thus, we had to place our sequence into higher taxa, as our resolution could not be matched by references in both BOLD and GenBank. The crustacean Order Decapoda contributed to the greatest resolution and highest frequency. The diet included both crabs and shrimps, represented by Portunidae and Penaeidae, respectively, which is consistent with the previously known feeding ecology of lionfish (Morris & Akins 2009).

The spatial and temporal distribution of lionfish also affects the prey items detected in the gut. Lionfish are habitat generalists (Cure et al. 2014), and can be found in any natural marine system, or artificial structure, including the seagrass–mangrove–reef continuum within inshore La Parguera. In contrast to other mobile predators (Appeldoorn et al. 2009), lionfish do not typically undertake diurnal feeding migrations between different habitats. However, they have been observed to venture off-structure to feed over sand, perhaps in response to intraspecific competition (Green et al. 2011, Dahl & Patterson 2014). In our study location, lionfish densities are relatively low (C. Harms-Tuohy pers. obs.), and intraspecific competition is likely minimal. A study of lionfish movement on a reef in La Parguera identified that lionfish did not move between nearby forereef habitat of the same depth and characteristics (Harms-Tuohy 2016). Considering that all lionfish were collected from the fore reefs of the sampling sites, we would expect their diets to resemble the prey communities dominant to these areas, and this was evidenced in our results. This further supports that the diet of lionfish observed in this study was driven by the spatial distribution of the prey.

Overall, this study successfully demonstrated the efficiency of the metabarcoding approach to identify the prey profile of lionfish. The most significant contribution of this method is between use of the digested materials in the guts, including what little remains within empty stomachs. We report a comparable resolution of species diversity obtained from the liquefied portion of the guts in comparison with that contributed by the tissues. Given that lionfish collection was performed at times most feasible to divers (08:00–14:00 h), the contents of the lionfish stomachs were almost entirely digested. However, in most cases, partially digested specimens could be identified taxonomically as either fish or invertebrate, but no further. Morphological identification of gut contents relies heavily on the digested state of the prey items (Baker et al. 2014). Regardless, this method has been used widely in lionfish feeding ecology. Visual assessment of gut contents from lionfish in the Bahamas reported up to 41 fish species (Albins & Hixon 2008, Morris & Akins 2009), while DNA barcoding of 157 lionfish gut contents in the Mexican Caribbean (Valdez-Moreno et al. 2012), and 130 lionfish gut contents from the Bahamas (Côté et al. 2013), reported 31 and 37 fish species, respectively. Although the yield of new species identified certainly decreases with increased sampling effort (see Morris & Akins 2009), our study reports 39 different fish species from only 63 lionfish stomachs, thus validating the small sampling effort and increased efficiency of DNA metabarcoding (Table 6).

Despite the efficiencies realized using metabarcoding for prey identification, our approach is not without caveats. There is a high initial investment regarding the purchase of primers with enough barcodes to differentiate each sample. However, in subsequent studies, the same barcodes can be reused, thus significantly reducing the cost associated with specimen capturing, DNA processing (e.g. extraction, amplification, gel extraction), and NGS. Additionally, there is no current method to differentiate among prey-of-prey (i.e. items that were consumed by a prey fish that the lionfish subsequently ate) and true prey. However, as our lionfish diet mostly comprised herbivores and planktivores with few piscivores, this scenario likely did not affect our results. There is currently no precise way to quantify prey in the stomach using metabarcoding. Unfortunately, it cannot be assumed that the number of sequences for each particular species represents the amount of DNA (or number of individuals) contributing to the sample because the quality of that DNA depends on many factors including degradation and digestion rates (Deagle & Tollit 2007, Troedsson et al. 2009, Valentini et al. 2009b). Thus, quantitative analyses at this time are limited to the frequency of prey occurrence. Percent composition by number can be calculated by conducting metabarcoding on experimental individuals fed a mixed but controlled number of prey sacrificed over several time periods of digestion, including complete digestion to the liquid phase. Nevertheless, identifying prey and their frequency of occurrence using metabarcoding is a significant step forward, allowing useful information to be obtained from a minimum number of samples (Taberlet et al. 2012) without the need to collect samples immediately after feeding events. To further enhance the resolution of sequences obtained from this method, species-specific primers could be generated to search for the presence of specific prey items that may be of concern (Pompanon et al. 2012). This is particularly useful if the prey are poorly represented in a diet. Predator blocking primers could also assist in a wider range of detected species, in that predator DNA many times overwhelms that of the prey (Pompanon et al. 2012). In addition, it is unlikely that our primers amplified every single prey. Thus, the fish diet presented here is not expected to be exhaustive of all taxa consumed by the lionfish.

Successful mitigation of the impacts of invasive species requires an understanding of how they are affecting native communities. Impacts can be defined as competition or predation with native species, habitat alteration, niche displacement, and hybridization among many other factors. The direct effect of predation can be assessed through gut content analysis and measured in terms of what species may be targeted, or what functional groups are at risk in a broader sense. Feeding ecology will continue to provide temporal and spatial snapshots of lionfish impacts on native communities, which can be compared regionally and annually to assess changes in prey assemblages.

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Table 6. Yield of new fish species identified in different methodological attempts. Comparison of visual identification, DNA barcoding, and DNA metabarcoding methods

No. of stomachs	No. of fish species	Method	Yield	Reference
1069	41	Visual ID	0.038	Morris & Akins (2009)
52	14	Visual ID	0.269	Albins & Hixon (2008)
157	31	Barcoding	0.197	Valdez-Moreno et al. (2012)
130	37	Barcoding	0.285	Côté et al. (2013)
63	39	Metabarcoding	0.619	This study

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Contribution to the Theme Section 'Invasion of Atlantic coastal ecosystems by Pacific lionfish'

Diet of invasive lionfish (*Pterois volitans* and *P. miles*) in Bermuda

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ABSTRACT: As a generalist and opportunistic predator, lionfish (*Pterois volitans* and *P. miles*) consume large quantities of juvenile reef fish and invertebrates, as well as the adults of small-bodied species. To better understand the impacts of these fishes upon invaded coral reef ecosystems, we describe the feeding habits of invasive lionfish in Bermuda based on stomach contents analysis, and the influence that environmental factors have on their diet via spatial and temporal changes in prey availability. Relative to other regions throughout the northwestern Atlantic, lionfish in Bermuda consume a greater proportion of crustaceans, and their diet appears to rely upon the relative abundance of available prey species. A poorly -known crustacean, the red night shrimp *Cinetorhynchus rigens*, is the species of greatest importance to the diet of Bermuda lionfish. Currently, herbivorous fishes do not make a major contribution to their diet, although the lionfish frequently target both ecologically (e.g. bluehead wrasse *Thalassoma bifasciatum*) and economically important species (e.g. Atlantic creolefish *Paranthias furcifer*).

KEY WORDS: Invasive lionfish · Feeding ecology · Stomach contents · Diet · Resource use

INTRODUCTION

Invasive species play a major role in human-induced environmental changes across the planet (Mack et al. 2000). In marine ecosystems, established invasive species pose a major threat to biological diversity (Vitousek et al. 1997) by impacting community structure and function and modifying ecosystem processes, which together may have long-lasting ecological and economic consequences throughout invaded regions (Molnar et al. 2008).

Lionfishes (*Pterois volitans* and *P. miles*), native to the Indian and Pacific Oceans, are recognized as the first invasive teleost to expand its range successfully into the Atlantic Ocean (Whitfield et al. 2002). Following their first documented sighting off Dania Point, Florida in 1985, lionfish dispersed rapidly northward along the US coast, through the Caribbean Sea, and into the Gulf of Mexico (Schofield 2010). Ferreira et al. (2015) reported the first capture of an invasive lionfish in Brazil in 2014, an event predicted by their thermal tolerance, generalist feeding behavior, habitat versatility, ability to exploit multiple habitats, and broad latitudinal range in the Indo-Pacific (Morris & Whitfield 2009, Luiz et al. 2013). Today, lionfish are one of the most common, and oftentimes most abundant mesopredators in parts of their invaded range (Whitfield et al. 2007).

The progression of the lionfish invasion in Bermuda appears to be unique compared to other locations in the northwestern Atlantic. The first lionfish recorded in Bermuda was collected in 2000 from a

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tide pool in Devonshire Bay. In the years following, additional lionfish were captured or sighted in low numbers around Bermuda, with only 12 reported between 2001 and 2003, and 18 reported during the summer of 2004 (S. Manuel, Bermuda's Department of Environment and Natural Resources, unpubl. data). Although Bermuda was the first country outside of the USA to report lionfish in the Atlantic, the lionfish population there appears to have grown more slowly than in other regions (C. Eddy unpubl. data). In the Bahamas, for example, lionfish densities reached approximately 400 fish ha^{-1} only 4 yr after their initial invasion began (Green & Côté 2009), and the mean lionfish density on natural reefs in the northern Gulf of Mexico reached 490 fish ha^{-1} within 3 yr of the first lionfish sighting (Dahl & Patterson 2014, Frazer et al. 2012). This difference could be attributed to the effects of strong seasonal temperature changes in Bermuda on reproduction (i.e. a relatively shorter spawning season due to cold winter sea temperatures; Morris 2009, Smith et al. 2013), limited recruitment from other regions due to Bermuda's isolation (Schultz & Cowen 1994), and ocean currents that may advect locally spawned eggs and larvae away from the island (R. Johnson, Bermuda Institute of Ocean Sciences, pers. comm.). Nonetheless, anecdotal evidence and catch data (e.g. public culling efforts and lobster fishery bycatch; Bermuda's Department of Environment and Natural Resources unpubl. data) from Bermuda suggests that the lionfish population continues to expand, and there is concern that Bermuda may still be in the early stages of invasive species population growth, characterized by a period of slow growth followed by a period of exponential growth (Sakai et al. 2001).

As a generalist and opportunistic invasive predator with highly effective and unique hunting strategies (i.e. ambush predation, cooperative hunting, palpation, herding, and directed water-jets) (Kendall 1990, Morris & Akins 2009, Albins & Lyons 2012), lionfish consume large quantities and a broad diversity of juvenile and small-bodied adult reef fish as well as small invertebrates (Morris & Akins 2009). Prey naïveté in the invaded range, along with the lionfishes' resemblance to more benign organisms, also appears to contribute to their hunting success (Cure et al. 2012). Considering these feeding characteristics, their rapid growth rate (Edwards et al. 2014), small size-at-maturity (Morris 2009, Gardner et al. 2015), high fecundity and high spawning frequency (Morris 2009, Gardner et al. 2015), and apparent lack of natural predators (Albins & Hixon 2013), there is great concern that if their populations are not properly managed, invasive lionfish could cause significant ecological disruption in Bermuda, through predation and resource competition. Elsewhere, Albins & Hixon (2008) showed that lionfish can reduce the recruitment of reef fish by nearly 80% in as little as 5 wk. Further, Lesser & Slattery (2011) suggest that lionfish are the cause of widespread declines of herbivorous reef fish and a subsequent phase shift that occurred at mesophotic depths around the Bahamas from a healthy, robust coral ecosystem to an algae-dominated community. Additionally, across study sites in the Bahamas, Green et al. (2012) showed that the biomass of lionfish prey species declined by 65% between 2008 and 2010, while lionfish biomass simultaneously increased from 23 to ~40% of total predator biomass. Thus, invasive lionfish can potentially negatively impact ecologically and economically important species, with consequent effects cascading through invaded ecosystems.

To explore the impact lionfish may have upon the coral reef communities of Bermuda, there is a need to understand their location-specific diet. Moreover, to better understand the impacts of these invasive species throughout the invaded range, there is a need to compare their feeding habits in different regions to highlight diet variation and to examine how environmental factors (e.g. depth and water temperature) may alter prey availability, with a subsequent effect on diet. As Bermuda's coral reefs are considered to be some of the healthiest in the Atlantic Ocean (Jackson et al. 2014), this is also an opportunity to study the potential impact of lionfish in an ecosystem with limited impacts from development, pollution, and overfishing. In this study, we describe the diet of Bermuda's invasive lionfish population to provide a more detailed understanding of their potential impact upon the coral reef ecosystem.

MATERIALS AND METHODS

Collections

Lionfish were collected from multiple locations around the Bermuda platform (32° 21′ N, 64° 48′ W) between January 2013 and February 2016 (Fig. 1). Specimens (n = 1508) were collected by commercial fishermen (lobster traps, n = 75), recreational fishermen (hook and line, n = 2), permitted lionfish cullers (pole-spear, n = 1045), researchers (pole-spear, n = 141), and fisheries management staff (experimental lionfish traps, n = 148; an additional 97 lionfish were delivered to researchers without a label to indicate a

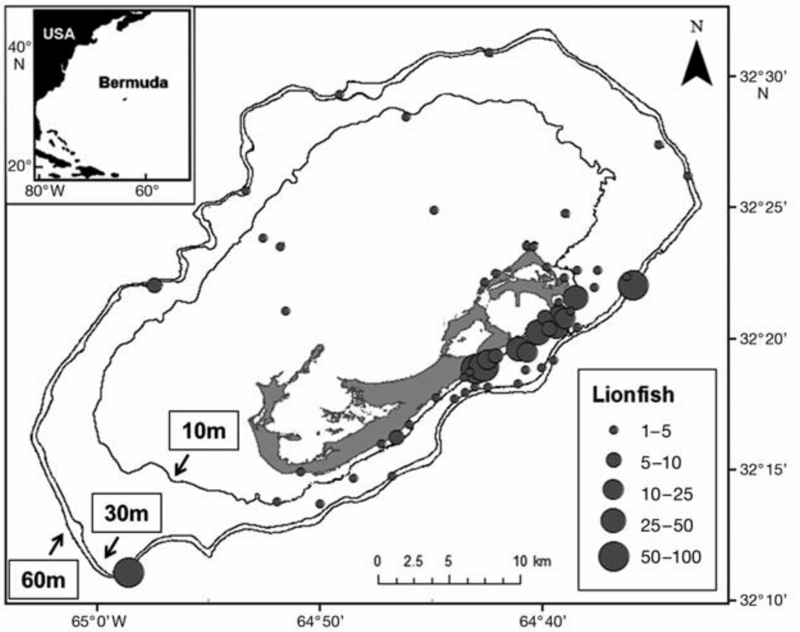

Fig. 1. Locations and relative number of lionfish (*Pterois volitans* and *P. miles*) captured between 2013 and 2015 for this study

method). Specimens were collected across a wide range of depths (0–10 m: n = 499; 10–20 m: n = 148; 20–30 m: n = 109; 30–40 m: n = 19; 40–50 m: n = 22; 50–60 m: n = 415; >60 m: n = 10). The remaining individuals (n = 199) were provided to the research team with no indication of the depth of capture. Overall, 229 lionfish were captured in 2013, 396 in 2014, 493 in 2015, and 159 in 2016. The remaining individuals (n = 134) were provided to the research team with no indication of the date of capture. Of those for which we have such data, 325 were captured in winter (January to March), 161 in spring (April to June), 677 in summer (July to September), and 114 in fall (October to December). Prior to dissection, total length (TL), standard length (SL), and body mass were recorded. Lionfish were placed on ice and dissected the same day as they were captured. If dissections had to be delayed, lionfish were frozen at −20°C for the interim.

Stomach contents analyses

Stomach contents were identified to the lowest taxonomic level possible, counted, and sorted into groups according to their identification. Well-digested prey items that could not be identified to species or family were labeled as 'unidentified' crustacean, crab, lobster, shrimp, or teleost. Occasionally, even this was not possible and items were labeled 'unidentified digested material'. In addition, each prey item was categorized by family and trophic guild (limited to 8 broad categories: cleaner, detritivore, herbivore, invertivore, omnivore, piscivore, planktivore, and zooplanktivore). For each stomach, the mass of each prey group was recorded and volume was measured by water displacement in a graduated cylinder. Neither prey mass nor volume were adjusted for partial digestion, thus these measurements are potentially underestimated. The contribution of each prey taxon to the overall diet of lionfish was quantified using 3 traditional metrics of prey quantity: percent frequency of occurrence (%F), percent composition by mass (%M), and percent composition by number (%N) (Hyslop 1980). For these metrics, omnivorous prey items that belong in multiple trophic guilds were classified as belonging to the guild that best describes the majority of its diet as reported in the literature. To examine the importance of each prey taxon (by species, family, and trophic guild), 3 indices of importance were calculated: the index of relative importance (IRI) (Pinkas et al. 1971), the index of preponderance (IOP) (Natrajan & Jhingran 1962), and the percent index of relative importance (%IRI) (Cortés 1997):

$$\mathrm{IRI}_a = F_a \times (N_a + M_a) \quad (1)$$

$$\mathrm{IOP}_a = \frac{(F_a \times M_a)}{\sum_{a=1}^{n}(F_a + M_a)} \quad (2)$$

$$\%\mathrm{IRI}_a = 100 \times \mathrm{IRI}_a \div \sum_{a=1}^{n}\mathrm{IRI}_a \quad (3)$$

where n is the number of prey types, F_a is the frequency of occurrence of species a, M_a is the percent composition by mass of species a, and N_a is the percent composition by number of species a.

Cumulative prey curve

A cumulative prey curve was used to assess whether our sample size was sufficient to accurately describe lionfish diet. Identified prey items were grouped by family, and the cumulative number of novel prey items was analyzed using 1000 randomizations of the data (Bizzarro et al. 2007). The mean number of novel prey items for each consecutive stomach (±95% confidence interval) was calculated, and these values were plotted to create the cumulative prey curve. Sample size sufficiency was assessed using the linear regression method of Bethea et al. (2011), where the slope from a linear regression of the last 4 stomach samples (i.e. an approximation of the rate at which novel prey items are encountered) is compared to a slope of 5% using a Student's *t*-test of equality of 2 population regression coefficients (Zar 1999). A sufficient sample size is indicated when the slope of the cumulative prey curve's endpoints is not significantly greater than a line with 5% slope (p-value > α). For all statistical tests, we used a significance value of α = 0.05.

Environmental factors

Canonical correspondence analysis (CCA) was used to investigate how diet was influenced by environmental variables (e.g. lionfish size, depth, season, and year of capture). The analysis was performed using the 'cca' script, available in the software package 'vegan' in R. We used 999 Monte Carlo permutations to evaluate the statistical significance of these explanatory variables in the ordination (ter Braak 1986). CCA examines the multivariate relationship between explanatory variables (e.g. environmental factors) and the weighted average of response variables (i.e. prey items) using a redundancy analysis, based on proportions and sample size (ter Braak 1986, 1987). Environmental variables that do not contribute significantly to diet variation are removed and the remaining relationships are expressed in reduced canonical space (ter Braak 1986, Jongman et al. 1987). CCA can be used to show how the change in environmental variables may affect the distribution of species at specific sites. When used with diet data, CCA explains how the distribution of prey items (i.e. 'species') collected in lionfish stomachs (i.e. 'sites') changes along gradients of these environmental factors, and can highlight the extent to which each drives diet variance. Using prey–environment bi-plots, CCA results are best understood by highlighting the amount of variance explained by each canonical axis and examining the correlation between the canonical axes and the explanatory variables (ter Braak 1986, 1987). The response variables were the prey items' contribution to lionfish diet by mass (i.e. %*M*), and the data were $\ln(x + 1)$-transformed to account for positive skewness.

RESULTS

Collected lionfish (n = 1508) ranged in size from 124 to 467 mm TL (mean ± SE: 335 ± 2 mm). A total of 2703 prey items were removed from 1352 stomachs and assigned to the lowest possible taxonomic level. The mass of prey by taxon was measured for 818 stomachs. Overall prey items were removed from a broad size-range of lionfish (100–150 mm: n = 9; 150–200 mm: n = 23; 200–250 mm: n = 66; 250–300 mm: n = 240; 300–350 mm: n = 396; 350–400 mm: n = 403; 400–450 mm: n = 207; >450 mm: n = 8). The number of prey items per stomach ranged from 0 to 27 (mean ± SE: 2.0 ± 0.08). A total of 15 stomachs were everted, while 141 stomachs had been removed prior to donation to the research team. In total, 36.0% of stomachs (n = 487) were empty. Overall, 28.1% of stomachs from lionfish captured in shallow water (<30 m) were empty, as were 61.6% of stomachs from lionfish captured in deep water (>30 m).

The cumulative prey curve suggests that a sufficient number of stomachs was analyzed to provide an accurate description of lionfish diet (slope < 5%; p = 0.04) (Fig. 2). There were 91 novel prey groups identified and, on average, 3 novel items were collected from the final 79 stomachs. Although rare prey items found in only a single stomach seem to prevent the cumulative prey curve from reaching a true asymptote, as originally described by Bizzarro et al. (2007), we examined more stomachs than similar studies (e.g. Morris & Akins 2009) and found a broadly similar prey base, so we feel confident that our results accurately describe the diet of lionfish in Bermuda.

Prey composition

In total, 22 families of teleosts, 14 families of crustaceans, and 3 families of mollusks contributed to the diet of lionfish (Table 1). Teleosts accounted for 55.5% of the lionfish diet by number (%*N*), 73.4% by mass (%*M*), and occurred in 51.9% of stomachs (%*F*). Other prey items included crustaceans (43.0%*N*, 23.4%*M*, 33.8%*F*), mollusks (0.2%*N*, 1.1%*M*, 0.4%*F*), and unidentified prey items (3.6%*N*, 2.1%*M*, 6.9%*F*).

Teleost prey included 44 identifiable species (Table 1). The most speciose families were Labridae (6 spp.), Monocanthidae (5 spp.), Serranidae (4 spp.), Apogonidae (4 spp.), and Pomacentridae (4 spp.). The 5 most common teleost prey families accounted for 20% of the overall diet by number and 66% of all identifiable teleosts items (Labridae: 7.0%*N*; Blennidae: 3.7%*N*; Holocentridae: 3.6%*N*; Scaridae: 2.8%*N*;

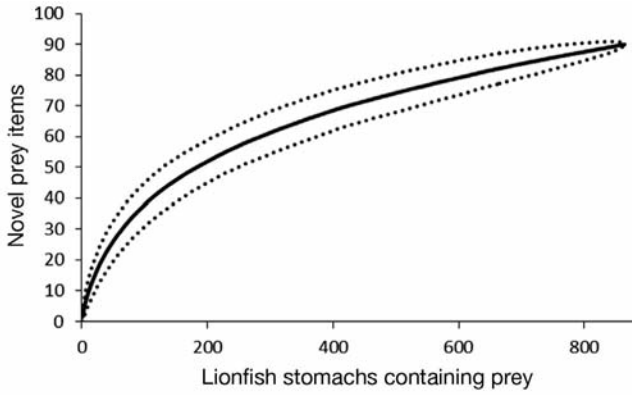

Fig. 2. Mean cumulative number of prey taxa per lionfish (*Pterois volitans* and *P. miles*) stomach sample ± 95% confidence interval (dotted lines)

and Serranidae: 2.5 %N). By mass, the 5 most common prey families accounted for 34 % of the overall diet and 71 % of all identifiable teleosts (Labridae: 10.7 %M; Holocentridae: 8.6 %M; Serranidae: 6.1 %M; Blennidae: 4.8 %M; and Haemulidae: 4.1 %M). The 5 families that occurred most frequently in lionfish stomachs included Labridae (6.6 %F), Blennidae (3.2 %F), Holocentridae (3.1 %F), Serranidae (2.3 %F), and Gobiidae (2.1 %F).

Crustacean prey included 17 species (Table 1). The most speciose families were Calappidae (3 spp.) and Grapsidae (3 spp.). The 5 most common crustacean prey families accounted for 28 % of the overall diet by number and 97 % of all identifiable crustaceans (Rhynchocinetidae: 13.7 %N; Munididae: 6.2 %N; Calappidae: 4.0 %N; Portunidae: 2.6 %N; and Grapsidae: 1.4 %N). By mass, the 5 most common prey families accounted for 20 % of the overall diet and 96 % of all identifiable crustaceans (Rhynchocinetidae: 14.0 %M; Grapsidae: 2.6 %M; Calappidae: 1.4 %M; Mithracidae: 1.0 %M; and Munididae: 0.6 %M). The 5 families that occurred most frequently included Rhynchocinetidae (11.1 %F), Munididae (3.3 %F), Portunidae (1.8 %F), Calappidae (1.4 %F), and Grapsidae (1.3 %F).

The red night shrimp *Cinetorhynchus rigens*, also known as the mechanical shrimp, was the most common prey item across all metrics (13.7 %N, 14.0 %M, and 11.1 %F) (Table 2). Depending upon the metric in use, the second most common species was either squat lobster *Munida simplex* (6.2 %N) or bluehead wrasse *Thalassoma bifasciatum* (7.6 %M and 3.4 %F). The third most common species also differed by diet metric and was either the bluehead wrasse (3.5 %N), reef squirrelfish *Sargocentron coruscum* (4.7 %M) or squat lobster (3.3 %F).

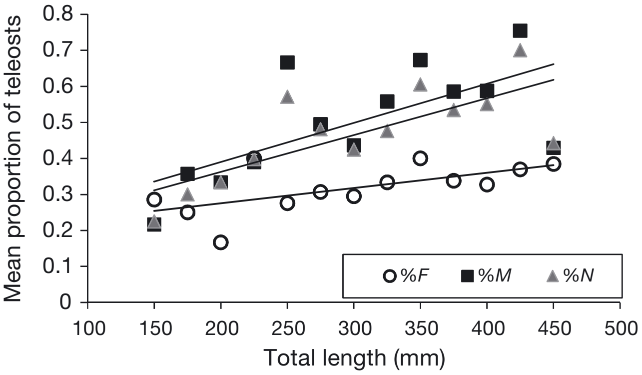

Fig. 3. Mean proportion of teleosts (MPT) in the diet of Bermuda lionfish (*Pterois volitans* and *P. miles*), separated into 25 mm size classes (total length, TL), as described by the 3 common diet metrics — percent composition by number (%N), percent frequency of occurrence (%F), and percent composition by mass (%M). Lines indicate a significant increase in the MPT with lionfish TL: $\text{MPT}_{\%F} = 0.0004(\text{TL}) + 0.1912$; $\text{MPT}_{\%M} = 0.0011(\text{TL}) + 0.1728$; $\text{MPT}_{\%N} = 0.001(\text{TL}) + 0.1584$

The contribution of teleosts within the lionfish diet increased significantly with lionfish size across all 3 diet metrics (%N: $R^2 = 0.5714$, $F_{1,13} = 13.96$, $p < 0.05$; %M: $R^2 = 0.4615$, $F_{1,13} = 9.65$, $p < 0.05$; %F: $R^2 = 0.3883$, $F_{1,13} = 6.40$, $p < 0.05$) (Fig. 3).

Indices of importance

In both %IRI and IOP, the top-ranked prey items, both by species and family, were relatively consistent (Table 3). Two species (red night shrimp and bluehead wrasse) and their corresponding 2 families (Rhynchocinetidae and Labridae) were ranked highest, suggesting they make a substantial and important contribution to the diet of lionfish in Bermuda. Nine species and 8 families were consistently ranked within the top 10 prey items (Table 3). There was little variation in the relative importance of prey items characterized by trophic position, with invertivores, detrivores, and herbivores consistently ranked highest (Table 3).

Environmental factors

Eigenvalues for the 4 multivariate axes were 0.49 (CCA1), 0.31 (CCA2), 0.23 (CCA3), and 0.14 (CCA4). Depth, season, and lionfish size significantly influence the diet of lionfish in Bermuda and generally correspond to CCA1 ($p = 0.001$), CCA2 ($p = 0.001$), and CCA3 ($p = 0.006$), respectively. CCA1 and CCA2 (Fig. 4a) accounted for 42.1 and 26.6 % of the explain-

Table 1. Contribution of lionfish (*Pterois volitans* and *P. miles*) prey by taxa, showing percent composition by number (%*N*), percent composition by mass (%*M*), and percent frequency of occurrence (%*F*). C: cleaner; D: detritivore; H: herbivore; I: invertivore; O: omnivore; P: piscivore; Pl: planktivore; Z: zooplanktivore. Trophic guild information collected from Bohlke & Chaplin (1993), Cartes (1993), Cervigón (1993), Chande & Mgaya (2005), Corredor (1978), Frick et al. (2004), Hughes & Elner (1989), Iversen et al. (1986), Lavalli et al. (2007), McEachran (2009), Puccio et al. (2006), Randall (1967), Robertson (1981), Romero et al. (2004), Ryan (1956), Samson et al. (2007), Sterrer (1986, 1992), Whiteman et al. (2007), and Zhang et al. (1998)

Taxon	Frequency (stomachs)	%*N* (n = 1352)	%*M* (n = 818)	%*F* (n = 1352)	Trophic guild
Unidentified	96	3.6	2.1	6.9	–
Mollusca					
Loliginidae					
Sepioteuthis sepioidea	2	0.1	0.9	0.1	P
Octopodidae					
Octopus spp.	2	0.1	0.2	0.1	I
Marginellidae					
Volvarina albolineata[b]	1	<0.1	<0.1	0.1	Pl, Z
TOTAL	5	0.2	1.1	0.4	
Crustacea					
Unidentified crustacean	5	0.2	<0.1	0.4	–
Unidentified shrimp	155	12.6	2.8	11.1	–
Unidentified crab	28	1.7	0.2	2.0	–
Unidentified lobster	1	<0.1	<0.1	0.1	–
Rhynchocinetidae					
Cinetorhynchus rigens	155	13.7	14.0	11.1	D
Munididae					
Munida simplex	46	6.2	0.6	3.3	D, H, I
Portunidae					
Portunus anceps	26	2.6	0.2	1.9	H, I
Grapsidae					
Percnon gibbesi	15	1.3	2.6	1.1	H
Plagusia depressa	1	<0.1	<0.1	0.1	H
Planes minutus	2	0.1	<0.1	0.1	O
Calappidae[a]	7	3.0	0.1	<0.1	I
Cryptosoma bairdii	16	0.9	1.2	1.1	I
Calappa gallus	2	0.1	0.1	0.1	I
Calappa ocellata	1	<0.1	<0.1	0.1	I
Stenopodidae					
Stenopus hispidus	5	0.2	0.2	0.4	C
Hippolytidae					
Lysmata grabhami	3	0.1	<0.1	0.2	C
Lysiosquillidae					
Lysiosquilla scabricauda	2	0.1	0.2	0.1	I, P
Gonodactylidae					
Neogonodactylus oerstedii	1	<0.1	0.1	0.1	I
Palaemonidae					
Brachycarpus biunguiculatus	1	<0.1	0.1	0.1	C
Palaemon northropi	1	0.1	<0.1	0.1	D
Mithracidae					
Mithraculus forceps	3	0.1	0.9	0.2	D
Penaeidae	1	0.1	<0.1	0.1	I
Scyllaridae					
Scyllarides nodifer	1	<0.1	0.1	0.1	I
Xanthidae	1	<0.1	<0.1	0.1	I
TOTAL	479	43.0	23.4	33.8	
Teleosts					
Unidentified teleost	326	25.6	25.3	23.4	–
Labridae[a]	9	0.6	0.2	0.6	–
Thalassoma bifasciatum	48	3.5	7.6	3.4	C, Z
Halichoeres garnoti	15	1.4	0.7	1.1	I, P
Halichoeres bivittatus	15	1.3	2.2	1.1	I, P
Xyrichtys martinicensis	3	0.1	<0.1	0.2	I
Halichoeres maculipinna	1	<0.1	<0.1	0.1	I, P
Clepticus parrae	1	<0.1	<0.1	0.1	Pl, Z
Serranidae					
Paranthias furcifer	24	2.1	4.5	1.7	Z
Liopropoma spp.	3	0.1	0.4	0.2	I, P

Table 1 continued on next page

Table 1 (continued)

Taxon	Frequency (stomachs)	%*N* (n = 1352)	%*M* (n = 818)	%*F* (n = 1352)	Trophic guild
Hypoplectrus puella	4	0.2	1.0	0.3	I, P
Liopropoma rubre	1	<0.1	0.3	0.1	I, P
Holocentridae[a]	26	1.9	3.2	1.9	
Sargocentron coruscum	13	1.5	4.7	0.9	I
Holocentrus adscensionis	1	<0.1	0.4	0.1	I
Sargocentron vexillarium	3	0.1	0.3	0.2	I, P
Bothidae	16	1.3	1.4	1.1	I, P
Gobiidae[a]	3	0.2	0.1	0.2	
Coryphopterus glaucofraenum	18	1.4	1.1	1.3	D, H, I
Coryphopterus personatus	4	0.2	0.1	0.3	Pl
Gnatholepis thompsoni	4	0.3	0.3	0.3	D, H, I
Scaridae[a]	12	1.7	0.6	0.9	
Sparisoma aurofrenatum	11	1.0	0.2	0.8	H
Scarus taeniopterus	4	0.1	0.4	0.3	H
Blennidae[a]	13	1.4	1.2	0.9	
Parablennius marmoreus	20	1.2	2.2	1.4	D, H, I, Pl
Entomacrodus nigricans	12	1.2	1.4	0.9	D, H, Pl
Haemulidae[a]	10	1.0	0.8	0.7	
Haemulon aurolineatum	5	0.6	0.2	0.4	I
Haemulon flavolineatum	8	0.7	3.0	0.6	I
Apogonidae[a]	11	0.5	0.9	0.8	
Apogon pseudomaculatus	1	<0.1	0.2	0.1	I, Z
Apogon townsendi	1	<0.1	0.1	0.1	I, Z
Apogon binotatus	1	<0.1	0.1	0.1	I, Z
Apogon maculatus	3	0.1	0.3	0.2	I, Z
Chaetodontidae[a]	7	0.4	0.1	0.5	
Chaetodon ocellatus	6	0.3	0.4	0.4	I
Chaetodon capistratus	2	0.2	<0.1	0.1	I
Acanthuridae	7	0.3	0.1	0.5	
Acanthurus chirurgus	1	<0.1	0.1	0.1	H
Acanthurus bahianus	4	0.2	0.5	0.3	H
Synodontidae[a]	4	0.2	0.1	0.3	
Synondus synodus	2	0.1	0.2	0.1	P, Z
Aulostomidae					
Aulostomus maculatus	4	0.1	<0.1	0.3	I, P
Mullidae[a]	1	<0.1	0.5	0.1	
Pseudupeneus maculatus	14	0.7	3.3	1.0	I
Mulloidichthys martinicus	1	<0.1	0.2	0.1	I
Pempheridae					
Pempheris schomburgkii	2	0.3	0.4	0.1	I, Z
Pomacentridae					
Stegastes variabilis	1	<0.1	0.2	0.1	D, H, I
Stegastes spp.	3	0.1	0.2	0.2	D, H, I
Chromis insolata	1	<0.1	0.1	0.1	Z
Chromis flavicauda	1	0.1	<0.1	0.1	Z
Monocanthidae					
Cantherhines spp.	1	<0.1	<0.1	0.1	H, I
Aluterus schoepfii	1	<0.1	<0.1	0.1	H
Monacanthus tuckeri	4	0.2	1.0	0.3	I, Pl
Monacanthus ciliatus	1	<0.1	<0.1	0.1	H, I, Pl
Aluterus scriptus	1	<0.1	0.1	0.1	H, I
Carangidae					
Decapterus spp.	1	<0.1	0.2	0.1	Z
Sparidae					
Diplodus bermudensis	1	<0.1	<0.1	0.1	H
Clupeidae					
Jenkinsia lamprotaenia	1	<0.1	<0.1	0.1	Z
Antennariidae	1	<0.1	<0.1	0.1	I, P
Tetraodontidae					
Sphoeroides spengleri	1	0.1	0.2	0.1	I
TOTAL	724	55.5	73.4	51.9	

[a] Stomach contents identified only to Family level (e.g. Calappidae[a] = unknown box crab)
[b] Likely captured incidentally

Table 2. Top 10 prey species of lionfish (*Pterois volitans* and *P. miles*) in Bermuda according to the relative metrics of prey quantity: percent composition by number (%N), percent composition by mass (%M), and percent frequency of occurrence (%F). [A] = crustacean; [B] = teleost

Rank	Species	%N	Species	%M	Species	%F
1	*Cinetorhynchus rigens*[A]	13.7	*Cinetorhynchus rigens*[A]	14.0	*Cinetorhynchus rigens*[A]	11.1
2	*Munida simplex*[A]	6.2	*Thalassoma bifasciatum*[B]	7.6	*Thalassoma bifasciatum*[B]	3.4
3	*Thalassoma bifasciatum*[B]	3.5	*Sargocentron coruscum*[B]	4.7	*Munida simplex*[A]	3.3
4	*Portunus anceps*[A]	2.6	*Paranthias furcifer*[B]	4.5	*Portunus anceps*[A]	1.9
5	*Paranthias furcifer*[B]	2.1	*Pseudupeneus maculatus*[B]	3.3	*Paranthias furcifer*[B]	1.7
6	*Sargocentron coruscum*[B]	1.5	*Haemulon flavolineatum*[B]	3.0	*Parablennius marmoreus*[B]	1.4
7	*Coryphopterus glaucofraenum*[B]	1.4	*Percnon gibbesi*[A]	2.6	*Coryphopterus glaucofraenum*[B]	1.3
8	*Halichoeres garnoti*[B]	1.4	*Halichoeres bivittatus*[B]	2.2	*Cryptosoma bairdii*[A]	1.1
9	*Percnon gibbesi*[A]	1.3	*Parablennius marmoreus*[B]	2.2	*Percnon gibbesi*[A]	1.1
10	*Halichoeres bivittatus*[B]	1.3	*Entomacrodus nigricans*[B]	1.4	*Halichoeres garnoti*[B]	1.1

able diet variance, respectively, while CCA3 (Fig. 4b) accounted for 19.2%.

DISCUSSION

In general, the diet of lionfish in Bermuda appears to be broadly similar to that of lionfish found in other locations around the world, consisting of a diverse range of teleost and crustacean prey. However, compared other parts of its invaded range, the contribution of teleosts by number (%N) to the diet of lionfish in Bermuda appears to be ~15 to 30% lower (55.5%N) than other regions (71.2%N in the Bahamas, 74.4%N in the Mexican Caribbean, and 84.1%N along the southeastern US coast; Morris & Akins 2009, Muñoz et al. 2011, Valdez-Moreno et al. 2012). In addition, crustaceans appear to play a larger role in the diet of lionfish in Bermuda (43.0%N) relative to the Bahamas (28.5%N), the Mexican Caribbean (25.6%N), and the southeastern US coast (13.9%N) (Table 4). The greater contribution of teleost prey items by mass (73.4%M) suggests that they make a more important contribution to the diet in terms of energy and nutrients compared to crustaceans (23.4%M). This scenario is similar to that

Table 3. Top lionfish (*Pterois volitans* and *P. miles*) prey species, families, and trophic guilds in Bermuda according to index of preponderance (IOP) and percent index of relative importance (%IRI) (values shown in parentheses). [A] = crustacean; [B] = teleost

Rank	Species		Family		Trophic guild	
	IOP	%IRI	IOP	%IRI	IOP	%IRI
1	*Cinetorhynchus rigens*[A] (0.81)	*Cinetorhynchus rigens*[A] (69.3)	Rhynchocinetidae[A] (0.81)	Rhynchocinetidae[A] (52.0)	Invertivore (1.99)	Invertivore (60.4)
2	*Thalassoma bifasciatum*[B] (0.14)	*Thalassoma bifasciatum*[B] (8.6)	Labridae[B] (0.37)	Labridae[B] (19.8)	Detrivore (0.89)	Detrivore (27.3)
3	*Paranthias furcifer*[B] (0.04)	*Munida simplex*[A] (5.1)	Holocentridae[B] (0.14)	Holocentridae[B] (6.3)	Herbivore (0.25)	Herbivore (6.9)
4	*Sargocentron coruscum*[B] (0.02)	*Paranthias furcifer*[B] (2.6)	Blennidae[B] (0.08)	Blennidae[B] (4.7)	Cleaner (0.17)	Cleaner (4.0)
5	*Pseudupeneus maculatus*[B] (0.02)	*Sargocentron coruscum*[B] (1.3)	Serranidae[B] (0.07)	Munididae[A] (3.8)	Zooplanktivore (0.06)	Zooplanktivore (1.4)
6	*Parablennius marmoreus*[B] (0.02)	*Portunus anceps*[A] (1.2)	Haemulidae[B] (0.04)	Serranidae[B] (3.4)	Planktivore (<0.01)	Planktivore (0.1)
7	*Percnon gibbesi*[A] (0.02)	*Parablennius marmoreus*[B] (1.1)	Mullidae[B] (0.02)	Haemulidae[B] (1.8)	Piscivore (<0.01)	Piscivore (<0.1)
8	*Halichoeres bivittatus*[B] (0.01)	*Percnon gibbesi*[A] (1.0)	Grapsidae[A] (0.02)	Scaridae[B] (1.3)		
9	*Munida simplex*[A] (0.01)	*Pseudupeneus maculatus*[B] (0.9)	Gobiidae[B] (0.02)	Gobiidae[B] (1.3)		
10	*Haemulon flavolineatum*[B] (0.01)	*Halichoeres bivittatus*[B] (0.9)	Scaridae[B] (0.01)	Calappidae[A] (1.3)		

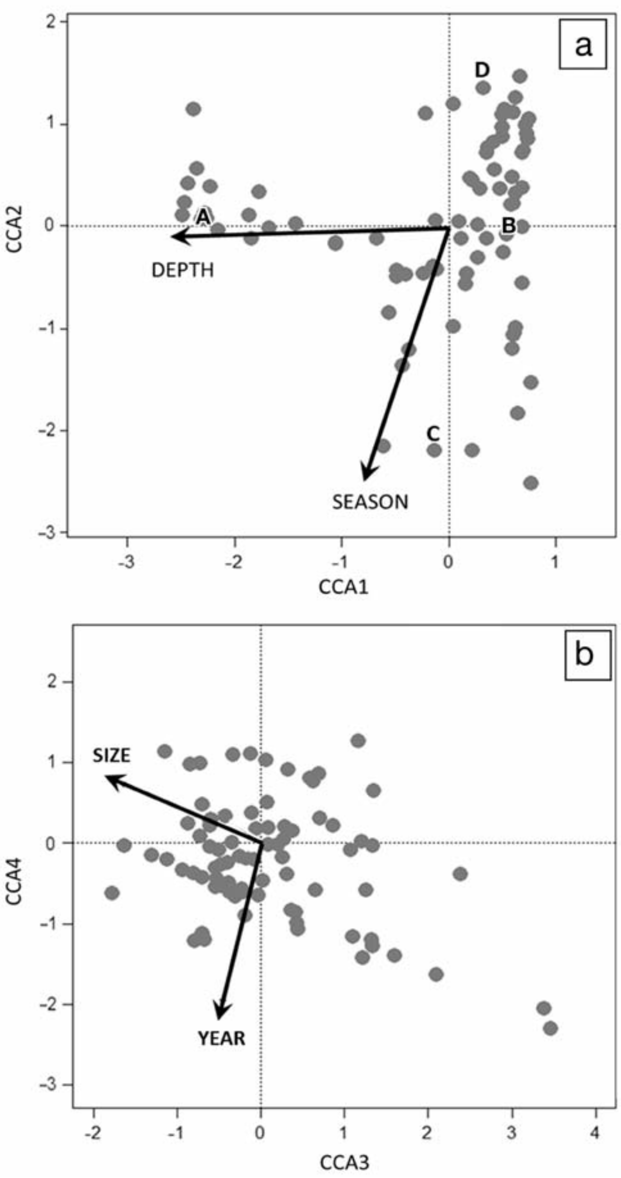

Fig. 4. Canonical correspondence analysis biplots. Data points represent individual prey groups. Arrows represent explanatory variables for (a) depth and season and (b) size and year, and their relationship to the distribution of prey groups. A correlation between an explanatory variable and a biplot axis is indicated by a small angle between them. A: squat lobster *Munida simplex*; B: nimble spray crab *Percnon gibbesi*; C: belted cardinalfish *Apogon townsendi*; D: pearl blenny *Entomacrodus nigricans*

reported by Morris & Akins (2009) in the Bahamas, where lionfish consumed more than 4 times the teleosts (78.0 % V[1]) relative to crustaceans (14.4 % V[1]).

Table 4. Comparison of the 3 common diet metrics — percent composition by number (%N), percent frequency of occurrence (%F), and percent composition by mass (%M) — of lionfish (*Pterois volitans* and *P. miles*) between different regions throughout the northwest Atlantic Ocean

		%N	%M	%F
Bermuda	Teleost	55.5	73.4	51.9
	Crustacean	43.0	23.4	33.8
Bahamas	Teleost	71.2	78.0[a]	61.6
	Crustacean	28.5	14.4[a]	24.7
Mexican-Caribbean	Teleost	74.4		
	Crustacean	25.6		
Southeast USA[b]	Teleost	84.1	86.5[a]	
	Crustacean	13.9	11.5[a]	

[a]This study used %V in lieu of %M
[b]Data from Muñoz et al. (2011)

Muñoz et al. (2011) reported an even greater contribution of teleosts to the diet of lionfish along the US coast (86.5 %M teleosts vs. 11.5 %M crustaceans). Nonetheless, crustaceans still make a more substantial contribution to the diet of lionfish in Bermuda than elsewhere. In total, 22 families of teleosts (44 species) and 14 families of crustaceans (17 species) were found in the stomach contents of lionfish in Bermuda. This substantial contribution of both teleosts and crustaceans, with a broad range of families and species in each category, supports the prevailing view that lionfish are generalist and opportunist predators (Muñoz et al. 2011, Layman & Allgeier 2012, Côté et al. 2013).

The contribution of key prey items was fairly consistent across the indices of importance, at the species and family level, and when considering trophic guild (Table 3). Despite some limited variation in the order by which items were ranked, 9 species and 8 families were consistently represented in each index, and the 2 top-ranked species and their families were consistent across all metrics (Table 3). Similarly, invertivores (60.4 %IRI) and detrivores (27.3 %IRI) were the 2 top-ranked trophic guilds consumed by lionfish, while herbivores ranked third (6.9 %IRI). The consistency of the rankings among the indices indicates that the red night shrimp *Cinetorhynchus rigens* is the most important prey species for lionfish in Bermuda (Table 3). This conclusion is reinforced by its substantial contribution in number, mass, and frequency and its ubiquitous presence in lionfish stomachs throughout the year and at all depths. Even at the family level, Rhynchocinetidae (to which the red night shrimp belongs) makes the greatest contribution to the lionfish diet (Table 3). This is the first

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[1] As the average density of prey items was approximately 1 g cm^{-3}, we assume the percent composition by volume (%V) and %M are equivalent, thus allowing the comparison of %M from our study with %V in others

study to show a crustacean species, and its related family, playing such a substantial and extensive role in the diet of lionfish.

The red night shrimp makes the biggest contribution to the diet of lionfish in Bermuda for any single prey item, approximately 2 to 3 times greater than the second item by all 3 diet metrics (Table 2). This is notable considering their minimal contribution (0.5 %N and 1.0 %V^1) recorded in the Bahamas (Morris & Akins 2009) and their absence from lionfish diet in the Gulf of Mexico and along the southeastern coast of the USA (Muñoz et al. 2011, Dahl & Patterson 2014). Because lionfish and red night shrimp both inhabit deep crevices and caves during the day and actively feed at night (Burkenroad 1939, Fishelson 1975), this spatial and temporal habitat overlap likely increases the frequency of encounters between the 2 species, potentially contributing to the high consumption of red night shrimp. Alternatively, in the absence of data that describe the populations of red night shrimp in Bermuda or elsewhere, this could potentially reflect their abundance in Bermuda, which lionfish exploit opportunistically. Considering their significant presence in the diet of lionfish in Bermuda, if these shrimp have not previously been the target of native predators, the possibility exists that lionfish may exert an unsustainable pressure on their population. On the other hand, if red night shrimp are consumed by native predators, there could be consequences for the other species that feed upon them, as they would now be competing with invasive lionfish for those resources. Given the rate at which red night shrimp are being consumed by lionfish in Bermuda, a further examination of their biology and ecology may be warranted to identify the impact lionfish may have on their population and any subsequent effects on Bermuda's coral reef ecosystem.

As in previous studies (Morris & Akins 2009, Muñoz et al. 2011, Dahl & Patterson 2014), our work shows that lionfish feed upon juveniles and small-bodied adults of many species. Teleost prey items are not just small, but often shallow-bodied (e.g. wrasse and gobies), a trait shown by Green & Côté (2014) to be correlated with an increased vulnerability to predation. It is harder to describe the characteristic traits of crustacean prey in a similar manner. For example, the nimble spray crab *Percnon gibbesi* are flat and wide-bodied, with a row of spines on each walking leg and, in general, do not present themselves as easily consumable prey. In addition, these shore crabs are found along rocky shorelines below the mean low-water level (Sterrer 1986) and often forage in water <30 cm deep (C. Eddy pers. obs.). Despite these potentially unappealing morphological traits and their shallow habitat, nimble spray crabs ranked as the 7th (IOP) and 8th (%IRI) (Table 3) relatively important prey item because they made a large contribution to diet for lionfish captured at one specific site (3 to 7 m deep) where a shallow rocky coastline is immediately adjacent to high-relief reefs.

Considering the contrasting morphological shapes and behaviors of teleost and crustacean prey, it seems other factors may be contributing to the increased consumption of the latter. A greater abundance of crustacean prey relative to teleosts at certain times of the day (e.g. crepuscular periods) or in certain locations (e.g. deep crevices and caves) is the most parsimonious answer. However, prey naïveté may also be contributing to the heavy predation on some of these otherwise risk-averse crustaceans. Cryptic and shy, red night shrimp shelter in recesses and amongst the spines of the long-spined sea urchin *Diadema antillarum* (Humann & DeLoach 1992, Hernández 2008). Similarly, the nimble spray crab, which is sometimes known as the urchin crab, is often found to associate with this same species (Hayes et al. 1998). To naïve prey with these sheltering instincts, the elongate dorsal and pectoral fins of lionfish may appear to be a similarly benign structural refuge, perhaps increasing their exposure to predation. Alternatively, as lionfish are visual predators (Fishelson 1997), the movement of some crustaceans (e.g. hurried motions, frequent rapid changes in direction) may stimulate an instinctive predatory response from the lionfish that increases their vulnerability.

Lionfish in Bermuda consume more than 60 different prey species that play important roles in coral reef communities. While the Atlantic creolefish *Paranthias furcifer* is the only economically valuable species among the top 10 prey items for lionfish in Bermuda (Tables 2 & 3), other prey items play vital ecological roles, such as juvenile bluehead wrasse *Thalassoma bifasciatum*, which provides a beneficial service to reef fish by cleaning them of dead skin and ectoparasites (Feddern 1965). Although herbivorous teleosts (e.g. juvenile scarids and acanthurids), which help maintain coral health and facilitate coral recruitment by grazing upon algae, seem to be consumed infrequently in Bermuda (at least at present), the possibility exists that lionfish diet may change from year to year as prey availability changes (Muñoz et al. 2011). While the ecological roles of some species, in particular the crustaceans, are relatively unknown, the taxa consumed by lionfish in Bermuda include a widely diverse assortment of scavengers and detritivores, zooplanktivores, planktivores, herbivores, and piscivores. The prey data in the current study show that most trophodynamic guilds are represented in the lionfish diet, reinforcing the concern that these invasive teleosts may have the potential to disrupt normal ecosystem function. Furthermore, many lionfish prey species could be essential prey for higher-level predators, including some of economic importance (e.g. snappers) (Muñoz-Escobar & Gil-Agudelo 2012).

Lionfish diet in Bermuda is affected by both depth and season, presumably due to spatial and temporal variation in community structure and, therefore, prey availability driven by those environmental factors (MacNeil & Connolly 2015). The first axis of the CCA bi-plot is correlated with depth, which is likely due to variations in habitat preferences and depth distributions of various prey species (Fig. 4a). For example, certain prey species (e.g. squat lobster) are only encountered at 60 m, while other species (e.g. nimble spray crab and seaweed blenny *Parablennius marmoreus*) were only found within stomachs of lionfish captured in shallow waters (i.e. <30 m). Similarly, Atlantic creolefish are abundant at 60 m and their juveniles are one of the most common prey items for lionfish captured at these depths. For this reason, it is important to consider that this species may be significantly impacted by lionfish, which occur in very high densities at these depths in Bermuda (Department of Environment and Natural Resources unpubl. data). The second axis of the CCA bi-plot (Fig. 4a) corresponds with season, suggesting that there are also seasonal trends in lionfish diet. For example, certain prey items (e.g. pearl blenny *Entomacrodus nigricans* and belted cardinalfish *Apogon townsendi*) were only found in the stomachs of lionfish during specific times of the year (e.g. mid-winter and mid-autumn, respectively), indicating that there may be seasonal changes in prey availability that could not be detected adequately with the visual prey surveys. This could also be indicative of a seasonal movement between different habitats, for which we already have anecdotal evidence, but which would have to be explored directly in the future.

Another factor that influences diet is the size of the lionfish. As the third CCA axis was significantly correlated with lionfish size (Fig. 4b) and the mean values of all 3 diet metrics (i.e. %N, %M, %F) showed an increase in the contribution of teleosts among larger lionfish (Fig. 3), it appears lionfish undergo an ontogenetic diet shift from crustaceans to teleosts. This pattern has been previously recognized in lionfish from the Bahamas (Morris & Akins 2009) and could be explained by a number of factors. Since lionfish are a gape-limited predator (Côté et al. 2013), the diet shift may reflect an increase in the maximum size of potential prey as lionfish gape increases, and a limit to the minimum size of prey as small items may escape through the gaps between gill rakers (Graham et al. 2007). Further, as larger lionfish have greater gross energetic requirements, a switch to teleosts may provide greater mass-specific nutritional benefits compared to crustaceans. It should be noted, however, that the current study found a large 308 mm (TL) lionfish with 27 squat lobsters in its stomach and another larger 442 mm (TL) individual with a single red night shrimp in its stomach. Thus, while larger lionfish may consume a greater proportion of teleosts, it does not preclude them from opportunistically feeding on crustaceans. We propose that the ontogenetic diet shift in lionfish reflects their capacity to exploit an increasing variety of resources, effectively expanding their dietary niche and spreading their impact across a broad range of species, both large and small.

Given the influence of depth on the diet of lionfish, it is important to note that stomachs of lionfish caught in deep water (>30 m) were more than twice as likely to be empty (61.6%) compared to those captured in shallow water (<30 m; 28.1%). As our surveys suggest potential prey items are abundant at both shallow and deep sites, the higher proportion of empty stomachs from individuals captured in deeper waters may have resulted from decompression-related barotrauma, regurgitation, and stomach eversion during the ascent following specimen collection (DeMartini et al. 1996). We thus suggest that future work aiming to develop a more complete understanding of lionfish diet and their potential impacts on the reef ecosystem should include methods that prevent the loss of stomach contents from lionfish captured in deep water.

Considering the broad diversity of prey items for lionfish in Bermuda and the large quantity of well-digested items that could not be identified in the stomachs, it is possible that some prey species were not recognized and accounted for, and that the diet of lionfish in Bermuda is even broader than characterized here. As such, the economically important species previously documented in the diet of lionfish elsewhere (e.g. juvenile groupers and snappers in the Bahamas; Morris & Akins 2009) may not be exempt from predation in Bermuda even though they went undetected in the current study. It is also possible that we simply did not sample lionfish from areas where juveniles of these species are common. The current study focused along the outer perimeter of

Bermuda's reef platform as there were very limited reports of lionfish in the lagoon and inshore waters (Fig. 1) compared to observations of higher densities along the outer and deep reefs. However, in Bermuda, juveniles of many teleost families recruit to inshore and lagoonal patch reefs far more frequently than to rim and terrace reefs (Smith et al. 2013). For this reason, additional efforts should be made to search for and collect lionfish from inshore and lagoonal habitats to more completely investigate their presence in these critical nursery areas and their potential impact on the biodiversity of both ecologically and economically important species. Taken together, these data suggest that if the lionfish population in Bermuda continues to expand, there will likely be no spatial or temporal refuge for potential lionfish prey, which may eventually include any juvenile or small-bodied fishes or crustaceans. This, in turn, suggests that the impacts of invasive lionfish may eventually be felt across a broad range of species and habitats, as has indeed been suggested by other studies (see for example Muñoz et al. 2011, Côté et al. 2013).

CONCLUSIONS

The extent to which invasive lionfish may impact Bermuda's coral reef ecosystem is still relatively unknown. While evidence suggests that, to date, their population has not experienced the explosive growth seen in other regions, there is concern that this may be coming soon, with wide-scale impacts on biodiversity, community structure, and ecosystem function. This study provides information to help resource managers evaluate which prey species may be most vulnerable to lionfish predation and therefore the species and habitats that may suffer the greatest impact. Our work suggests that small crustacean species will experience considerable impacts, but the consequences of the intensive selection for these species remains unknown. Overall, the diet of lionfish seems to be spread across a broad range of species, perhaps minimizing their current impact on individual species. As the lionfish population grows in Bermuda, this may change. Although lionfish are consuming some ecologically important teleosts, herbivorous species (e.g. parrotfish) appear to be targeted less often, suggesting a phase shift to an algae-dominated community may not be an immediate concern. However, a commercially important serranid (Atlantic creolefish) is highly-targeted in its juvenile deep reef habitat (i.e. 60 m). If this remains the case, there may be less of a direct impact upon the health of the coral reef itself, although the negative influence upon other teleosts, including top predators, may increase. Of course, if the progress of the lionfish invasion in Bermuda accelerates, these scenarios may change dramatically.

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Contribution to the Theme Section 'Invasion of Atlantic coastal ecosystems by Pacific lionfish'

Experimental assessment of lionfish removals to mitigate reef fish community shifts on northern Gulf of Mexico artificial reefs

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ABSTRACT: Substantial declines in reef fishes were observed at northern Gulf of Mexico artificial reef sites between 2009–2010 and 2011–2012, a period that bracketed the appearance of invasive lionfish in this ecosystem. Small demersal reef fishes, the predominant prey of lionfish in other systems, displayed the greatest declines. However, a confounding factor during this time was the *Deepwater Horizon* Oil Spill (DWH) in summer 2010. In some areas, targeted lionfish removals have been demonstrated to mitigate negative effects on native fishes. Therefore, we conducted a 2 yr experiment to examine the effectiveness and ecological benefits of targeted lionfish removals at artificial reefs (n = 27) off northwest Florida, USA, where lionfish densities reached the highest recorded in the western Atlantic by 2013. All lionfish were removed via spearfishing from 17 reefs in December 2013, 9 of which were periodically re-cleared of lionfish through May 2015. Remaining sites served as uncleared controls. Both juvenile and adult lionfish quickly recruited to cleared reefs, with lionfish reaching pre-clearance densities in <1 yr on reefs cleared only once. Removal treatment significantly affected reef fish community structure at experimental reefs, but removal effort was insufficient to achieve substantial gains for most taxa, and declines in several taxa were observed throughout, regardless of treatment. It is unclear whether chronic effects of the DWH or regionally high lionfish densities were more important factors in explaining trends observed in reef fish communities, but small-scale targeted lionfish removal efforts had few positive impacts overall on native reef fish communities in this study.

KEY WORDS: Lionfish · Reef fish · Invasive species · Gulf of Mexico

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INTRODUCTION

Indo-Pacific lionfishes, *Pterois volitans/miles* complex (hereafter 'lionfish'), have exhibited an extensive and rapid invasion in the western Atlantic Ocean, thus earning the species the distinction of being the most successful marine fish invader to date (Whitfield et al. 2002, Morris & Akins 2009, Albins 2013). Lionfish are so abundant and broadly distributed in their invaded range that their eradication is thought to be unachievable (Côté et al. 2013). At the time of this writing, lionfish have established an invaded range of over 7 million km^2, in diverse habitat types beyond their native coral reefs across tropical and sub-tropical western Atlantic Ocean waters, including the Caribbean Sea and Gulf of Mexico (GOM) (Schofield 2009, Côté et al. 2013, Schofield et al. 2014).

The GOM is the most recently invaded basin, where lionfish were first reported in 2009 off the northern Yucatan Peninsula, Mexico (Aguilar-Perera & Tuz-Sulub 2010), in the Florida Keys, USA (Rutten-

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berg et al. 2012), and along the west Florida shelf (Schofield 2010). By late 2010, lionfish had been observed in eastern, northern and western regions of the GOM (Schofield 2010, Fogg et al. 2013, Dahl & Patterson 2014, Nuttall et al. 2014). In the short span of time since initial observations, lionfish populations in the northern GOM (nGOM) have increased exponentially and have reached high densities (>20 fish 100 m^{-2}) on artificial reefs, yet their densities on natural reefs remain 2 orders of magnitude lower (Dahl & Patterson 2014).

The first sightings of lionfish in the nGOM coincided with another significant event in the region: the Deepwater Horizon Oil Spill (DWH). The spill released more than 200 million gallons (~7.6×10^8 l) of oil over several months beginning in April 2010. Effects of the DWH on various biological communities have been documented, yet it is unclear how resilient the nGOM ecosystem will be to this large-scale disturbance (Graham et al. 2010, DeLaune & Wright 2011, Williams et al. 2011, Whitehead et al. 2012). Among reef fishes, reported DWH impacts include changes in diet and trophic position (Tarnecki & Patterson 2015, Norberg 2015), and shifts in community structure (NOAA-NRDA 2015) following exposure to toxic petroleum compounds (Murawski et al. 2014). Recent ecosystem modeling simulations have indicated that depleted reef fish stocks in the region could have contributed to the rapid increase in lionfish density and biomass (Chagaris et al. 2015). While the DWH may not be the singular factor initiating fish declines, the negative effects of disturbance on native reef fish communities may have similarly increased the system's vulnerability to lionfish invasion.

While the full extent of chronic impacts of the DWH on reef fishes in the nGOM remains unclear, the literature on invasive lionfish in the western Atlantic suggests they pose a clear long-term threat to nGOM reef fishes. Lionfish impacts on native communities have been reported from invaded regions, with a consensus that lionfish alter reef fish community and trophic structure in regions where they have become abundant (Lesser & Slattery 2011, Albins & Hixon 2013, Albins 2015). Lionfish are novel predators that consume a broad range of fish and invertebrate prey (Albins & Hixon 2008, Morris & Akins 2009, Muñoz et al. 2011), including the juvenile stages of ecologically and economically important fishes (Lesser & Slattery 2011, Dahl & Patterson 2014). Lionfish predation has caused substantial declines in the abundances of small adult reef fishes, as well as juvenile recruits of larger reef fish species (Albins & Hixon 2008, Green et al. 2012, Albins 2015, Benkwitt 2015). Furthermore, lionfish have caused significant and rapid declines in prey fish biomass (Green et al. 2012) and species richness (Albins 2013) following their arrival on both continuous reefs and patch reefs. Native predator–prey dynamics may also be destabilized in the presence of lionfish (Ingeman & Webster 2015), where the invaders can cause nearly 3-fold greater prey mortality when compared with native mesopredators (Albins 2013). Larger native reef fish species may also be affected via indirect processes such as competition for resources. Dietary overlap of lionfish with native mesopredators, or even apex predators, may lead to decreases in the abundances of those species (Layman & Allgeier 2012). Predation by and lack of predation on lionfish ultimately results in the diversion of resources from higher trophic levels to an energetic dead end. Additionally, lionfish may alter the behavior of native reef fish and invertebrates via competition for space and shelter (Curtis-Quick et al. 2014, Raymond et al. 2015).

The speed of the lionfish invasion coupled with negative impacts to recipient ecosystems has motivated researchers to work towards developing best management practices to mitigate impacts to native communities. There is consensus among researchers and managers that lionfish control is desirable to mitigate their negative effects on marine ecosystems and economies, given that lionfish are now considered to be permanent members of western Atlantic fish communities (Morris & Whitfield 2009, Arias-González et al. 2011). However, the potential benefits as well as the costs of targeted lionfish removal programs remain unclear. All lionfish management strategies hinge on the goal of a reduction of lionfish populations and thus their corresponding impacts. Targeted removals of lionfish have gained considerable attention in recent years and in some cases have reduced both the numbers and mean size of individuals (Frazer et al. 2012, de León et al. 2013). However, lionfish populations have shown an ability to recover quickly from removal efforts, requiring repeated and substantial harvesting effort to maintain low abundances (Arias-González et al. 2011, Barbour et al. 2011). Partial culling has been effective in some cases for stopping the loss of native prey fish biomass with lower effort than would be required for complete lionfish removal (Green et al. 2014). However, other cases have reported that all lionfish must be removed to see substantial conservation gains (Benkwitt 2015). Promotion of the species as a food fish is also gaining popularity and could be a way to increase the geographical scale of lionfish removals (Ferguson & Akins 2010, Morris et al. 2011b, Côté et al. 2013).

Here, we report results of a lionfish removal experiment conducted at artificial reef sites in the nGOM. The objectives of the study were to evaluate the effectiveness of targeted lionfish removals as a means to control lionfish densities, as well as to evaluate the effectiveness of lionfish removal for native reef fish community recovery. Pre-invasion community structure data enabled us to examine shifts in native reef fish communities that occurred after lionfish were observed on study reefs in 2010, and then to examine whether lionfish removal efforts facilitated recovery of native fishes. However, the occurrence of the DWH in 2010 presented a confounding factor for initial changes in reef fish communities, and also patterns seen in reef fish communities following lionfish removals. Therefore, we interpret study results with respect to experimental treatments, as well as within the context of potential effects of the DWH on nGOM reef fishes.

MATERIALS AND METHODS

Study region and experimental reefs

Study sites consisted of 27 artificial reefs within the Escambia East-Large Area Artificial Reef Site (EE-LAARS; 260 km^2), which is located approximately 32 km south of Pensacola, FL, USA (Fig. 1). The same reefs were used for both the observational and the experimental component of this study. Reefs were originally deployed on the seabed (depth range 27–41 m) by the Florida Fish and Wildlife Conservation Commission in 2003 and consist of 3 different design types: single pyramid, paired tetrahedrons, and paired cylinders with rounded tops (Dance et al. 2011). The composition of all reefs was principally concrete, although pyramid reefs had sides composed of steel rebar in a lattice configuration. Reef volume ranged from 4.09 to 5.68 m^3.

Three reefs of each design type were randomly selected for inclusion in one of 2 lionfish removal treatments or a control group during the removal experiment. Nine reefs were selected for a single lionfish removal event (clear-once treatment) in early 2014, and 9 additional reefs were selected to be repeatedly cleared of lionfish via triannual removal events (maintain-clear treatment) through May 2015. The remaining 9 sites were selected for a control treatment with no lionfish removed over the study. However, one of the clear-once reefs was mistakenly not cleared of lionfish in winter 2014; thus, there was one more control reef (n = 10), and one less clear-once site (n = 8), than originally planned.

ROV video sampling and analysis

We sampled study reefs with a VideoRay Pro4 remotely operated vehicle (ROV) to estimate reef fish community structure for both components of the study, albeit on different time scales. Video sampling

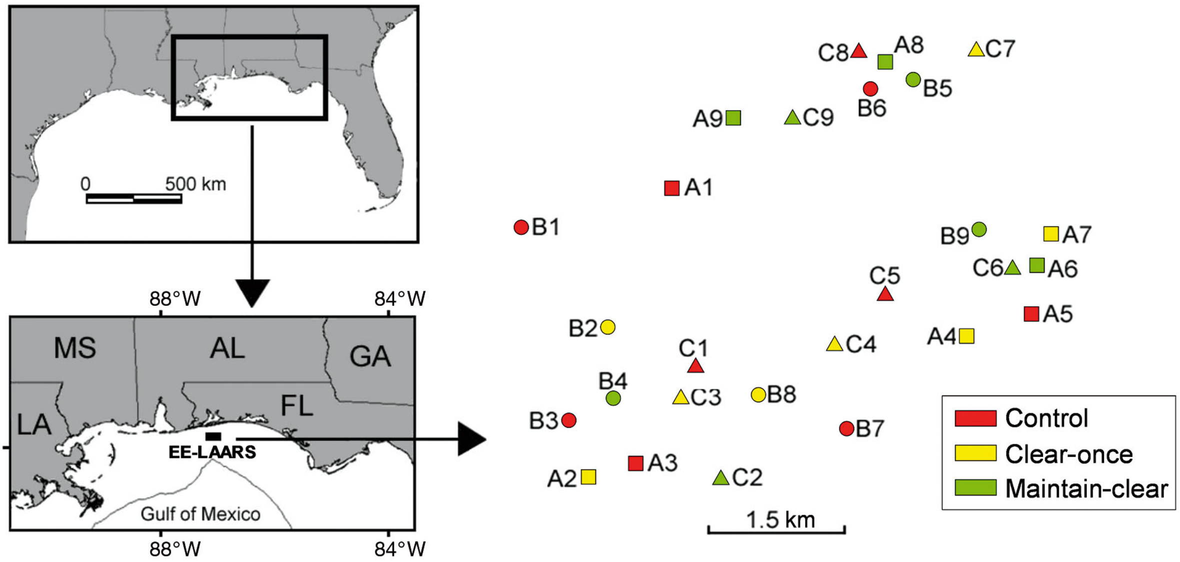

Fig. 1. Map of the northern Gulf of Mexico indicating the locations of the Escambia East-Large Area Artificial Reef Site (EE-LAARS) and the 27 experimental reefs examined in the current study. Experimental reefs were located in the northeast quadrant of the 260 km^2 area. Symbols denote different reef types: triangles (paired cylinders with rounded tops), circles (paired tetrahedrons) and squares (single pyramid)

was conducted quarterly in 2009–2010, which provided baseline data on reef fish community structure prior to lionfish being observed in the nGOM, and again in 2011–2012, the year following the start of the lionfish invasion. During the removal experiment, we conducted triannual (i.e. approx. every 4 mo) video sampling from December 2013 through August 2015. Specifically, ROV sampling for the removal experiment was performed in December 2013 (fall 2013), March 2014 (spring 2014), July 2014 (summer 2014), December 2014 (fall 2014), May 2015 (spring 2015), and August 2015 (summer 2015).

The VideoRay Pro4 ROV (dimensions: 36 cm long, 28 cm tall, 22 cm wide; mass = 4.8 kg) has a depth rating of 170 m, a 570-line color camera with wide-angle (116°) lens, and is equipped with a red laser scaler to estimate fish size. The laser scaler consists of two 5 mW , 635 nm (red) class IIIa lasers mounted in a fixed position 75 mm apart, allowing for estimation of fish size using the ratio of the distance between lasers to the distance between snout and fork length of fishes observed onscreen (Patterson et al. 2009, Dance et al. 2011). The ROV was tethered to the surface and controlled by a pilot via an integrated control box that contains a 38 cm video monitor to observe and capture the digital video feed from the ROV's camera. Additionally, a GoPro Hero4 high definition (1080p at 120 fps) digital camera was mounted to the forward view of the ROV to provide high definition video for reef fish community surveys.

The ROV-based point-count sampling method described by Patterson et al. (2009) was employed to estimate reef fish community structure in a 15 m wide cylinder with reefs at the center of the cylinder's base. High definition video samples were viewed in a darkroom on a Sony LMD-2110W high-resolution LCD monitor to enumerate and identify reef fishes to the lowest taxonomic level possible. A second video reader independently analyzed randomly selected video samples (n = 16) to estimate reader agreement. Differences between reader estimates were evaluated by computing the average percent error (APE) for each taxon in a given sample, following Beamish & McFarlane (1983). The mean of site-specific APEs across all taxa was computed to produce an overall APE between readers.

Total length (TL) was estimated for lionfish struck by the laser scaler if lionfish orientation was estimated to be less than 20° from perpendicular to lasers in order to minimize measurement error (Patterson et al. 2009). Fish size was estimated by first multiplying the length of a fish measured in a video frame by the known distance between lasers (75 mm), and then dividing that product by the distance measured between lasers in the frame. Patterson et al. (2009) estimated a mean negative bias of 3 % (SD = 0.6) from this method; thus, our estimated lionfish TL was bias-corrected based on a random probability draw and normally distributed bias with a mean equal to 3 % and a SD of 0.6 %. Total length distributions of lionfish on control sites were gathered from ROV estimates of TL. A 1-factor ANOVA was computed to test whether estimates of mean lionfish length estimated with the ROV laser scaler were different among removal treatments at the start of the study in the fall of 2013.

Targeted removals of lionfish

Divers removed lionfish from study reefs via spearfishing. Divers were able to capture and remove all lionfish present from reef structure and surrounding seabed during removal events. Initial removals on clear-once and maintain-clear sites were performed in January and February 2014, and then repeated on maintain-clear sites in July and August 2014, and in February and May 2015. Poor weather conditions prevented February and May 2015 removal events from occurring closer in time. Lionfish were speared immediately posterior to the skull-spinal column juncture and then placed in a saltwater ice slurry to euthanize. Each lionfish removed was weighed to the nearest 0.1 g and measured to nearest mm TL. The growth function reported by Barbour et al. (2011) for lionfish in USA waters was then solved for age and used to predict age distributions from the TL data obtained from culled fish. A linear regression was computed between lionfish counts from the ROV video samples and lionfish subsequently removed by divers at clear-once or maintain-clear reefs to test for bias in the ROV-derived lionfish counts.

Data analysis

Pre-removal experiment

Permutational analysis of variance (PERMANOVA) models were computed with the Primer statistical package (ver. 6; Anderson et al. 2008) to test for differences in reef fish community structure. Taxa-specific fish densities (fish 100 m^{-2}) were the dependent variables in PERMANOVA models, which were computed with standardized (by total sample abundance) untransformed fish density data, using

Bray-Curtis dissimilarities with 10 000 permutations. Models tested whether the pattern in the similarity matrices between levels of factors was significantly different from random. Single-factor PERMANOVA models were computed to test for differences in reef fish community structure between samples collected in 2009–2010 versus 2011–2012 for all fishes, as well as separately for exploited species (e.g. snappers, groupers, porgies, triggerfish and jacks; Table S1 in the Supplement at www.int-res.com/articles/suppl/m558p207_supp.pdf) and small demersal reef fishes (e.g. damselfishes, cardinalfishes, blennies, wrasses, gobies; Table S1 in the Supplement). In the model, reef surveys were nested within reefs across time to account for repeated sampling of individual reefs over time. This partitioning of variance accounted for differences within individual reefs and resulted in a residual error term inappropriate to test the effect of differences among individual reefs (Zar 1999, Hinkle et al. 2003), nor was this effect of primary interest to the study. Thus, *F*-ratios and p-values were not interpreted for effects of individual reefs in all repeated measures analyses.

Species-specific contrasts were performed for the 25 most abundant species with single-factor permutational ANOVA models computed in Primer (ver. 6; Anderson et al. 2008) to test for differences in fish density between 2009–2010 and 2011–2012 time periods. In the models, reef surveys were nested within reefs across time to account for repeated sampling of individual reefs over time. One-factor repeated measures ANOVAs also were computed to test the effect of time on reef fish diversity indices of species richness (number of species present), diversity (Shannon-Wiener H'), and evenness (Pielou's J'), as well as number of individuals (individuals from all species). For all ANOVA models, assumptions of normality and equal variances were assessed with Shapiro-Wilks (stats package) and Levene's (Fox & Weisberg 2011, 'car' package) tests, respectively, within R (version 3.1.1; R Core Team 2015). Data met the assumption of equal variances in all models, but normality was occasionally violated. Given ANOVA is robust to minor departures from normality (Schmider et al. 2010), models were computed with untransformed data.

Removal experiment

A single-factor PERMANOVA model was computed to test for differences in reef fish community structure between treatments among 27 reefs at the beginning of the removal experiment. Two-factor PERMANOVA models were computed to test the effect of removal treatment, sample timing, and their interaction on reef fish community structure among all fishes, as well separately for exploited species (Table S1 in the Supplement) and small demersal reef fishes (Table S1). Reef sites were nested within treatment to account for repeated sampling of reefs over time. Given only one sample (reef survey) occurred at each site during each time period, the highest order interaction, 'site(treatment) × time', was excluded from the model (Anderson et al. 2008). For any significant main effect (at $\alpha = 0.05$), post-hoc pairwise tests were computed with 10 000 permutations.

Two-factor repeated measures ANOVAs were computed to test the effect of removal treatment, sample timing, and their interaction on reef fish diversity indices of species richness (number of species present), H' and J', as well as number of individuals (individuals from all species) and lionfish density. Pairwise multiple comparison procedures (Tukey's tests) were used to test which levels were different when a main effect was detected. One-factor repeated measures ANOVAs also were computed to test whether reef fish diversity indices (species richness, H' and J') and the number of individuals (individuals from all species) were different among all time periods (2009–2010, 2011–2012, and 2013–2014). The 2013–2014 time period included data from all reefs prior to removals and only control sites following removals.

Video samples collected with ROV at clear-once reefs following lionfish removal in January and February 2014 enabled the estimation of lionfish recolonization rate over the remainder of the study. A linear mixed-effects regression was fit using restricted maximum likelihood to estimate the relationship between lionfish density and time since removal (Pinheiro et al. 2016, 'nlme' package). In the model, estimated lionfish density was the response variable, with days since removal as a fixed effect and reef site as a random effect to account for non-independence among repeat samples of the same reefs. The model formula in R is therefore: estimated lionfish density ~ days since removal + (1 | reef), where '1' assumes different intercepts for each reef (i.e. multiple responses dependent on reef). R^2 was calculated to describe the proportion of variance explained by both fixed and random factors (Nakagawa & Schielzeth 2013). Confidence intervals (95 %) were calculated for model estimates of intercept and slope.

RESULTS

We collected a total of 299 video samples at study reefs, with 221 965 fish observed among 109 taxa (96.0% identified to species). Of these samples, 137 were collected in 2009–2010 and 2011–2012 (83 967 fish among 85 taxa), and 162 were collected during the removal experiment (137 998 fish among 80 taxa). Fish counts were compared between readers for 16 samples, which produced 191 taxa-specific paired comparisons. The overall APE between readers was 5.7% among these 16 video samples.

Pre-removal experiment

No lionfish were observed in 2009–2010 video samples, but lionfish were observed when sampling resumed following the DWH event. Thus, lionfish first appeared on study reefs sometime between winter 2010 and fall 2011. There was a significant difference in reef fish community structure between 2009–2010 and 2011–2012 (PERMANOVA, $p = 0.015$) (Table 1). There also were differences in community structure of fishery species (PERMANOVA, $p = 0.042$) (Table 1) and small demersal species (PERMANOVA, $p < 0.001$) (Table 1) between 2009–2010 and 2011–2012, as well as in species richness and diversity (ANOVA, $p \leq 0.002$; (Table 2, Table S2 in the Supplement). Higher diversity, as well as approximately 50% more species, were observed at study reefs in 2009–2010 than in 2011–2012; however, there was a general increase in number of individuals across all taxa in the latter time period (Fig. 2). The general increase in the mean number of individuals observed during 2011–2012 is mostly attributed to increases in small (<150 mm TL) pelagic planktivores (e.g. mackerel scad *Decapterus macarellus*) and tomtate *Haemulon aurolineatum* (Tables 2 & 3, Fig. S1 in the Supplement). Declines observed in species richness and diversity between 2009–2010 and 2011–2012 were beginning to stabilize relative to pre-invasion values by 2013–2014, but trends were not statistically significant for richness (Fig. 2, Table S2). General trends were declines in mean density, which were observed for 19 of the 25 most abundant reef fish species from 2009–2010 to 2011–2012 (Table 3). Out of these, statistically significant declines were observed in vermilion snapper *Rhomboplites aurorubens* ($p = 0.026$), twospot cardinalfish *Apogon pseudomaculatus* ($p = 0.001$), blue runner *Caranx crysos* ($p = 0.007$), red porgy *Pagrus pagrus* ($p = 0.001$), slippery dick *Halichoeres bivitta-*

Table 1. PERMANOVA results of the model computed to test for differences in reef fish community structure (species composition and relative abundance) between samples collected in 2009–2010 versus 2011–2012 estimated from video samples collected with a remotely operated vehicle at study reefs. Significant ($\alpha < 0.05$) p-values are in **bold**

Model	Source	df	Type III SS	MS	pseudo-*F*	p
All fishes	Time	1	21114	21114	3.46	**0.015**
	Site (Time)	34	2.13×10^5	6257		
	Residual	101	1.86×10^5	1838		
	Total	136	4.20×10^5			
Exploited reef fishes	Time	1	14049	14049	2.42	**0.042**
	Site (Time)	34	2.01×10^5	5927		
	Residual	101	2.50×10^5	2479		
	Total	136	4.67×10^5			
Small demersal reef fishes	Time	1	27318	27318	4.37	**<0.001**
	Site (Time)	34	2.17×10^5	6393		
	Residual	101	2.63×10^5	2604		
	Total	136	5.09×10^5			

Table 2. One-way repeated measures ANOVA results for models computed to test the effect of timing, 2009–2010 versus 2011–2012, on reef fish diversity indices and number of individuals. Significant ($\alpha < 0.05$) p-values are in **bold**

Index	Source	df	Type III SS	MS	*F*	p
Species richness	Between subjects	17	154.91	9.11		
	Between treatments	1	130.34	130.34	65.10	**<0.001**
	Residual	17	30.04	2.00		
	Total	35	319.29			
Shannon-Wiener diversity, *H'*	Between subjects	17	3.414	0.201		
	Between treatments	1	0.766	0.766	13.462	**0.002**
	Residual	17	0.967	0.056		
	Total	35	5.148			
Pielou's evenness, *J'*	Between subjects	17	0.339	0.020		
	Between treatments	1	0.019	0.019	1.284	0.168
	Residual	17	0.158	0.009		
	Total	35	0.517			
No. of ind. (across taxa)	Between subjects	17	3.29×10^6	1.94×10^5		
	Between treatments	1	7.75×10^4	7.75×10^4	1.267	0.276
	Residual	17	1.04×10^6	6.12×10^4		
	Total	35	4.41×10^6			

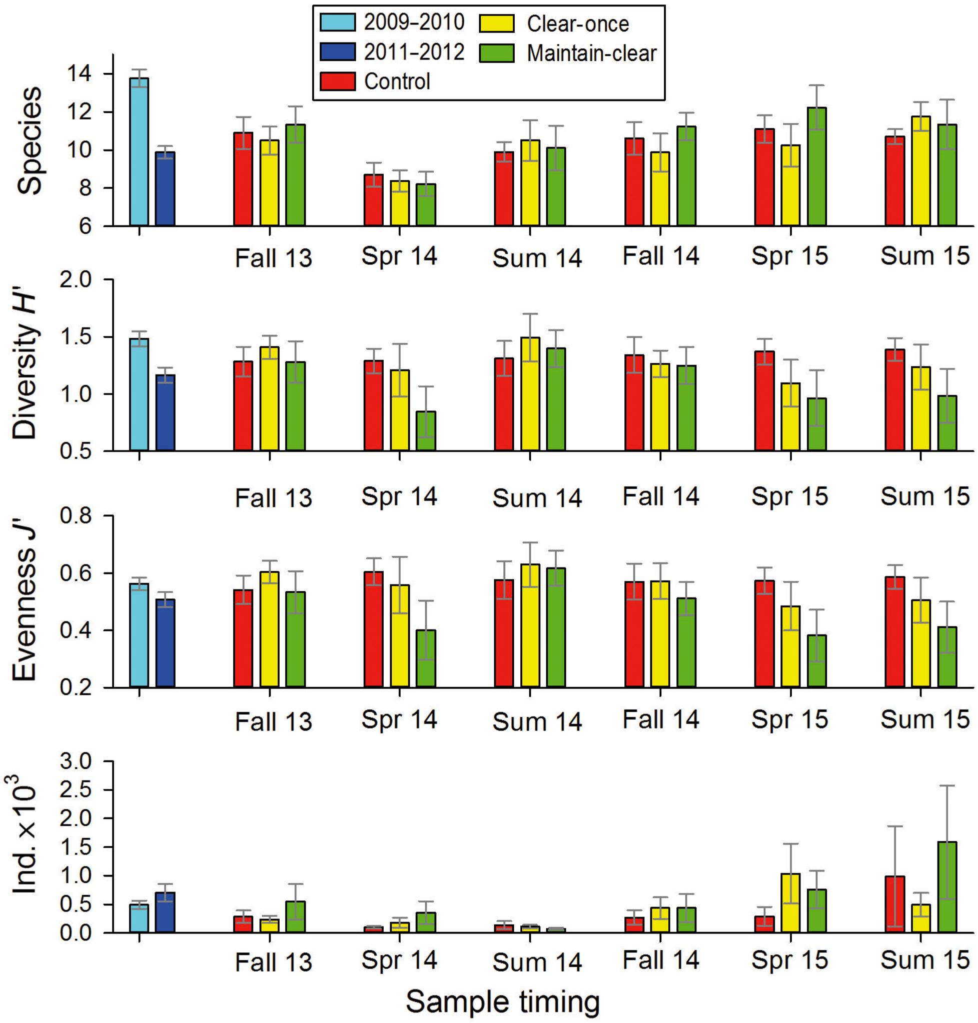

Fig. 2. Species diversity indices and number of individual fish across taxa on study reef sites during spring 2009–winter 2010, fall 2011–summer 2012, and then computed during the lionfish removal experiment from fall 2013–summer 2015. Values are means ± SE. The diversity index *H′* is Shannon-Wiener diversity, and the evenness index *J′* is Pielou's evenness. Removals occurred between fall 2013 and spring 2014 (maintain-clear and clear-once), summer 2014 and fall 2014 (maintain-clear), and fall 2014 and spring 2015 (maintain-clear)

tus (p = 0.001), seaweed blenny *Parablennius marmoreus* (p = 0.001), yellowtail reeffish *Chromis enchrysura* (p = 0.001), scaled sardine *Harengula jaguana* (p = 0.018), gulf flounder *Paralichthys albigutta* (p = 0.006), and lesser amberjack *Seriola fasciata* (p = 0.001) (Table 3). While declines were observed for some larger predatory reef fishes (e.g. snappers, jacks, triggerfish) during 2011–2012, the biggest declines were seen in small (<100 mm) demersal planktivores and invertivores (Table S1, e.g. blennies, damselfishes, wrasses). The density of many of these small demersal species declined by >90% between the 2009–2010 and 2011–2012 time periods (Table 3, Fig. S1).

Removal experiment

Six triannual ROV sampling events were conducted at study reefs from fall 2013 to summer 2015 for the lionfish removal experiment. A linear regression relating numbers of lionfish removed and lionfish numbers counted in ROV samples was statistically significant ($F_{1,34}$ = 283.4, $p < 0.001$, R^2 = 0.90, lionfish removed = 1.47 + 1.29 × lionfish count). The slope of 1.29 indicates that on average 29% more lionfish were removed from study reefs during removal events than had been estimated to exist on those reefs from ROV video samples. Therefore, ROV-based lionfish counts were scaled upward by a factor of 1.29 to account for incomplete detectability in ROV samples.

Unscaled lionfish counts in video samples ranged from 1 to 184 during the study, which translates to a density range of 0.7 to 103 fish 100 m^{-2}. Estimated initial mean ± SE lionfish density was not different among control, clear-once, and maintain-clear reefs and ranged from 28.9 ± 12.3 to 31.8 ± 5.7 fish (Fig. 3 & Table 4). Divers removed 1575 individual lionfish from clear-once and maintain-clear study reefs, including 564 fish during the 2 follow-up removals at

Table 3. Mean density (fish 100 m^{-2}) and percent change in the 25 most abundant fishes observed at study artificial reef sites in 2009–2010 prior to lionfish presence versus in 2011–2012 after lionfish presence was confirmed. Significant ($\alpha < 0.05$) p-values in mean density between the time periods indicated with an asterisk (*)

Species	Common name	2009–2010		2011–2012		% Change
		Mean density	SE	Mean density	SE	
Decapterus macarellus	Mackerel scad	82.1	33.8	236.9	69.7	188.7
Haemulon aurolineatum	Tomtate	82.1	20.3	102.9	29.6	25.4
Lutjanus campechanus	Red snapper	21.1	3.1	15.7	2.6	–25.8
*Rhomboplites aurorubens**	Vermilion snapper	19.2	5.6	10.2	3.7	–46.8
*Apogon pseudomaculatus**	Twospot cardinalfish	11.1	6.2	0.2	0.1	–98.5
*Caranx crysos**	Blue runner	7.8	4.1	0.1	0.1	–99.2
Centropristis ocyurus	Bank sea bass	7.4	1.9	2.8	0.8	–62.4
*Pagrus pagrus**	Red porgy	7.0	1.4	0.5	0.2	–93.3
*Halichoeres bivittatus**	Slippery dick	6.9	1.9	0.5	0.2	–92.6
Seriola dumerili	Greater amberjack	5.0	0.9	6.8	2.1	36.3
Balistes capriscus	Gray triggerfish	5.0	0.9	3.4	0.6	–31.4
*Parablennius marmoreus**	Seaweed blenny	3.7	0.9	0.1	–	–97.7
Lutjanus griseus	Gray snapper	3.3	0.7	3.3	1.0	1.8
Lutjanus synagris	Lane snapper	3.3	1.0	0.5	0.2	–84.6
Rypticus maculatus	Whitespotted soapfish	2.8	0.8	1.3	0.2	–53.8
*Chromis enchrysura**	Yellowtail reeffish	2.0	0.4	0.0	–	–99.6
Equetus lanceolatus	Jacknife fish	1.7	0.5	1.2	0.3	–30.0
Apogon sp.	Unidentified cardinalfishes	0.8	0.6	0.0	–	–100.0
*Harengula jaguana**	Scaled sardine	0.7	0.4	0.0	–	–100.0
Canthigaster rostrata	Sharpnose puffer	0.5	0.1	0.8	0.2	62.0
*Paralichthys albigutta**	Gulf flounder	0.5	0.2	0.0	–	–100.0
*Seriola fasciata**	Lesser amberjack	0.4	0.1	0.0	–	–100.0
*Mycteroperca phenax**	Scamp	0.4	0.1	0.8	0.1	88.8
Chaetodon ocellatus	Spotfin butterflyfish	0.4	0.1	0.3	0.1	–15.8
Epinephelus morio	Red grouper	0.3	0.1	0.2	–	–42.4

maintain-clear reefs. There was a significant interaction between the effects of removal treatment and sample timing on lionfish density (ANOVA; $p < 0.001$) (Table 4 & Fig. 3). Densities of lionfish on control reefs significantly increased over the study period from 31.1 ± 5.7 fish 100 m^{-2} in fall 2013 to 49.2 ± 7.9 fish 100 m^{-2} in summer 2015 ($p = 0.042$) (Table 4) despite a brief decline in abundance in December 2014 (Fig. 3). Across all sample periods, control reefs held higher densities of lionfish than maintain-clear ($p \leq 0.027$) (Table 4) reefs following the initial removal event. Control reefs held higher densities than clear-once sites for only 2 surveys immediately following lionfish removal ($p \leq 0.004$) (Fig. 3 & Table 4). On maintain-clear reefs, lionfish density was only significantly different between December 2013 and March 2014, the sampling periods immediately prior to and following removal ($p = 0.003$) (Fig. 3 & Table 4), and May 2015 ($p = 0.020$). Following their removal from clear-once and maintain-clear sites in January and February 2014, lionfish densities increased to 4.1 ± 2.0 fish 100 m^{-2} at maintain-clear and 5.2 ± 1.6) fish 100 m^{-2} at clear-once reefs by March 2014 (Fig. 3). Estimates of lionfish density on maintain-clear sites averaged 9.7 ± 1.5 fish 100 m^{-2} among all ROV sampling events that on average occurred 2.3 mo after lionfish removal events (Fig. 3). Lionfish density steadily increased in the year following the single removal event on clear-once reefs, and densities recovered to pre-removal levels by July 2014 ($p = 0.153$) (Fig. 3 & Table 4). The initial, pre-removal mean lionfish density on clear-once reefs was surpassed by the end of the study (Fig. 3).

There was no difference among treatments in the initial fall 2013 estimates of mean lionfish size estimated with the ROV laser scaler (ANOVA, $F_{2,18} = 2.77$, $p = 0.089$), which ranged from 207 mm on maintain-clear reefs to 242 mm on control reefs. Estimated TL of lionfish ($n = 222$) on control reefs over the study period ranged from 134 to 456 mm (Fig. S2 in the Supplement), but the size distribution of fish mostly fell between 150 and 350 mm TL. Length frequency distributions from removal events at clear-once and maintain-clear treatments were computed from the 1575 culled individual lionfish. Among all sites, total lengths of removed lionfish ranged from 74 to 376 mm (Fig. S3 in the Supple-

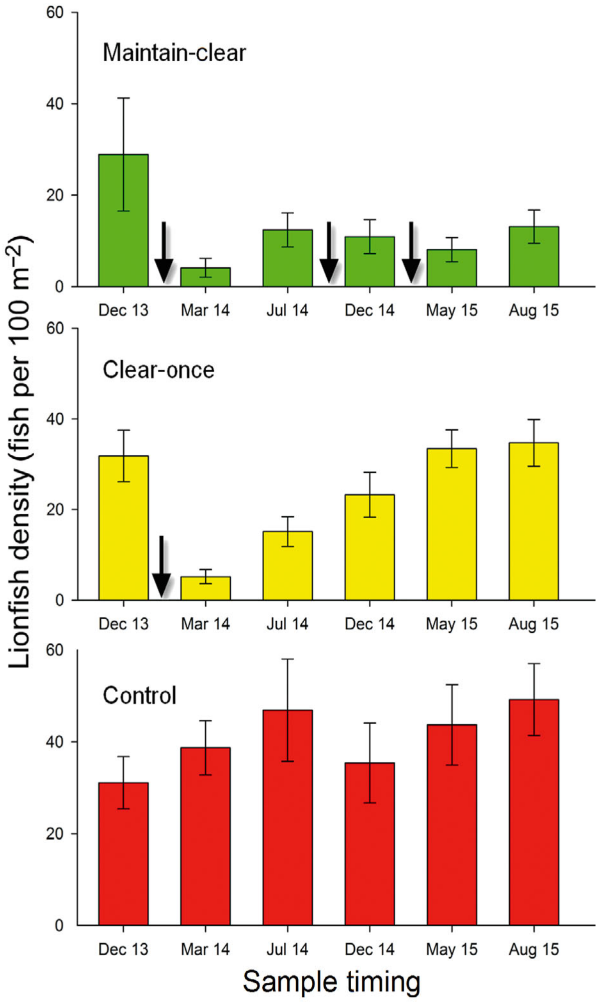

Fig. 3. Mean (±SE) lionfish density estimated from counts made with a remotely operated vehicle (ROV) and then scaled (×1.29) to correct for incomplete detectability. Arrows indicate timing of lionfish removal efforts between ROV samples

ment). Length distributions for clear-once and maintain-clear reefs from the initial removal event were similar and skewed toward larger-sized lionfish (>200 mm TL; Fig. S3A,B). Age distributions estimated with the growth function reported by Barbour et al. (2011) indicated the majority of lionfish from both treatments were 1 and 2 yr old fish (Fig. S3F,G). The size distribution of lionfish removed from maintain-clear reefs in July 2014 had 2 distinct modes, with the predominant mode centered on 150 mm TL (Fig. S3C). Therefore, fish that recruited to cleared reefs following the initial removal events in January and February 2014 likely consisted of a large percentage of age-0 fish, as well as individuals as old as 4 yr (Fig. S3G). The final removal events conducted at maintain-clear reefs in February–May 2015 had fewer small (<200 mm TL) fish than the previous removal event, but more than were originally removed in early 2014 (Fig. S3E).

Reef fish communities were not different between treatments at the beginning of the experiment prior to removals. Removal treatment had significant effects on reef fish community structure. For the PERMANOVA model containing all reef fish taxa, both treatment (p = 0.021) and sample timing (p = 0.001) were significant, but their interaction was not (p = 0.254, Table 5). Reef fish communities on control reefs were significantly different from both removal

Table 4. Two-factor repeated measures ANOVA results for model computed to test the effects of lionfish removal treatment (Treat: control, clear-once, maintain-clear) and sample timing on lionfish density (fish × 100 m^{-2}) estimates at study artificial reefs. Post hoc pairwise multiple comparisons (Tukey) for significant main test results follow. Significant ($\alpha < 0.05$) p-values are in **bold**

Source	df	Type III SS	MS	*F*	p
Treat	2	22586	11293	7.71	**0.003**
Site(Treat)	24	35154	1465		
Timing	5	4727	945	5.05	**<0.001**
Treat × Timing	10	7034	703	3.75	**<0.001**
Residual	120	22484	187		
Total	161	91494			

Test	Sample timing					
	Dec 13	Mar 14	Jul 14	Dec 14	May 15	Aug 15
Control vs. maintain-clear	0.970	**0.001**	**0.001**	**0.027**	**<0.001**	**<0.001**
Control vs. clear-once	0.997	**0.002**	**0.004**	0.412	0.528	0.285
Clear-once vs. maintain-clear	0.953	0.994	0.958	0.419	**0.031**	0.076

Timing	Dec 13	Mar 14	Jul 14	Dec 14	May 15
Control					
Mar 14	0.815				
Jul 14	0.110	0.763			
Dec 14	0.982	0.994	0.420		
May 15	0.316	0.964	0.995	0.752	
Aug 15	**0.042**	0.525	0.999	0.220	0.946
Clear-once					
Mar 14	**0.002**				
Jul 14	0.153	0.693			
Dec 14	0.811	0.096	0.843		
May 15	1.000	**0.001**	0.089	0.673	
Aug 15	0.998	**<0.001**	0.056	0.553	1.000
Maintain-clear					
Mar 14	**0.003**				
Jul 14	0.116	0.793			
Dec 14	0.067	0.898	1.000		
May 15	**0.020**	0.989	0.986	0.998	
Aug 15	0.149	0.729	1.000	0.999	0.971

Table 5. PERMANOVA results for models computed to test the effects of lionfish removal treatment (Treat: control, clear-once, maintain-clear) and sample timing on reef fish community structure (species composition and relative abundance) estimated from video samples collected with a remotely operated vehicle at study reefs. Significant ($\alpha < 0.05$) p-values are in **bold**

Model	Source	df	Type III SS	MS	pseudo-*F*	p
All fishes	Treat	2	51242	25621	2.70	**0.021**
	Timing	5	16587	3317	2.24	**0.001**
	Site(Treat)	24	2.27×10^5	9474		
	Treat × Timing	10	16770	1677	1.13	0.254
	Residual	120	1.77×10^5	1482		
	Total	161	4.90×10^5			
Exploited reef fishes	Treat	2	10677	5338	0.71	0.641
	Timing	5	27020	5404	4.42	**0.001**
	Site(Treat)	24	1.83×10^5	7621		
	Treat × Timing	10	13149	1315	1.07	0.356
	Residual	116	1.42×10^5	1224		
	Total	157	3.76×10^5			
Small demersal reef fishes	Treat	2	19348	9674	0.88	0.489
	Timing	5	20130	4026	3.37	**0.001**
	Site(Treat)	24	2.62×10^5	10948		
	Treat × Timing	10	9874	987	0.83	0.805
	Residual	119	1.42×10^5	1195		
	Total	160	4.54×10^5			

Table 6. Two-way repeated measures ANOVA results for models computed to test the effects of lionfish removal treatment (Treat: control, clear-once, maintain-clear) and sample timing on reef fish diversity indices and number of individuals. Significant ($\alpha < 0.05$) p-values are in **bold**

Index	Source	df	Type III SS	MS	*F*	p
Species richness	Treat	2	8.34	4.17	0.214	0.809
	Site(Treat)	24	468.44	19.52		
	Timing	5	149.33	29.87	7.252	**<0.001**
	Treat × Timing	10	26.83	2.68	0.652	0.767
	Residual	120	494.17	4.12		
	Total	161	1147.61			
Shannon-Wiener diversity *H'*	Treat	2	1.35	0.68	0.877	0.429
	Site(Treat)	24	18.50	0.77		
	Timing	5	1.64	0.33	2.015	0.081
	Treat × Timing	10	1.56	0.16	0.962	0.481
	Residual	120	19.50	0.16		
	Total	161	42.41			
Pielou's Evenness *J'*	Treat	2	0.31	0.16	1.36	0.275
	Site(Treat)	24	2.77	0.12		
	Timing	5	0.28	0.06	1.911	0.097
	Treat × Timing	10	0.28	0.03	0.951	0.490
	Residual	120	3.52	0.03		
	Total	161	7.13			
No. of ind. (across taxa)	Treat	2	2.38×10^6	1.19×10^6	0.399	0.676
	Site(Treat)	2	7.15×10^7	2.98×10^6		
	Timing	5	1.54×10^7	3.09×10^6	3.216	**0.009**
	Treat Timing	10	6.29×10^6	6.29×10^5	0.655	0.764
	Residual	120	1.15×10^8	1.30×10^6		
	Total	161	2.11×10^8			

treatments (PERMANOVA, $p \leq 0.034$), but removal treatments were not significantly different from each other (PERMANOVA, p = 0.333) (Table S3 in the Supplement). Models computed for exploited species and small demersal fishes produced different results wherein the effect of sample timing was significant, but treatment and the interaction between the main effects were not (Table 5). Substantial gains in abundance were not observed for most taxa regardless of lionfish removal effort (Fig. S1 in the Supplement). Modest increases in mean density were seen for bank sea bass *Centropristis ocyurus*, pelagic planktivores (e.g. scads, sardines), small demersal fishes (e.g. damselfishes, cardinalfishes) and slippery dick *Halichoeres bivittatus* from targeted lionfish removals (Fig. S1). Removal treatment did not affect any of the reef fish diversity indices measured (Table 6, Fig. 2). The effect of sample timing was significant for species richness, but not for diversity or evenness (Table 6, Table S4 in the Supplement, Fig. 2). Differences in numbers of individuals across taxa stemmed from low numbers in spring and summer 2014 compared to increases in numbers in summer 2015 (Fig. 2, Table S4). There were no significant interactions between removal treatment and sample timing on any reef fish diversity index (Table 6).

The linear mixed model regression fit to lionfish density versus experiment day for clear-once reefs was significant with a slope (±95% CI) of 0.063 ± 0.011 lionfish 100 m^{-2} d^{-1} (Fig. 4). Therefore, the density of lionfish on cleared reefs was estimated to increase by 1 fish 100 m^{-2} approximately every 16 d.

DISCUSSION

Extensive baseline data on reef fish community structure at both natural and artificial reefs in the nGOM (e.g.

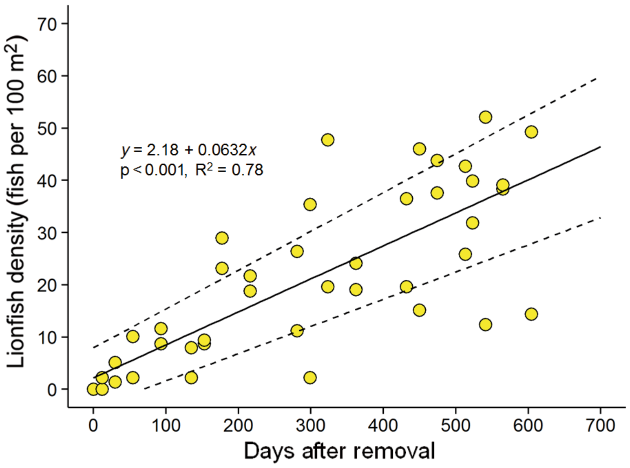

Fig. 4. Scatterplot of estimated lionfish density (scaled upward by a factor of 1.29 for incomplete detectability) versus days after lionfish removal for clear-once experimental artificial reef sites and the line fit to the significant fixed effect of days after removal. The intercept (±95% CI) is the average of coefficients from individual reefs in the model. The slope (±95% CI) is the recolonization rate of lionfish to all cleared reefs, taking individual reef variation into account

Dance et al. 2011, Patterson et al. 2014) have enabled us to track the lionfish invasion in this region (Dahl & Patterson 2014). Results presented here for artificial reef study sites off northwest Florida clearly demonstrate that shifts in reef fish community structure occurred between 2009–2010 and 2011–2012, time periods which bracket the appearance of lionfish in the nGOM region (Dahl & Patterson 2014). Taxa-specific differences were most pronounced for small demersal reef fishes, such as damselfishes, cardinalfishes, wrasses, and blennies, which have been documented as predominant prey of lionfish (Albins & Hixon 2008, Morris & Akins 2009, Dahl & Patterson 2014). In fact, local depletion or extirpation of these taxa due to lionfish predation has been reported in other systems (Green et al. 2012, Albins 2015). Lionfish densities on nGOM artificial reefs were among the highest in the western Atlantic by fall 2013 (Dahl & Patterson 2014), and mean density had already reached nearly 10 fish 100 m^{-2} on our study reefs by fall 2012. This is significant in that such densities are above threshold values where ecological impacts have been predicted to occur on Caribbean reefs (Green et al. 2014, Benkwitt 2015).

Observed shifts in reef fish community structure following the arrival of lionfish in the nGOM seem like compelling evidence of lionfish effects, especially given similar declines attributed to lionfish in other parts of their invaded range. However, the occurrence of the DWH in summer 2010 is a confounding factor in drawing inference about potential ecological impacts of lionfish in this region. Estimates of the spatial extent of DWH surface oil extended over our study area periodically from April to August 2010 (Goni et al. 2015). There is clear evidence that some nGOM reef fishes were exposed to toxic petroleum compounds released during the spill (Murawski et al. 2014), with documented impacts on fishes including genetic effects, shifts in trophic position, declines in size at age, and changes in community structure (Whitehead et al. 2012, Norberg 2015, NOAA-NRDA 2015, Tarnecki & Patterson 2015). Therefore, it is possible that reef fish declines observed at study reefs in 2011–2012 were initially driven by the DWH. Declines observed in larger species, such as snappers and gray triggerfish, during 2011–2012 versus 2009–2010 could have resulted from mortality due to the spill or emigration from spill-affected areas. Few of these species settle directly on reefs, but instead recruit to reefs following months to years in intermediate nursery habitats, such as *Sargassum* wracks, seagrass beds, or shell rubble reefs. Therefore, it is unlikely that lionfish directly consumed these groups on our study reefs, a conclusion supported by diet data (Dahl & Patterson 2014). Small demersal fishes, such as damselfishes, cardinalfishes, wrasses, blennies, and gobies, are obligate reef residents, settle directly from the plankton onto reef habitat, and are much more site-attached than the larger taxa described above. Therefore, localized effects of lionfish were more likely to have affected small demersal fishes directly as opposed to larger and more mobile species.

Our inability to definitely state that reef fish community shifts predated lionfish becoming well-established on study reefs partly stems from the fact that no data on fish community structure were collected at the study reefs during the year immediately following the DWH when mean lionfish density (~5 fish 100 m^{-2}) was less than predicted threshold values from the Caribbean (Dahl & Patterson 2014, Green et al. 2014). However, declines in the number of species and lower species diversity observed in 2011–2012 relative to 2009–2010 showed signs of stabilizing by 2013, with the possibility of a reversing trend. This occurred while mean lionfish density on study reefs increased to over 30 fish 100 m^{-2}, and mean mass of individuals had nearly doubled over what was observed in fall 2011 (Dahl & Patterson 2014). Therefore, despite an increasing lionfish population in the region, and specifically on our study reefs, fish communities had somewhat stabilized from declines observed following 2009–2010. One group that did not experience density or diversity increases during 2013, however, was small demersal fishes. Given these taxa are the predominant prey of lionfish in this and other systems, exponentially increasing lionfish populations after 2012 may have then suppressed any resiliency these groups may have otherwise shown in recovery from the DWH event or limited lionfish presence.

Regardless of the ultimate cause(s) of reef fish community structure shifts observed between 2009–2010 and 2011–2012, a central goal of this study was to conduct lionfish removal events to examine what level of effort would be required to facilitate recovery in affected communities. Targeted removals from nGOM artificial reefs did significantly reduce lionfish density. However, reductions were short-lived as juvenile and adult lionfish rapidly recruited to cleared reefs. Lionfish were observed on cleared reefs as early as a week after removing all lionfish, and more than 500 individuals were removed from maintain-clear reefs during follow-up removal events during the year following initial culling. One year after lionfish removal, clear-once sites had lionfish densities comparable to those of control reefs, and mean lionfish density on clear-once reefs eventually surpassed the levels initially observed in fall 2013. When accounting for incomplete detectability, our estimates of lionfish density illustrate the extent to which the nGOM region is invaded. Mean densities from our control sites throughout the study, and clear-once sites at the conclusion of the study, were 8- to 10-fold higher than the mean density (4.4 fish 100 m^{-2}) reported by Hackerott et al. (2013) in a meta-analysis of lionfish densities on Caribbean reefs. This may explain why we failed to see lasting lionfish reductions in both population numbers and size. Indeed, results reported here are consistent with models that predict sustained removal efforts are required to control lionfish populations (Arias-González et al. 2011, Barbour et al. 2011, Morris et al. 2011a), perhaps at intensities greater than has been performed elsewhere in the invaded range (Frazer et al. 2012, de León et al. 2013, Benkwitt 2015).

A reduction in the mean size of lionfish present in the system would be a desirable management outcome as it could reduce cumulative predation on vulnerable reef fishes given that lionfish diet shifts with ontogeny and proportionally more fish are consumed at larger sizes (Morris & Akins 2009, Dahl & Patterson 2014). Larger, mature individuals also have higher energetic demands and consume prey at higher rates than smaller sized fish (Cerino et al. 2013). Recruitment and settlement of juvenile lionfish onto previously cleared reefs was high following the first removal event in early 2014, effectively lowering the mean size of individuals at both maintain-clear and clear-once reefs. However, the size distribution of lionfish present at maintain-clear reefs from the final removals in February and May 2015 had shifted back toward larger adults, thus mostly negating early reductions in mean size of lionfish.

The early life history and recruitment dynamics of invasive lionfish are not well understood. Therefore, little information exists to evaluate whether juveniles that recruited to cleared reefs were more likely to have local or distant sources. Adult lionfish also quickly recruited to cleared reefs, which means they had to swim long (>300 m) distances over open substrate to study reefs that were isolated from any natural reef habitat (>5 km) and located between 300 m and 1 km from adjacent artificial reefs. This inference contrasts with reports of limited adult or post-settlement movement in estuarine (Jud & Layman 2012), southeast Atlantic natural hardbottom (Bacheler et al. 2015), and Caribbean patch and continuous coral reef ecosystems (Akins et al. 2014), where site fidelity of lionfish has been reported to be high, and may explain the higher degree of success of targeted removal efforts in such areas (Frazer et al. 2012). Our findings support recent work that indicates that lionfish display lower site fidelity under high-density conditions (Tamburello & Côté 2015). Lionfish densities observed on control reefs throughout our study represent the highest values reported across their invaded range; thus, intraspecific competition for prey resources may be prompting movement on greater scales than has been reported previously (Tamburello & Côté 2015). Consistent with that hypothesis is the fact that non-reef benthic fishes (e.g. lizardfishes, flounders, sea robins) and invertebrates constituted significantly greater proportions of lionfish diet at nGOM artificial reefs versus lionfish recovered from natural reefs (Dahl & Patterson 2014). Therefore, lionfish associated with artificial reefs are clearly spending time away from reefs foraging on non-reef associated prey. The extent of these movements and the area over which lionfish are utilizing prey resources is currently unknown, but conventional or acoustic tagging approaches could be employed to examine those questions.

The rapid recolonization rate of juvenile lionfish settling from the plankton and/or adults immigrating from nearby habitat onto cleared reefs resulted in lionfish densities that were rarely below thresholds proposed by others to mitigate ecological impacts to native fishes despite substantial removal effort (Green et al. 2014, Benkwitt 2015). This may be why our lionfish removals did not translate into significant gains for most fish taxa, though previous studies were mostly focused on small fishes likely to be consumed by lionfish. For larger species included in our analyses, the effects of lionfish were not apparent but may have been undetectable on the timescale studied, especially if impacts are indirect, competitive trophic interactions resulting in reduced growth or reproduction (Albins 2015). Additionally, this study differs from other removal experiments in that small demersal reef fishes (e.g. damselfishes, cardinalfishes, wrasses, blennies), which constitute high proportions of lionfish diet in systems or habitats where they are abundant, were already nearly absent from study reefs at the start of the experiment. While these species did increase somewhat following lionfish removals in this study, their densities remained less than 25% of the values observed in 2009–2010, or those reported by Dance et al. (2011) for even earlier time periods. Benkwitt (2015) reported that even single lionfish were able to negate substantial gains in lionfish prey species on small (1 m^3) patch reefs in The Bahamas, and Green et al. (2014) reported that approximately 90% lionfish removal was required to foster ecological resiliency for native prey fish communities on larger (100–150 m^2) Bahamian patch reefs. No such estimate yet exists for the nGOM region of a threshold lionfish density necessary to mitigate lionfish effects and foster ecosystem resiliency, but recolonization rates of lionfish following experimental removals at study reefs could be used hereafter to predict the level of harvesting effort that would be required to keep lionfish densities suppressed below some threshold. Indeed, our results predict that to maintain lionfish densities <5 fish 100 m^{-2}, all lionfish must be harvested from reefs approximately every 2 mo, about twice the frequency performed in this study.

The extraordinary and continued success of invasive lionfish in the nGOM may be attributable to mechanisms of decreased biotic resistance or resilience. Disturbed ecosystems, regardless of causation, have been shown to be more vulnerable to invasion (Stachowicz et al. 2002). Indeed, recent trophic dynamic ecosystem simulations computed with an Ecopath with Ecosim model of the west Florida Shelf ecosystem indicate that depleted biomass of top predators (e.g. groupers, snappers) can influence the relative invasion success of lionfish (Chagaris et al. 2015). Evidence of native western Atlantic species preying on lionfish is rare; thus, top predators in the Chagaris et al. (2015) model were assumed not to prey upon lionfish. The model also assumed no lionfish cannibalism; thus, no direct lionfish control was present in the model. Despite this, their results suggest that lionfish invasion success can be influenced through competitive trophic interactions. Historic overexploitation (i.e. overfishing) of top predators in the nGOM region, coupled with declines following the DWH, may have compromised ecosystem resistance to the initial invasion success of lionfish and contributed thereafter to their exponential increases in abundance and biomass.

Localized lionfish removal efforts in this study did not result in substantial gains in native reef fish abundance, but sustained removal efforts were somewhat effective at limiting lionfish densities to relatively low levels on nGOM artificial reefs. Unfortunately, regionally high lionfish densities may require more frequent removal efforts than we attempted, or on much larger spatial scales, to effect meaningful reductions in lionfish density and biomass. If expansive lionfish culling efforts could be accomplished on the shallow (<40 m depth) shelf, lionfish populations associated with mesophotic reefs on the outer shelf and upper continental slope (i.e. below traditional recreational diving limits, 40 m), or other areas that receive little to no control efforts, might still serve as constant sources of new lionfish recruits. Efforts to reduce lionfish biomass at those depths will be logistically challenging and expensive. Therefore, to see beneficial effects on local reef fish communities, lionfish removals going forward will require an effort high enough to offset recolonization from difficult to reach source populations. Ongoing ecosystem modeling efforts that are aimed at evaluating the ecological impacts of lionfish in the nGOM should be coupled with economic models to estimate the expense and feasibility of lionfish removal or harvesting efforts that will be required to accomplish the goal of minimizing lionfish impacts in the northern Gulf of Mexico.

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Contribution to the Theme Section 'Invasion of Atlantic coastal ecosystems by Pacific lionfish'

Invasive lionfish *Pterois volitans* reduce the density but not the genetic diversity of a native reef fish

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ABSTRACT: The Indo-Pacific red lionfish *Pterois volitans* has spread throughout the western Atlantic causing declines in biomass and diversity of native species at local reefs; worst-case scenarios predict species extinctions and ecosystem phase shifts. While reductions in reef fish population density and recruitment are evident, it is not known whether lionfish are reducing genetic diversity of native species, a major driver of extinction in natural populations. A before-after control-impact experiment was used to determine whether lionfish removals cause an increase in density of native species and genetic diversity in one species, the bicolor damselfish *Stegastes partitus*. We found that removing lionfish significantly augmented the density of several reef fish species. However, while allelic frequencies in bicolor damselfish recruits changed after removals, genetic diversity did not increase substantially despite a 3-fold increase in recruit density. Responses to lionfish removal differed among native species; rare species with small population sizes may be more susceptible to recruitment failure and diversity loss as a result of lionfish predation than widespread species with large populations.

KEY WORDS: Lionfish · Invasive predator effects · Genetic diversity · Native prey · Targeted removals

INTRODUCTION

Biological invasions are among the greatest threats to biodiversity, costing billions of dollars annually in damages to infrastructure and decimating local populations (Pimentel et al. 2005). Invasive predators reduce local prey through direct interactions but also affect non-prey species through competition for prey or trophic cascades (Johnson et al. 2009). Changes to community structure and trophic organization associated with invasions are of major concern for the structure and function of native communities and ecosystems and the services they provide. Negative effects of invasive species on native ecosystems have been described; however, the potential effect of invasive species on native genetic diversity has received less attention (Parker et al. 1999, Vilà et al. 2011).

Loss of genetic diversity in populations is troubling, because it hinders the ability for populations to respond to environmental changes and stressors (Booy et al. 2000). In a meta-analysis, Spielman et al. (2004) reported that out of 170 pairs of taxa, 77% of endangered or threatened species showed an average of 35% lower genetic diversity (i.e. heterozygosity) than comparable non-endangered or threatened species in the same taxonomic group. Frankham (1995) named biological invasions along with habi-

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tat loss as a primary factor that causes extinction for genetic reasons; biological invasions can lead to increased inbreeding depression, accumulation of deleterious mutations, and loss of diversity in native populations due to genetic drift.

Invasive predators can have 2 main effects on genetic populations, viz. selection and genetic drift. Selection occurs when predators remove prey non-randomly from a population, where prey with a certain trait are more or less likely to be removed. Selection is an ongoing process and begins as soon as prey removal begins. The strength of selection and size of the prey population will determine the rate at which observable changes in gene frequencies and diversity appear in the prey population. Even rapid evolutionary changes are expected to take years to decades to manifest themselves in the gene frequencies of the populations (Thompson 1998). Additionally, selection is expected to act only to change gene frequencies of the specific genes under selection, leaving unaffected or unlinked genes unaltered. Drift, on the other hand, is the random removal of genotypes from the population (Hartl & Clark 2007). Drift is also an ongoing process in every population and at low levels; in large genetic populations, it will not significantly change genetic diversity of a population. However, in situations where predation is severe and the predator removes a large proportion of the population creating a genetic bottleneck, predation can act as a strong drift effect and cause genetic diversity reductions. In the case of drift, changes in gene frequencies are expected to happen across the genome (Hartl & Clark 2007). When such predation bottlenecks occur on a recruitment cohort (e.g. Doherty et al. 2004), the effect on gene frequencies and genetic diversity can occur over short periods of time (days to months) as the cohort of recruits passes through the predation gauntlet (Larson & Julian 1999).

To our knowledge, only 2 studies have investigated the effect of invasive predators on genetic diversity or patterns of genetic differentiation of native prey species (Gasc et al. 2010, Iwai & Shoda-Kagaya 2012). Iwai & Shoda-Kagaya (2012) concluded that predation by invasive mongoose has driven genetic differentiation among populations of the Japanese Otton frog *Babina subaspera*. Gasc et al. (2010) found a decrease in genetic diversity (i.e. heterozygosity and allelic richness) of brown anole lizards *Anolis sagreii* in the Bahamas after the invasion of a rat predator. The impact of invasive predators on genetic diversity of native species may be a more widespread phenomenon than is currently appreciated; more studies are needed to assess this.

The invasive lionfish *Pterois volitans* was first observed in the western Atlantic in southern Florida in the mid-1980s and has since spread as far north as New York (USA) and as far south as Brazil (Ferreira et al. 2015). In some places, such as the Bahamas, the lionfish is among the more abundant fish species (Morris & Whitfield 2009), reaching densities of 450 ind. ha^{-1}, more than an order of magnitude higher than in its native range (Kulbicki et al. 2012). The lionfish exhibits various traits that contribute to its success: it grows and matures quickly (Morris & Whitfield 2009), and it is a voracious, generalist predator that consumes over 70 species of small reef fish as well as shrimps and other invertebrates (Morris & Akins 2009).

Some prey species in the invaded range appear to be highly susceptible to lionfish predation. For example, Kindinger (2015) found that the native Caribbean damselfish *Stegastes planifrons* fails to recognize the lionfish as a predator and does not respond to lionfish with evasive behavior. In studies of small patch reef sites, lionfish have been shown to reduce the abundance of newly recruited reef fishes by up to 90% (Albins & Hixon 2008) and reduce prey species richness up to 2.4 times more than a native predator (Albins 2013). Lionfish have been predicted, in the worst-case scenarios, to cause reef fish extinctions (Albins & Hixon 2013). This novel predator's voracious appetite, coupled with the naïveté of native prey species, could effectively reduce prey population sizes sufficiently to lower population genetic diversity as other invasive predators have been shown to do (Gasc et al. 2010), exacerbating extinction risk for these species. However, whether lionfish are affecting the genetic diversity of native species is currently unknown.

Here, we experimentally determined the effect of lionfish on the densities of several reef fishes and on the genetic diversity of a common Caribbean native, the bicolor damselfish *S. partitus*. We hypothesized that if lionfish are sharply reducing the abundance of recruits of this (and other) species, then they may create a genetic bottleneck within recruitment cohorts and, therefore, reduce the genetic diversity of those cohorts as they pass through the predation gauntlet. We use a before-after control-impact experiment to determine whether the removal of lionfish can result in increased density and diversity of the bicolor damselfish in Panama. We also compared genetic diversity of bicolor damselfish populations pre- and post-lionfish invasion in Panama.

MATERIALS AND METHODS

Study location

We implemented a before-after control-impact design to study the effects of lionfish removal on the density and genetic diversity of native reef fishes (Fig. 1). We established 1 control (C) and 1 treatment site (T1) in October 2013, and a second treatment site (T2) in May 2014 for additional replication of the study of genetic diversity only. All sites contained lionfish at the time of first survey. Lionfish were removed from the treatment sites during the course of the experiment; the densities of lionfish were not manipulated at the control site. We established these sites at Tiger Rock, located northeast of the Bocas del Toro province of the Republic of Panama (Fig. 1A). The treatment and control sites were chosen based on their close proximity (~1–2 km) to one another and their similar biological structure (see below for description) to standardize naturally occurring differences in currents, recruitment, and substrate class over the archipelago. The habitat is a chain of discrete calcium carbonate reef formations. The 2 western-most sites (C: 9° 13′ 15.94″ N, 81° 56′ 46.95″ W; and T1: 9° 13′ 06.47″ N, 81° 56′ 27.09″ W) are ~100 m long on their seaward sides where surveys were conducted, with a circumference of ~500 m. The reefs extend to near 35 m deep, and each island is separated by ~1 km of sand habitat from the nearest reef. The third site (T2: 9° 12′ 47.77″ N, 81° 55′ 34.44″ W) is ~300 m long on its seaward side and 1 km in circumference, 30 m deep, and 1 km away from the next adjacent reef. All sites experienced a strong northward-flowing current heading out to sea. The benthic reef community is sponge-dominated and home to many reef fish, crinoids, sharks, and, since 2009, invasive lionfish (Schofield 2010).

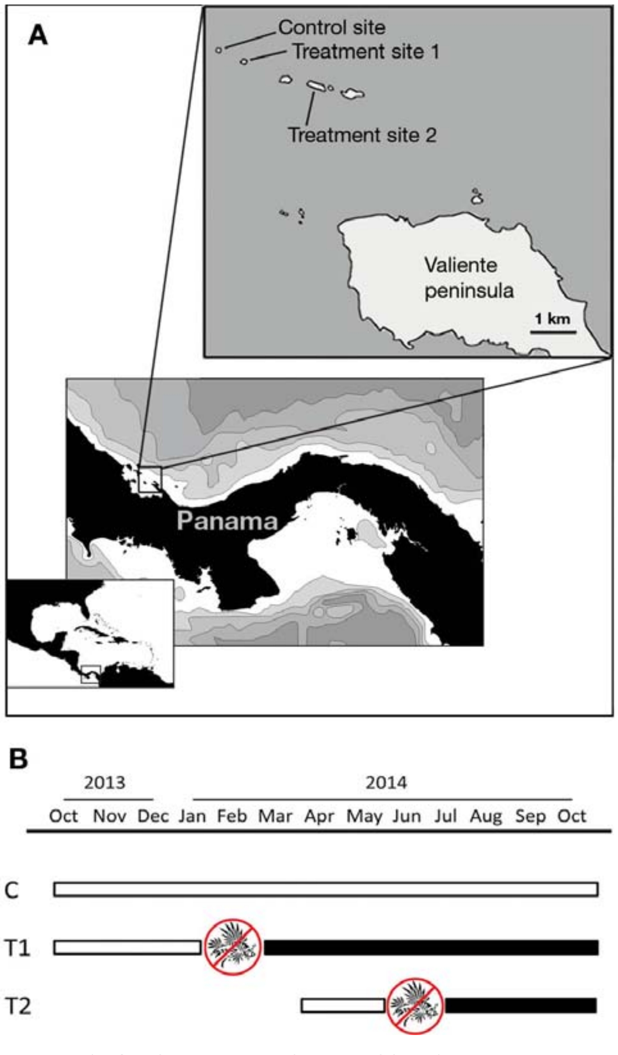

Fig. 1. (A) Study region in the Republic of Panama. Bottom left inset shows the northwestern Atlantic; upper inset shows the study sites at Tiger Rock in relation to the Valiente Peninsula. (B) Timeline for each of 3 sites: Control (C) and Treatments 1 and 2 (T1, T2). White bars: time period during which lionfish densities were unmanipulated; black bars: post-lionfish removal periods

Lionfish surveys and removals

To gauge the efficacy of lionfish removals, we surveyed lionfish densities approximately monthly (weather permitting) at each site with a lionfish-focused survey approach using 4 transects (each 3 m × 30 m) at each site. Divers would swim in a sinusoidal pattern along the transect, roving ~1.5 m on either side of the transect tape. Transects were conducted at depths of 10, 12, 15, and 18 m, parallel to the reef crest. We collected baseline ('before') data at sites C and T1 for 3 mo prior to the experimental removal of lionfish starting in October 2013. At T2, we surveyed baseline lionfish density in May and June 2014. The lionfish removal began in February 2014 at T1 and in June 2014 at T2. Surveys continued until October 2014 (Fig. 1B).

At T1 and T2, lionfish removals occurred in 1 bout with continued monitoring and 'clean-up' of any lionfish found after the major removal effort in February 2014 and June 2014, respectively (Fig. 1B). Divers on SCUBA harvested lionfish using pole spears until none were visible. Lionfish were removed first from the transect area, starting at the deepest part of the wall and proceeding shallower on the seaward (north-facing) sides. To prevent migration of lionfish into the transect area, we removed lionfish from buffer areas adjacent to the study site located to the western, eastern, and southern sides of T1 (500 m × 35 m buffer area) and T2 (1 km × 30 m buffer area). After the initial removals, any lionfish observed in the treatment sites were removed to maintain low densities throughout the course of the experiment.

Reef fish surveys

To assess whether lionfish removals caused an increase in the density of 2 size classes of reef fishes (recruits and adults), we surveyed densities at all sites before and after lionfish removals on an approximately monthly basis (weather permitting). New recruits of each species were defined by size class, ≤2.5 cm total length (TL). We chose this size class because individuals of this size are typically less than 1 mo old (Hogan 2007). Counting ~1 mo old recruits ensures independence of our recruit densities between monthly surveys. 'Adults' were defined as all individuals >2.5 cm, which also includes non-reproductive, juvenile individuals, but for brevity, we call this size class 'adults.' Sizes were estimated by surveyors on SCUBA to the nearest 0.5 cm size class. Surveyors calibrated size estimations prior to the beginning of the experiment by estimating fish sizes underwater and then catching the same fish and measuring them. Recruit surveys were conducted at each site along 1 m × 30 m transects (n = 4) per site; 1 transect was surveyed parallel to the reef crest at each of 4 depths (10, 12, 15, and 18 m) within each site. Adult densities were estimated using 3 m × 30 m transects to account for the larger territory of adult fishes. A PVC T-bar was implemented to ensure that transect widths were consistent. As with the lionfish density surveys (see above), we collected baseline data on the densities of native species at C and T1 for 3 mo starting in October 2013 prior to lionfish removal in February 2014; at T2, baseline surveys were conducted in May and June 2014 prior to lionfish removal in late June 2014. Surveys continued until October 2014 (Fig. 1B).

The densities of 3 different species were recorded: bicolor damselfish *Stegastes partitus*, yellowhead wrasse *Halichoeres garnoti*, and bluehead wrasse *Thalassoma bifasciatum*. We chose these species for their ease of identification, their common occurrence in the reef community, and their known susceptibility to lionfish predation (Morris & Akins 2009).

We used a model selection approach based on the deviance information criterion (DIC) (Spiegelhalter et al. 2002) to determine what, if any, response native species had to the removal of lionfish. We fit 6 models to the data for each species and size-class:

$$D_{j,k} = \alpha + \varepsilon_{j,k} \tag{1}$$

$$D_{j,k} = \alpha_k + \varepsilon_{j,k} \tag{2}$$

$$D_{j,k} = \alpha + \beta I_j + \varepsilon_{j,k} \tag{3}$$

$$D_{j,k} = \alpha_k + \beta_k I_j + \varepsilon_{j,k} \tag{4}$$

$$D_{j,k} = \alpha + \beta I_j t_j + \varepsilon_{j,k} \tag{5}$$

$$D_{j,k} = \alpha_k + \beta_k I_j t_j + \varepsilon_{j,k} \tag{6}$$

where $j = 1,2,\ldots,11$ indexes the measurement dates; $k = 1,2,3,4$ indexes the measurement depths; $D_{j,k}$ is the difference in native fish densities between the treatment and control site for measurement j at depth k; t_j is the difference in days between the date of observation j and the date of removal of lionfish; I_j is an indicator variable for the removal of lionfish from the treatment transects for measurement j; $I_j = 0$ before first removal, 1 afterwards; $\varepsilon_{j,k}$ represents a random error term, described below; and α and β are also described below. Modeling the difference in densities between the treatment and control transects should remove the influence of any annual/seasonal trend in the populations under study.

Model (1) is the null model: the removal of lionfish from the treatment transect had no effect on the difference in density of native species at any depth or time: α, the difference in density due to treatment, remains the same throughout the experiment. Model (3) suggests a step effect: removal of lionfish from the treatment transect resulted in a single constant change in the difference in densities of native species. Here, α becomes the difference in density before the intervention, while β represents the constant change in density after the removal. Model (5) suggests that the difference in densities of native species changed linearly starting with the date of removal of lionfish. α remains the difference in density before the intervention, while β now represents the rate of change in density after the removal. Finally, Models (2), (4), and (6) are similar to models (1), (3), and (5), respectively, except that the size of any effect was allowed to differ by depth k. Thus, the single α and β from Models (1), (3), and (5) are replaced by different α_k and β_k for each depth k. Our primary parameters of interest are the β values, since they estimate the change in density differences that occurs due to lionfish removal.

To account for potential lack of statistical independence by time and/or depth, we estimated for each model a correlation matrix $\mathbf{V}$ for $\varepsilon_{j,k}$ using a separable exponential formula that decomposes the relationship between 2 observations into a product of exponential functions of their distance apart in time and space (Mitchell & Gumpertz 2003):

$$\mathrm{corr}(\varepsilon_{j_1,k_1}, \varepsilon_{j_2,k_2}) = \phi_t^{|t_{j_1} - t_{j_2}|} \times \phi_d^{|d_{k_1} - d_{k_2}|} \qquad (7)$$

where ϕ_t and ϕ_d represent temporal and spatial correlations, respectively. Thus, the error terms $\varepsilon_{j,k}$ were modeled as $\boldsymbol{\varepsilon} \sim N(\mathbf{0},\boldsymbol{\Sigma})$ where $\boldsymbol{\Sigma} = \mathbf{S} \times \mathbf{V} \times \mathbf{S}$, and $\mathbf{S}$ was a diagonal matrix of standard deviations σ_k for the observations. σ_k was assumed to be constant by depth k for each model.

We fit Models (1) to (6) using Markov chain Monte Carlo (MCMC) methods. Using $N(x,y)$ to represent a normal distribution with mean x and variance y, and Unif(x,y) to represent a uniform distribution (Unif) bounded between x and y, here is a list of our parameters and their (uninformative) priors:

- $\alpha \sim N(0,100^2)$ and $\beta \sim N(0,100^2)$; the same priors were used for models where α and β varied individually by depth
- $\sigma_k \sim \mathrm{Unif}(\frac{1}{b},b)$ for each depth k, with $(b - 1) \sim \mathrm{Exp}$ (0.001); by using partial pooling of the standard deviations, we guard against overfitting the data (Gelman et al. 2014)
- $\phi_t \sim \mathrm{Unif}(0,1)$ and $\phi_d \sim \mathrm{Unif}(0,1)$

Convergence of the MCMC chains was monitored using the Gelman-Rubin diagnostic (Gelman & Rubin 1992) and visual inspection of the traceplots of the chains for the parameters α and β. The Raftery-Lewis diagnostic (Raftery & Lewis 1992) was used to monitor precision of quantile estimates. Models were compared using Gelman's estimate of DIC (Spiegelhalter et al. 2002, Gelman et al. 2014), with smaller DIC indicating a better model, and models having ΔDIC < 4 considered to be of similar quality. Where multiple models had similar DIC values, the most parsimonious model was chosen.

Models were fit using the statistical software JAGS version 4.1.0 (Plummer 2003) and R version 3.2.5 (R Core Team 2016), including R packages coda version 0.18-1 (Plummer et al. 2006), rjags version 4.4 (Plummer 2016), and R2jags version 0.5-7 (Su & Yajima 2015).

Genetic diversity

We chose the bicolor damselfish as a model species for this study because of its ubiquity on Caribbean reefs, ease of capture, documented interactions with lionfish (Morris & Akins 2009), and the availability of molecular genetic markers. Additionally, previous genetic data are available for this species from Panama for comparison to our results (Salas et al. 2010). To determine the effect of lionfish predation on the genetic diversity of bicolor damselfish, we opportunistically collected ~50 individuals of the recruit size class (≤2.5 cm) from all sites at depths from 10 to 18 m before and after lionfish removal; there was no systematic difference in the depths of collection among sites. We focused on the recruit size class because lionfish preferentially target smaller individuals (Green & Cote 2014). Also, we independently sampled each recruit cohort by focusing on fish of a certain size, whereas adults are an amalgam of multiple separate genetic cohorts. Pre-removal genetic samples were taken in January 2014 for T1 and C and in June 2014 for T2. Post-removal genetic samples were taken in October 2014 for all sites. Divers collected samples using a clove oil mixture (9:1; 70% isopropanol:pure clove oil) and hand nets. Fish were collected within the bounds of each site (~360 m^2) but not specifically from the transect areas where surveys were conducted. Each fish was euthanized humanely, measured for TL, and a fin clip was taken for DNA analysis and stored in 95% ethanol. Samples were brought back to the lab at Texas A&M University – Corpus Christi for genomic DNA extraction (Qiagen DNeasy Blood and Tissue Kit) and genetic analysis. The timing of the before and after sampling (i.e. months apart) along with the lethal sampling of juveniles ensured that we did not sample the same individual twice and minimized the possibility of resampling from the same recruitment cohort in the before and after sampling, thereby ensuring the independence of the samples for statistical purposes.

We chose 12 microsatellite loci from Williams et al. (2003) and Thiessen & Heath (2007) to estimate genetic variation in this species (see Tables S1 & S2 in the Supplement at www.int-res.com/articles/suppl/m558p223_supp.pdf). We used polymerase chain reaction (PCR) to amplify microsatellite loci using dye-labeled forward primers in 10 µl reactions comprising ca. 100 ng template DNA, 10 µM of dye-label forward primer, 10 µM of unlabeled reverse primer, 200 µM of each dNTP, 0.1 U Flexi GoTaq (Promega) polymerase, 1× PCR buffer, and locus-specific concentrations of $MgCl_2$. PCR conditions were 94°C for 2 min, followed by 29 to 40 cycles of 94°C for 15 s, locus-specific annealing temperatures (see Williams et al. 2003, Thiessen & Heath 2007 for details) for 15 s, 72°C for 30 s, and a final extension of 72°C for 90 s. The sizes of the PCR products were estimated using an ABI 3730xl genetic analyzer. We used GeneMapper v. 4.0 software for genotyping microsatellite fragments (Applied Biosystems). We calculated standard indices of genetic diversity, including observed and expected heterozygosity (H_O and H_E) and allelic richness (A).

To test the hypothesis that lionfish predation was strong enough to cause declines in genetic diversity of bicolor damselfish recruit cohort populations, we first tested for changes within sites in allelic frequencies between pre- and post-removal samples with an exact *G*-test using GENEPOP on the web (version 1.2, dememorization: 1000, number of batches: 100, iterations per batch: 1000; Raymond & Rousset 1995); p-values were adjusted for multiple comparisons using the sequential Bonferroni method. A significant change in allele frequencies may indicate an effect of removal. Secondly, we calculated the change (difference) in genetic diversity indices (H_O, H_E, and A) between before and after lionfish removals at all 3 sites. If lionfish are having an impact on genetic diversity of these recruit cohorts, then we expect to see a positive change in diversity indices at the treatment sites where lionfish predation has been alleviated, but no significant change at the control site. To test if changes in diversity indices were significantly different from 0, we used 1-sample *t*-tests in R (R Core Development Team); p-values were corrected for multiple comparisons using the sequential Bonferroni method. Lastly, to test whether the invasion of the lionfish has led to declines in genetic diversity in Panamanian populations of bicolor damselfish, we compared our measured values of H_O, H_E, and A to the same diversity indices published previously by Salas et al. (2010). They sampled bicolor damselfish before the lionfish invasion in 2009 from fringing and patch reef sites in the same bay system in Bocas del Toro, Panama, named 'Coral Key' and 'Bocas' and spaced ~20 and 45 km, respectively, from our sites at Tiger Rock. They used 9 of the same microsatellite markers that we used in our study, and we compared the same diversity indices with a 1-sample *t*-test in R. We pooled our control and treatment site samples together for this comparison. If lionfish predation has influenced bicolor damselfish genetic diversity since the invasion, our samples should be lower in diversity than those of Salas et al. (2010). We used 1-sample *t*-tests implemented in R to test the hypothesis that genetic diversity has declined in Panamanian populations since the invasion of the lionfish in 2009; p-values were corrected for multiple comparisons using the sequential Bonferroni method.

RESULTS

Lionfish density

The average densities of lionfish prior to removal were 0.02, 0.03, and 0.04 fish m^{-2} at T1, T2, and C, respectively. These densities are comparable to natural reefs in the Bahamas (0.04 m^{-2}; Green & Cote 2009) but lower than artificial reefs in the Gulf of Mexico (0.14 m^{-2}; Dahl & Patterson 2014). After the removal, lionfish density at both treatment sites was reduced by an order of magnitude (Fig. 2; see Table S3 in the Supplement for all fish densities). Control site density decreased by 27%. Post-removal, lionfish density at this site was an order of magnitude greater than at T1 and T2 (Fig. 2). After initial lionfish removal efforts, no lionfish were seen at T1 for 6 mo or at T2 for remainder of the experiment (3 mo; Fig. 2).

Effects of lionfish on the density of native reef fishes

After the removal of lionfish at T1, the average recruit density of bicolor damselfish more than doubled (130% increase, Fig. 3A; Table S3). The control site, by comparison, experienced a 14% increase in density over the same period (Fig. 3A). The post-removal density of recruits at T1 was more than double (122% more) the density at C (Fig. 3A). The model that exhibited the best combination of fit and parsimony was Model (5), which indicates a linear increase in the difference in densities between the control and treatment site after removal (Fig. 3A; Table S4). A 95% credible interval for the slope of the increase is (0.06, 0.30) recruits m^{-2} mo^{-1}.

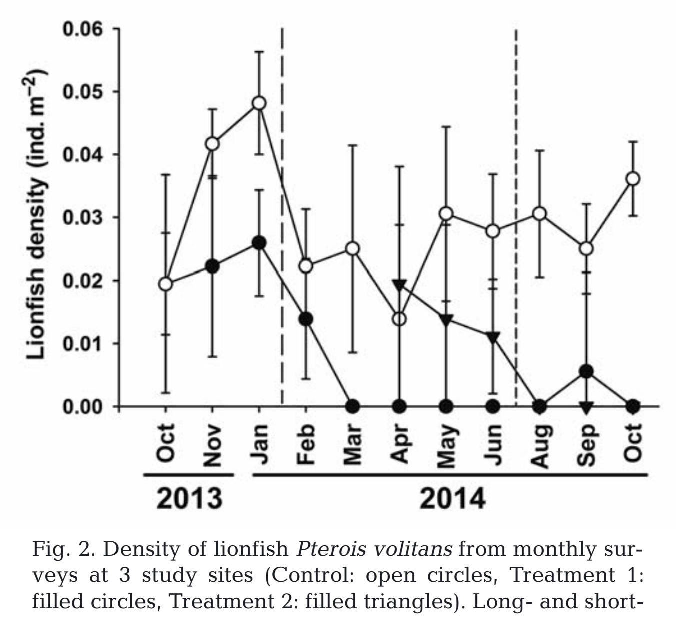

Fig. 2. Density of lionfish *Pterois volitans* from monthly surveys at 3 study sites (Control: open circles, Treatment 1: filled circles, Treatment 2: filled triangles). Long- and short-dashed vertical lines indicate the timing of lionfish removal in Treatments 1 and 2, respectively. Error bars indicate SD. Sites could not be surveyed in December 2013 and July 2014 due to weather; these dates are omitted for simplicity

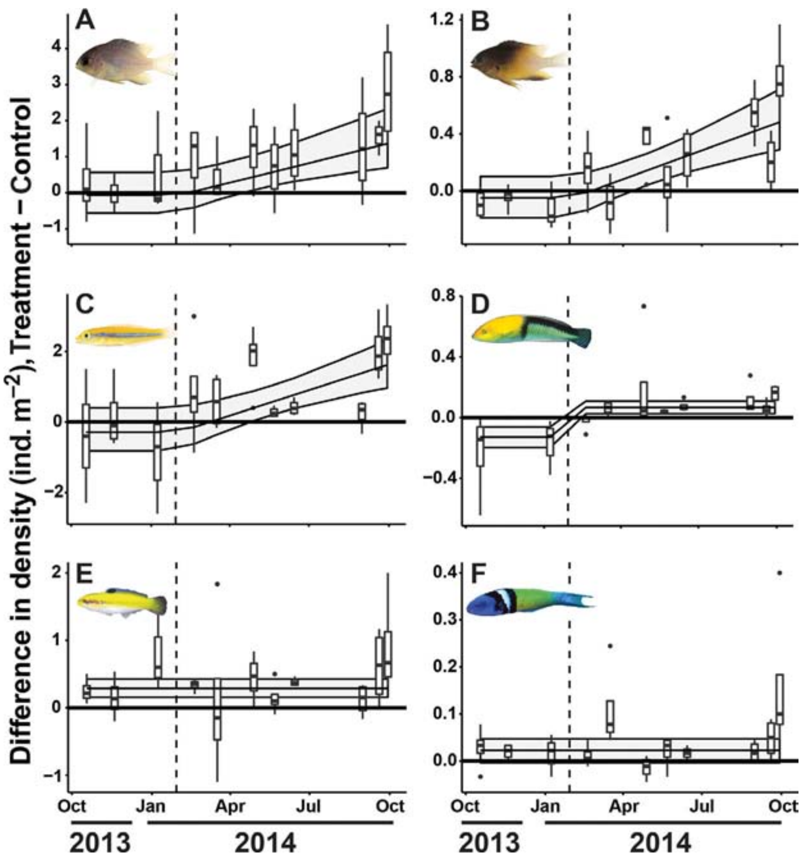

Fig. 3. Difference in density of recruits and adults of 3 reef fish species between 2 sites (Treatment 1 and Control) from monthly surveys. (A,B) *Stegastes partitus*, (C,D) *Halichoeres garnoti*, (E,F) *Thalassoma bifasciatum*; recruits and adults, respectively. Vertical dashed lines indicate the timing of lionfish removal from the treatment site. Note the differences in the scales on the *y*-axes. Box-plots were constructed using the standard quantile-based definition (e.g. Quinn & Keough 2002, p. 60). Shaded regions around trend lines are 95% credible intervals for the mean

Bicolor damselfish adult density increased by 520% at T1 after lionfish removal (Fig. 3B). In comparison, density of adults increased by 65% at C (Fig. 3B). The post-removal density of bicolor adults at T1 was 97% greater than the density at C over the same period (Fig. 3B). The model chosen for bicolor damselfish adults was again Model (5), a linear increase in the difference in densities between the control and treatment site after removal. The difference in density between T1 and C increased rapidly after lionfish removal (Fig. 3B). A 95% credible interval for the slope of the increase is (0.03, 0.10) adults m^{-2} mo^{-1}.

In contrast, mean recruit density of yellowhead wrasse remained stable (~2% increase) between pre- and post-removal of lionfish at the treatment site (Fig. 3C). However, the density of recruits dropped markedly (74%) at Site C after the removal period (Fig. 3C). As with bicolor damselfish, the post-removal density of yellowhead wrasse recruits at T1 was nearly double their density at C (188% greater; Fig. 3C). The model that exhibited the best combination of fit and parsimony was Model (5), which indicates a linear increase in the difference in densities between the control and treatment site after removal (Fig. 3C, Table S4). A 95% credible interval for the slope of the increase is (0.12, 0.36) recruits m^{-2} mo^{-1}.

Yellowhead wrasse adult density increased by 150% at T1 following lionfish removal. In contrast, density decreased by nearly an order of magnitude (850%) at C (Fig. 3D). The density of adults was 400% greater at T1 compared to C after lionfish removal (Fig. 3D). The model chosen for yellowhead wrasse adults was Model (3), a single constant increase in the difference in densities between the control and treatment site after removal (Fig. 3D, Table S4). A 95% credible interval for the increase is (0.12, 0.28) adults m^{-2} mo^{-1}.

After the removal of lionfish at T1, the average recruit density of bluehead wrasse increased slightly by 34% (Fig. 3E). However, the density of recruits also increased 88% at the control site after the removal period (Fig. 3E). As a result, the difference in recruit density between the treatment and control sites was nearly constant before and after removal of lionfish, and the preferred model was the null model (Model 1) (Fig. 3E). Bluehead wrasse adult density was quite low across all sites and times but did increase by 75% after lionfish removal at T1; densities did not change at C (Fig. 3F). Again, the preferred model was the null model (Model 1).

Effects of lionfish on the genetic diversity of *Stegastes partitus*

We found little evidence of systematic changes in allele frequencies in response to lionfish removals. We found small, significant changes in allele frequencies in some loci in samples from C and T2, but not T1 after the removal of lionfish based on exact

G-tests (Table S2). Only 6 of 36 site-by-locus comparisons indicated a significant change in allele frequency after lionfish removal; this was reduced to 3 significant comparisons after correction for multiple comparisons.

Additionally, there was no clear increase in diversity at the treatment sites compared to the control site after lionfish removals (Fig. 4, and see Tables S1, S5, S6 & Fig. S1 in the Supplement). The multi-locus average H_O showed small changes from pre- to post-removal, with all sites showing a mean increase in diversity ranging from 1 to 5% change in the frequency of observed heterozygotes (Fig. 4A). However, none of the changes was significant, and the responses of individual loci varied (i.e. both negative and positive changes were seen; see Fig. S1). Mean H_E increased very slightly (<1%) at C and T2 after removals, but declined (<1%) at T1 after removals; the effect was only significant for the control site at $\alpha = 0.05$; however, after correction for multiple comparisons, this was no longer significant (Fig. 4; H_E: *t*-test: df = 10, $t = 2.47$, $p = 0.033$; see Table S5 for all tests). As with H_O, individual loci varied in their response to removals exhibiting increases and decreases in H_E (Fig. S1). Lastly, allelic richness increased after lionfish removals from C and T2, but decreased after removals from T1. The effect was only significant at $\alpha = 0.05$ for the control site, and the effect remained significant after Bonferroni correction (df = 10, $t = 3.43$, $p = 0.007$). As observed with H_E and H_O, individual loci varied in their response to removals, exhibiting increases or decreases in allelic richness depending on the locus (Fig. S1).

We found that all 3 multi-locus diversity indices were on average very slightly higher in this study compared to previous samples taken by Salas et al. (2010) before the lionfish invasion in 2009 (Fig. 5 and see Fig. S2); none of these comparisons was significant at $\alpha = 0.05$ (Table S6). Again, responses of individual loci varied for each diversity index, with some loci showing increases and some showing decreases in diversity compared to the samples from Salas et al. (2010).

DISCUSSION

Predators can have 2 kinds of effects on their prey species populations, affecting their genetic composition through selection and genetic 'drift' effects. If predation is severe and a large portion of the prey population is removed, changes in gene frequencies and a reduction in genetic diversity can be observed across the entire genome (drift) caused by a genetic bottleneck. In this study, we looked for evidence of a genetic bottleneck in bicolor damselfish as a result of lionfish predation; however, we did not find one despite a large suppression effect of lionfish predation on the population.

In order for a genetic bottleneck to occur, lionfish must reduce the size of the populations substantially (Peery et al. 2012). Recruit and adult densities of bicolor damselfish rose linearly and significantly after lionfish removal at our treatment sites, indicating that lionfish were having a significant effect on their population demographics. The lionfish effectively reduced recruit populations by 55% and adult populations by 84%. If lionfish have an effect on genetic diversity, we would expect to see increases in diversity indices or changes in allelic frequencies between

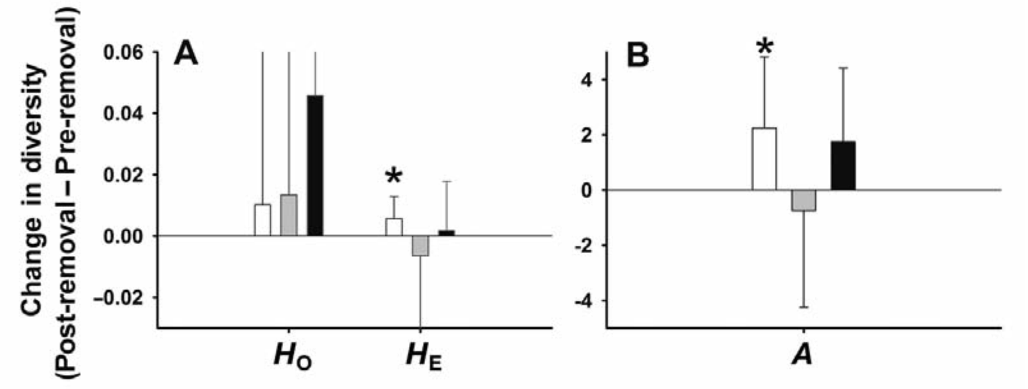

Fig. 4. (A) Changes in genetic diversity of *Stegastes partitus* pre- to post-removal of lionfish *Pterois volitans* across all sites. (A) Differences in mean observed heterozygosity (H_O) and expected heterozygosity (H_E); (B) differences in mean allelic richness (A) for 12 microsatellite loci between pre- and post-removal samples. Control: white bars; Treatment 1: grey bars; Treatment 2: black bars. Error bars are multi-locus SD. Asterisks (*) indicate significant changes within sites between the pre- and post-removal periods

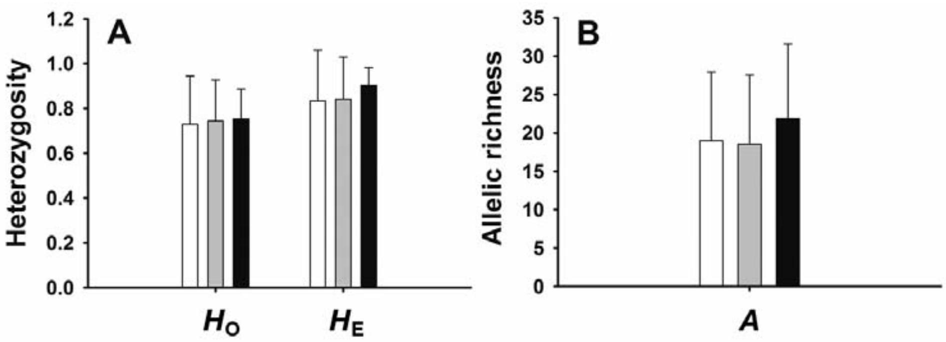

Fig. 5. Genetic diversity indices of Panamanian samples of *Stegastes partitus* pre- and post-invasion of lionfish *Pterois volitans*. (A) Mean observed heterozygosity (H_O) and expected heterozygosity (H_E); (B) mean allelic richness (A), for 12 microsatellite loci. Pre-invasion samples from Salas et al. (2010): Coral Key (white bars); Bocas Island (grey bars). Post-invasion samples from this study: mean for all Tiger Rock samples (black bars). Error bars are multi-locus SD

pre- and post- lionfish removal within treatment sites. While the allelic frequencies changed significantly in the control site, they did not change significantly in either treatment site. Similarly, genetic diversity increased slightly at the control site after lionfish removal but not at the treatment sites as expected.

Several factors may explain why bicolor damselfish populations have not experienced a significant decline in genetic diversity despite obvious predation effects of lionfish. The bicolor damselfish is a highly fecund species that reaches maturity quickly, which can lead to rapid population replenishment (Wilson & Meekan 2002). High fecundity and short generation times can result in sufficiently large genetic population sizes that a 55% reduction in density is not extreme enough to leave a detectable genetic signal. Peery et al. (2012) reported that many studies fail to detect bottlenecks in populations known to have experienced significant population declines. They stated that populations must be reduced by 2 to 3 orders of magnitude before a bottleneck can be detected through heterozygosity and allelic richness measures. The magnitude of reductions in population size observed in this study is not this extreme; therefore, we conclude that the effect of lionfish predation was not sufficient to cause a genetic bottleneck in these recruit cohorts.

It is possible that lionfish populations that have been established in this region since 2009 (Schofield 2010) have already caused a reduction in genetic diversity of bicolor damselfish in all possible recruit source populations—given their widespread nature—thereby limiting the possibility of genetic diversity recovering after lionfish removal. However, the levels of genetic diversity found in our samples were slightly higher, albeit not significantly, than estimates of genetic diversity of Panamanian populations of bicolor damselfish taken in the Bocas del Toro region prior to the lionfish invasion (Salas et al. 2010). This suggests that there has not been a widespread decline in genetic diversity of this species associated with the lionfish invasion in this region. In contrast to our findings, previous studies have demonstrated that reductions in genetic diversity of native prey species can be caused by invasive predators (Gasc et al. 2010, Iwai & Shoda-Kagaya 2012). However, these studies were conducted on terrestrial species on islands, where dispersal is more limited and population sizes are smaller than in the marine environment (Kinlan & Gaines 2003). Bicolor damselfish, like many marine fishes, have a high dispersal capability. With a pelagic larval stage duration of 27 to 31 d (Wellington & Victor 1989), the larvae of this species disperse on average 77 km, and up to 180+ km, from the natal reef (Hogan et al. 2012). Due in part to this tremendous dispersal capacity, the genetic effective population size of this species must be very large, with genetic populations spanning much of the Caribbean (Purcell et al. 2009). Connectivity among populations of this species is likely strongest within the same ecoregion (southwestern Caribbean; Schill et al. 2015) from locations 10s to 100s of km away (Hogan et al. 2010, 2012, Salas et al. 2010). Therefore, the genetic diversity in Panama (and elsewhere in the Caribbean) can be readily replenished from dispersal from sometimes distant source populations. Thus, marine populations of this size might be well buffered from reductions in genetic diversity caused by the introduction of invasive species.

Many species in the Caribbean have large effective population sizes and have widespread genetic connectivity, similar to the bicolor damselfish, which may make them particularly immune to genetic reductions from lionfish predation. Yellowhead and bluehead wrasse, studied here, both show high levels of genetic connectivity and diversity across large areas (Rocha 2004, Purcell et al. 2006). However, many species show population subdivision within the Caribbean (Shulman & Bermingham 1995, Purcell et al. 2006), and some have small populations and may be endemic to a particular region or reef system (Taylor & Hellberg 2003). Life-history traits that affect dispersal and recruitment may also have an effect on recovery from predation and can affect genetic diversity loss. Bicolor damselfish and yellowhead wrasse have shorter pelagic larval durations (28 and 26 d, respectively; Victor 1986, Wellington & Victor 1989) than bluehead wrasse (49 d; Victor 1986), perhaps contributing to their more rapid recolonization responses. Furthermore, recruitment patterns can also differ; some species experience regular bouts of recruitment, while others experience more of a boom-and-bust recruitment. For example, Victor (1982) showed that most summertime recruitment of bluehead wrasse in San Blas, Panama, occurred over a 2 wk period in late June/early July, with very low levels of recruitment outside of that window. The recruitment of bluehead wrasse in our study appeared to be episodic with low levels of recruitment year round except for large increases in recruitment in March and again in October 2014 and at both treatment and control sites. Species with episodic or low levels of recruitment, those that show lower standing diversity, have short dispersal distances, have restricted ranges, and have strong population structure within the Caribbean may be more susceptible to genetic diversity loss as a result of lionfish predation.

Additionally, species clearly differ in their response to lionfish predation, which may make them more or less susceptible to reductions in genetic diversity. Here we observed variable responses to lionfish predation among the 3 native species that we monitored. Juvenile and adult densities of bicolor damselfish and yellowhead wrasse increased when lionfish predation was alleviated; however, bluehead wrasse densities were not affected by removals. Species characteristics may play a large role in susceptibility to lionfish predation and subsequent diversity loss. Small, shallow-bodied, solitary fishes found resting on or just above reefs appear to be most susceptible to lionfish predation (Green & Cote 2014). Our 3 species here all have traits making them susceptible to lionfish: they are all small and shallow-bodied (at least in the juvenile form), and they are all closely associated with the reef. Bicolor damselfish are solitary and territorial, while bluehead wrasse tend to be solitary or aggregate in small shoals, and yellowhead wrasse tend to aggregate in small shoals of conspecifics (J. D. Hogan pers. obs.).

In conclusion, predation by the invasive lionfish was not strong enough to cause a genetic bottleneck in populations of the prey species investigated here. The size of these populations and the scale of genetic connectivity in the bicolor damselfish may have buffered against widespread losses in genetic diversity perpetrated by the lionfish. Species that demonstrate a susceptibility to invasive predators and those that have small, geographically restricted genetic populations may be at greater risks of reductions in genetic diversity from predation. However, this is not to say that this invasive predator has not left a genetic mark on these populations. Lionfish predation had a significant effect on population size, and lionfish could impose a selective effect on the genetic structure of these prey populations. Future studies should look for evidence of selection effects of invasive predators on prey species. These effects could be observed at lower rates of predation than drift effects. Next-generation sequencing technologies could be used to discover single nucleotide polymorphisms in genes under selection by predators.

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Contribution to the Theme Section 'Invasion of Atlantic coastal ecosystems by Pacific lionfish'

Lionfish cause increased mortality rates and drive local extirpation of native prey

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ABSTRACT: As predators play a central role in prey population regulation, predicting the impact of a novel predator requires determining how the invader affects the compensatory dynamics that underlie native prey persistence. The Indo-Pacific lionfish *Pterois volitans* is an invasive mesopredator that voraciously consumes native coral-reef fishes of the tropical western Atlantic and Caribbean. The fairy basslet *Gramma loreto* is a common prey of lionfish, and pre-invasion research has demonstrated that basslet populations undergo regulating density-dependent mortality due to predation. To unequivocally measure lionfish effects on prey mortality and to test whether prey survival remained density-dependent when exposed to predation by the invader, a controlled field experiment was conducted wherein both fairy basslet settlement density and lionfish presence were manipulated by divers on natural coral reefs. On reefs with and without lionfish, fairy basslet populations were repeatedly censused over the 28 d experimental period and mortality rates across a gradient of prey densities were quantified. Per capita loss of fairy basslet was density-dependent on reefs with and without introduced lionfish; however, the magnitude of this loss was significantly higher on reefs with the invader present. High mortality rates at low prey density resulted in local extinction of 2 of 14 fairy basslet populations exposed to the invader, a phenomenon observed only on lionfish reefs. Further, 9 out of 14 lionfish-exposed prey populations showed loss rates of >50% compared with just 3 prey populations with such rates on native-only reefs.

KEY WORDS: Coral reefs · Density dependence · Invasive species · Marine fishes · Population regulation · Predator recognition · Predator–prey

INTRODUCTION

Anthropogenic species introductions have been identified as a top conservation priority (Wilcove et al. 1998), as invasions can lead to altered community structure and ecosystem function, and to native species loss (Gurevitch & Padilla 2004, Pimentel et al. 2005, Vilà et al. 2011). Invasive predators have caused some of the most severe impacts of introductions (Salo et al. 2007, Jones et al. 2008) and have precipitated numerous extinctions via strong, direct, and consumptive effects (Blackburn et al. 2004, Kumschick et al. 2015). These invasive, predator-mediated extinctions necessarily imply a change in the processes that have previously ensured persistence of regulated prey populations. One condition of regulation is a compensatory response in one or more demographic rates to changes in prey density, causing populations to increase when rare and to decrease when abundant (Murdoch 1994, Hixon et al. 2002). Therefore, predicting the impact of a novel predator requires an understanding of whether and how the invader alters the existing compensatory dynamics that underlie native population regulation.

Demersal marine fishes have been instrumental in the detection and quantification of such demographic

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density dependence, as these populations are often amenable to the local-scale manipulations that can provide insight into the mechanisms behind density-mediated effects (Hixon & Webster 2002). Consensus has emerged that post-settlement mortality of demersal marine fishes often displays direct density dependence — a positive relationship between prey density and per capita mortality (Hixon 1998, Hixon & Webster 2002, White et al. 2010). Numerous studies have demonstrated that predation is often the proximate cause of density-dependent (hereafter DD) mortality (Hixon & Carr 1997, Anderson 2001, Carr et al. 2002, Holbrook & Schmitt 2002, Hixon 2015) and that this mechanism can lead to temporal population regulation (Steele 1997, Carr et al. 2002, Webster 2003, Hixon et al. 2012).

Of vital importance for understanding the implications of a predator introduction is characterizing the effects of multiple predators on patterns of prey mortality. Previous work on patch reefs has demonstrated emergent, synergistic effects of resident and transient predators on prey mortality patterns. Hixon & Carr (1997) showed that the effects of predators with differing hunting modes and scales of foraging (resident ambush piscivores versus transient pelagic hunters) — which separately caused density-independent (DI) mortality — interacted to produce DD mortality only when both predator types were present. Thus, the addition of a predator to an existing community has the potential to qualitatively alter the relationship between prey density and predation risk.

As the role of native predators in causing DD mortality is well understood in demersal fish communities, the introduction of a novel piscivore provides the opportunity to test how mortality patterns are altered by an invader. Theory predicts that density dependence (at some life stage and at some spatial scale) is a necessary condition for regulation (Murdoch 1994, Hixon et al. 2002), and simulations suggest that the effects of introduced predators on prey consumption rates may be particularly pronounced at low prey densities (Saul & Jeschke 2015), so the potential for a novel predator to weaken or even reverse DD demands study. Previously, Ingeman & Webster (2015) used manipulative field experiments — replicated before and after the introduction of the Indo-Pacific lionfish *Pterois volitans* to western Atlantic marine habitats — to measure changes in the density–mortality patterns of a common reef fish, the fairy basslet *Gramma loreto*. Per capita loss in fairy basslet remained DD after the invasion despite an increase in overall loss rates since the introduction of the novel predator (Ingeman & Webster 2015). However, the authors could not unequivocally attribute the altered mortality patterns to lionfish since the presence of the invader was confounded by possible environmental or biotic changes (e.g. increased native predator abundance and/or consumption rates) in the interval between experiments.

Therefore, in order to detect the effects of an invasive predator on the relationship between density and predation risk in native prey, I conducted a controlled field experiment on natural coral reefs in the Bahamas, manipulating both prey density and invasive predator presence, such that differences in loss rates are attributable to predation by the invader alone. On reefs with and without invasive lionfish, I compared (1) the immediate post-settlement and longer-term changes in density over the 28 d experimental period, (2) the magnitude of per capita loss due to predation between repeated censuses, and (3) the presence or absence of density dependence in populations of fairy basslet.

MATERIALS AND METHODS

Study species

The fairy basslet *Gramma loreto*, family Grammatidae, is a common aquarium fish inhabiting coral-reefs throughout the tropical western Atlantic (Böhlke & Randall 1963). Like most reef fishes, the fairy basslet has a bipartite life-cycle with pelagic larvae and demersal juveniles and adults (Böhlke & Chaplin 1994). Fairy basslet are typically found on the ceilings of caves, outcrops, and open reef ledges (hereafter 'ledges' collectively), where they feed opportunistically on passing plankton (Randall 1967). Individuals form dense aggregations, with the largest individuals occupying prime feeding positions nearest the outer edge of the ledge (Freeman & Alevizon 1983). Population size at the local level is tightly regulated by high and DD mortality caused by aggregating mesopredators (Webster 2003). Tagging studies have confirmed static membership of local aggregations and demonstrated that juveniles and adults rarely move farther than 3 m from their home ledge, such that post-settlement immigration is negligible and where each ledge supports a distinct local population (Webster 2003).

The Indo-Pacific lionfish *Pterois volitans/miles*, family Scorpaenidae, is an invasive mesopredator introduced in the mid-1980s that has rapidly spread throughout the region from an invasion locus near

Southeast Florida (Whitfield et al. 2002) and now inhabits most of the western Atlantic and Caribbean, including the Gulf of Mexico (Schofield 2010). These voracious, generalist predators have strong direct effects on native prey fishes via consumption of newly settled recruits and adults of small species (Albins & Hixon 2008, Albins 2013, 2015, Côté et al. 2013, Benkwitt 2015, Ingeman & Webster 2015) and have the potential to alter invaded reef ecosystems directly through consumption of ecologically important native fishes and via the indirect effects of predation (Albins & Hixon 2013). To date, few biotic controls have been identified in the invaded range: Atlantic lionfish are relatively free of parasites (Sikkel et al. 2014) and do not experience increased mortality or emigration even at extreme densities (Benkwitt 2013). Lionfish possess an impressive array of traits that may render them difficult to detect and/or may confuse prey (Lönnstedt & McCormick 2013, Marsh-Hunkin et al. 2013, Black et al. 2014) and are themselves well-defended from predation by venomous dorsal spines (Halstead et al. 1955). As such, predation on lionfish in the invaded range, although reported, is irregular and thus far insufficient to control their densities (Hackerott et al. 2013), which have been reported as high as 393 ind. ha^{-1} (Green & Côté 2009). Fairy basslet are common prey of the invader, which actively stalks juveniles and adults with large pectoral fins extended, herding individuals before striking rapidly (Albins & Lyons 2012). Anecdotally, fairy basslet individuals do not employ as robust an anti-predator response (fleeing into small refugia in the reef) to lionfish as toward native mesopredators, and experiments with other native Atlantic prey species have demonstrated a suboptimal response to the threat of predation by this novel predator (Black et al. 2014).

Study area

This study was conducted on coral patch reefs near the Cape Eleuthera Institute, Eleuthera, Bahamas. Patch reefs of highly variable structure occur on a 2–30 m deep shelf lining the 1500 m deep Exuma Sound to the southwest of Cape Eleuthera. Prior to initiation of the experiment, teams of divers on SCUBA identified 14 patch reefs ranging in surface area from 137 to 1290 m^2 at depths of 4–20 m, surrounded by sand and seagrass, and separated from all other hard substrate by at least 80 m. Experimental patch reefs were roughly cylindrical in shape and of variable diameter (10–30 m) and height (2–18 m). The benthos was dominated by small coral heads, algae, sponges, and soft corals of various species scattered over highly convoluted dead coral surfaces.

Experimental design

To determine the effects of invasive lionfish predation on prey density–mortality patterns, local fairy basslet populations were manipulated to create a range of prey densities on reefs with and without the introduced predator. Because the home ranges of adult lionfish span multiple local populations of fairy basslet prey, which restrict their movements to individual reef ledges, this study employed a split-plot design, whereby predator treatments where maintained at a larger scale (reef) than basslet density treatments (ledges within a reef). Reefs were paired by proximity, as well as similarity in size, depth, vertical relief, and relative coral cover, to form 7 experimental reef pairs. One reef in each pair was assigned by randomization to receive periodic lionfish removals (with randomization constrained to avoid excessive clustering of this treatment; 'native-only reef'); the other reef received variable levels of lionfish addition with the goal of achieving a standardized lionfish density ('lionfish reef'; see subsection 'Density manipulations'). Within each reef, 2 fairy basslet populations were chosen based on similarity in initial population size, ledge area, proximity to reef margin, and orientation to prevailing currents. In order to maximize the demographic isolation of experimental fairy basslet populations, only discrete ledges that were >3 m from other occupied ledges were chosen. Divers then performed an initial baseline census of all fairy basslet individuals on each experimental ledge and measured ledge surface area (0.4–1.5 m^2) to determine unmanipulated densities (6.3–18.9 fish m^{-2}). One fairy basslet population from each reef was then randomly chosen to receive artificially enhanced recruitment sufficient to increase density to levels commonly observed after a recruitment event (Webster 2003, Ingeman & Webster 2015). Fairy basslet additions rather than removals were employed to avoid artificially inflating extirpation rates by lowering prey density below ambient levels. Natural variation in the densities of unmanipulated populations created a continuous density-gradient that was thus extended by diver-enhanced artificial recruitment (manipulated population densities: 13.6–31.1 fish m^{-2}, see subsection 'Density manipulations').

Density manipulations

To maintain native-only reefs, divers conducted removals as needed, capturing lionfish using hand-nets where possible and employing pole spears where conditions made live-capture impossible. While the target for native-only reefs was complete removal of lionfish, the cryptic nature of this species, highly protected reef refugia, and occasional immigration resulted in low but non-zero densities on removal reefs. To maintain treatment densities on lionfish reefs, divers periodically captured juvenile and adult lionfish (8–38 cm total length [TL]) from native-only reefs and non-experimental habitats then translocated them to distant (>500 m) lionfish reefs. A target density of 300 lionfish ha^{-1} was chosen to represent a realistic average lionfish density based on observations of unmanipulated reefs in the region and other parts of the invaded range (Green & Côté 2009, Albins 2015). Transplant effects and variable emigration throughout the study duration necessitated repeated 'stocking' of lionfish reefs. However, targeted censuses indicated a strong density gradient between predator treatment levels, with lionfish reefs maintaining approximately 6 times higher densities (240.4 ± 35.7 lionfish ha^{-1}) compared to native-only reefs (40.1 ± 18.3 lionfish ha^{-1}).

To enhance the natural range in fairy basslet density, recruits (approximately 1–2 wk post-settlement and <2.0 cm TL) were captured using dip nets and anesthetic clove oil, transferred into seawater-filled plastic bags, and translocated to target populations. Recruits were captured from distant locations to minimize emigration from study ledges. Small numbers of recruits (<10) were added to a population during any single dive, and additions were conducted over several days in order to simulate a natural recruitment pulse and to minimize immediate (pre-census) loss of basslet transplants to aggregating predators. Censuses for the experiment commenced 24 h after recruit manipulations, thereby allowing a day for transplanted fish to recover from any handling effects and ensure that transplanted individuals did not suffer disproportionate mortality compared to resident fish.

Following the establishment of treatments and baseline censuses, a minimum of 2 divers re-censused each population after 2 d, 4 d, and weekly thereafter, with a final census after 4 wk. During each census, divers recorded the size of each fairy basslet individual, the total population size, and any predators within 2 m of the target basslet ledge. Observations of fairy basslet populations and individual sizes were highly congruent between divers, indicating that observation error was negligible.

Statistical analysis

All fairy basslet populations were censused prior to artificial recruitment enhancement, and initial densities were checked for systematic bias by both predator treatment and assignment to recruitment enhancement. Mean densities among treatment groups were compared using Welch's 2-sample *t*-tests, with no assumption of equal variance. These comparisons were repeated for fairy basslet observations at the first post-manipulation census to ensure that (1) mean fairy basslet density differed significantly among recruitment-enhanced versus unmanipulated populations and that (2) differences in fairy basslet density were not biased among reefs with and without lionfish. Additionally, cumulative population-level effects of fairy basslet on prey density were assessed by comparing the 4 resulting categorical treatment levels created by cross-factoring predator treatment (lionfish versus native-only reefs, 7 reefs each) and fairy basslet recruitment regimes (enhanced versus unmanipulated, 14 ledges each, 28 ledges total).

Prey per capita loss was defined as the proportional change in abundance accumulated between intervals.

$$(1 - N_{t+1}/N_t) \qquad (1)$$

Thus, positive values for per capita loss at a given time-step indicate that the total number of individuals decreased since the previous census, and this value is scaled to the previously observed abundance. Natural recruitment of fairy basslet was observed in between the 3rd and 4th censuses, indicated by reduced net loss and even population increases on some ledges. Uncontrolled recruitment means that net loss (as an aggregate measure of population change) likely underestimates mortality but is not likely to systematically bias results, since fairy basslet recruitment has been shown to be density-independent. I did not explicitly account for the difference in length of time intervals, which is likely to increase variability around estimates of per capita loss. However, time intervals were identical among treatment groups so this would not bias comparisons among groups or introduce a spurious effect of lionfish on patterns of mortality. To detect lionfish-induced changes in the magnitude of mortality and the presence of density dependence in fairy basslet per capita loss, I employed linear mixed effects models (LMMs) with 'ledge' nested within 'reef' as random effects, 'lionfish presence' and 'time-step' as categorical fixed effects, and 'prey density' (Note: this term represents the density at the beginning of each sampling interval and not the initial prey density.) as a continuous fixed effect. In order to test the significance of lionfish presence on per capita loss at each census, I included a 'lionfish × time-step' (fixed) interaction term. A significant interaction between lionfish and time-step would indicate a lionfish-induced change in the magnitude of mortality in interval since the previous census. Further, to measure an effect of lionfish on density dependence, I incorporated a 'lionfish × basslet density' (fixed) term. This inclusion allows separately fitted slopes of the response of fairy basslet loss to prey density on lionfish and native-only reefs. A slope coefficient for lionfish reefs that does not differ from zero would be consistent with the hypothesis that lionfish predation eliminates regulating density dependence.

I fitted full models (including all fixed effects and interactions) with and without random effects using restricted maximum likelihood estimation (REML) and compared them using likelihood ratio test (LRTs) with an adjustment for testing on the boundary (Zuur et al. 2009). The inclusion of a random intercept at the 'ledge' level resulted in a better fit than a fixed effects only model (L-ratio = 9.43, p = 0.001). Visual inspection of the residuals of the resulting models showed no departures from the assumptions of homogenous variance and normality among populations. However, there was evidence of temporal autocorrelation in the residuals, and inclusion of an AR1 structure substantially improved the model (ΔAIC $\gg$ 2). Re-examination of the residuals indicated that all assumptions had been met. After selecting the optimum random effects and correlation structure (see Appendix), I refit the competing models using maximum likelihood (ML) and tested the significance of fixed effects using LRTs. Where LRTs indicated that interaction terms were not significant, they were dropped from the model and the main effects were tested using LRTs. Finally, I estimated parameters and effect sizes from the final model using REML (Zuur et al. 2009). All statistical analyses were conducted in the R language and software environment, v. 3.2 (R Development Core Team 2015) using add-on packages nlme v. 3.1-128 (Pinheiro et al. 2014).

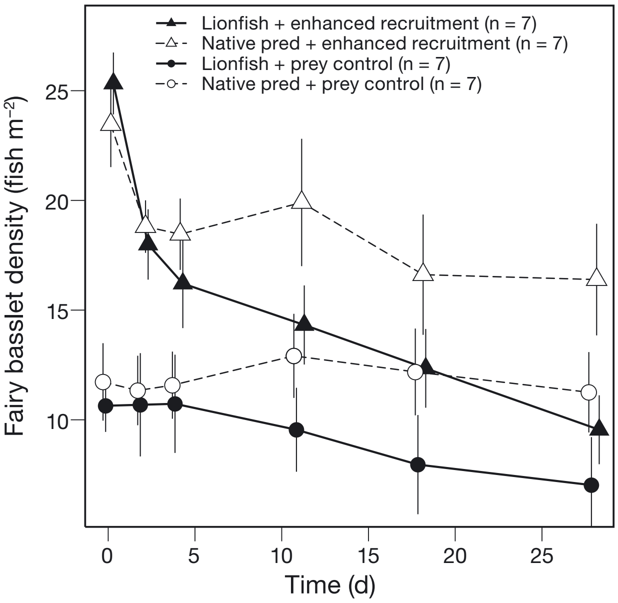

Fig. 1. Time series of fairy basslet *Gramma loreto* density (mean ± SE) over the 28 d experimental period on reefs with lionfish *Pterois volitans* (filled symbols and solid lines) and with native predators only (open symbols and dashed lines). Fairy basslet populations with artificially enhanced recruitment (triangles) were at significantly higher densities at the beginning of the experiment compared to unmanipulated populations (circles). However, high and directly density-dependent mortality reduced the difference in final densities within each predator treatment. Further, prey populations on lionfish reefs (filled symbols, far right) achieved lower final densities than native-only reefs (open symbols, far right), regardless of initial density

RESULTS

Prior to diver manipulation, fairy basslet densities showed no systematic bias by lionfish treatment (t = –0.26, p = 0.79) nor by assignment to enhanced recruitment treatment (t = –0.73, p = 0.47). In contrast, during the initial census (24 h post-manipulation) the fairy basslet population that received enhanced recruitment showed significantly higher densities of 24.1 fish m^{-2} compared to 11.2 fish m^{-2} in unmanipulated populations (t = 25.0, $p < 0.0001$; Fig. 1: circles versus triangles at initial census). Within each prey recruitment level, initial post-manipulation densities did not vary significantly by predator treatment (t = –0.51, p = 0.62 and t = 0.80, p = 0.44 for unmanipulated and recruitment-enhanced fairy basslet populations, respectively; Fig. 1: open versus filled symbols at initial census). Over 4 wk and across all reefs, net change in fairy basslet population density ranged from –22.7 fish m^{-2} (negative values indicating a decrease in density) to 5.5 fish m^{-2}, with far greater average decreases observed on lionfish reefs compared to native predator only reefs. This pattern was true of both recruitment-enhanced fairy basslet populations — where a decrease in density was approximately 140 % greater on reefs with lionfish present (mean change in density −15.8 versus −6.6 fish m^{-2}) — and at unmanipulated populations, with lionfish reefs experiencing 97 % greater decreases compared to native-only reefs (mean change in density −3.62 with lionfish present versus −1.83 fish m^{-2} on native-only reefs). Over the course of 4 wk and despite initial differences in prey density, fairy basslet populations on reefs with the invader were lower than those subject to predation by natives only (Fig. 1: filled symbols versus open at $t = 28$).

Cumulative per capita loss of fairy basslet over 28 d was DD for both native-only reefs and those with lionfish present (Fig. 2; circles versus triangles). That is, recruitment-enhanced populations experienced greater per capita loss compared to unmanipulated basslet populations on both native-only reefs (24.8 % versus 6.5 % loss) and on lionfish reefs (60.0 % versus 33.8 % loss). However, the magnitude of this loss was substantially greater on lionfish reefs regardless of prey density (Fig. 2; filled versus open symbols). Notably, unmanipulated (low-density) fairy basslet experienced slightly higher mean loss rates on lionfish reefs even compared to recruitment-enhanced (high-density) populations at native predator-only reefs (Fig. 2; filled circle versus open triangle). High mortality rates on lionfish reefs resulted in extirpation of 2 out of 14 fairy basslet populations; no fairy basslet populations on native-only reefs reached zero abundance. Further, 9 out 14 prey populations exposed to the invader showed loss rates of >50 % over 4 wk. In contrast, only 3 prey populations demonstrated such high mortality rates on native-only reefs.

Modeling interval per capita loss as a function of fairy basslet density using LMMs, there was a significant effect of 'prey density' (LRT, $p < 0.001$, see Table 1 for fixed effects selection criteria), indicating the presence of density dependence in per capita rates of prey loss (see Table 2 for parameter coefficients and variance). Further, I found no evidence to suggest that lionfish eliminated the presence of density dependence (LRT for the 'lionfish × prey density'

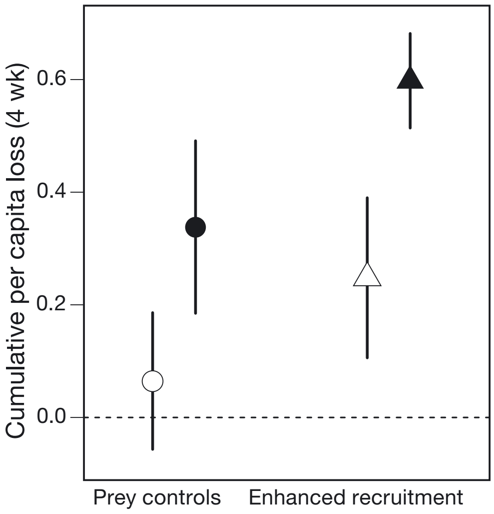

Fig. 2. Cumulative per capita loss (proportional change in abundance) for cross-factored treatment groups over 28 d (group means ± SE). At both unmanipulated and recruitment-enhanced fairy basslet *Gramma loreto* populations (circles and triangles, respectively), per capita loss was higher on reefs with lionfish *Pterois volitans* compared to native-only reefs (closed versus open symbols). Per capita loss at unmanipulated prey populations subject to lionfish predation were similar to recruitment-enhanced populations on native-only reefs (comparing filled circle to open triangle), suggesting that lionfish cause high mortality even at low prey density

Table 1. Selection criteria for fixed effects. Likelihood ratio and associated p-values comparing models with each potential explanatory variable (with all other fixed effects and optimal random structure in place) to a reduced model without the focal parameter. ΔAIC indicates the change in model fit associated with retaining the variable in the model; p-values <0.05 (and negative ΔAIC values) provide evidence for retaining the variable. Fixed effects retained in final model are indicated in **bold**

Explanatory variable	Likelihood ratio	p	ΔAIC
Lionfish (presence)	**8.330**	**0.004**	**−6.330**
Prey density	**11.124**	**<0.001**	**−9.124**
Lionfish × Prey density	0.018	0.975	1.982
Native predator biomass	0.867	0.352	4.017
Time step	**13.274**	**0.021**	**−3.274**
Lionfish × Time step	**11.197**	**0.003**	**−1.197**

Table 2. Summary of fixed effects for final model. Model coefficients and variance estimated using restricted maximum likelihood for all variables retained in final linear mixed effects model

Effect	Value	SE	df	*t*	p
Intercept	−0.351	0.165	128	−2.12	0.035
Prey density	0.021	0.008	128	2.48	0.014
Lionfish	0.123	0.227	26	0.54	0.590
Prey density:Lionfish	−0.008	0.011	128	−0.711	0.478
Time Step 2	0.076	0.097	128	0.788	0.432
Time Step 3	0.069	0.097	128	0.716	0.475
Time Step 4	−0.05	0.097	128	−0.525	0.601
Time Step 5	0.057	0.097	128	0.588	0.557
Time Step 6	0.143	0.097	128	1.481	0.141
Lionfish:Time Step 2	0.083	0.137	128	0.605	0.546
Lionfish:Time Step 3	0.124	0.137	128	0.906	0.366
Lionfish:Time Step 4	0.297	0.137	128	2.174	0.032
Lionfish:Time Step 5	0.323	0.137	128	2.380	0.020
Lionfish:Time Step 6	0.330	0.137	128	2.262	0.018

interaction, p = 0.98; Table 1). The coefficient for the effect of lionfish on the density–mortality relationship was small relative to the slope coefficient itself (0.008 and 0.021, respectively), and the confidence interval for this effect includes zero (Table 2). Together, these results indicate that prey loss was DD regardless of predator treatment. The effect of lionfish was mediated by time-step as indicated by a significant 'lionfish × time-step' interaction (LRT, p = 0.003), precluding the interpretation of the main effect of lionfish presence singularly across the duration of the experiment. However, in the final model, after accounting for prey density, during Time-Steps 4, 5, and 6 (11, 18, and 28 d post manipulation) per capita loss was higher on reefs with lionfish than on those without (Table 2).

DISCUSSION

Density dependence in vital rates represents a crucial component of population regulation, and the detection of density dependence and the identification of the mechanisms that lead to density dependence remain relevant areas of study in population ecology (reviews by Hixon et al. 2002, Lande et al. 2002, Osenberg et al. 2002, Brook & Bradshaw 2006, White et al. 2010, Lebreton & Gimenez 2013, Thorson et al. 2015). As predation is often the proximate cause for this compensatory pattern in reef fishes (Hixon 2015), understanding how an introduced marine piscivore may alter patterns of density-mediated mortality in native prey is an important step in predicting the ultimate effects of invasion, including the risk of local or global extinction of native species. In this experiment, I found evidence that fairy basslet mortality remains DD in the presence of invasive lionfish. However, lionfish predation caused an overall increase in prey mortality and contributed to the local extinctions of 2 of 14 of prey populations; in contrast, no fairy basslet populations were extirpated on native-only reefs. Both extirpated populations began the experiment at low initial density, suggesting that, unlike native piscivores alone, the invader can cause high per capita loss rates at low prey density. Further, 9 out 14 fairy basslet populations that were exposed to the invader — across a range of initial densities — had a per individual predation risk of >50% across the experimental period compared with 3 prey populations showing such mortality rates on native-only reefs. Thus, while patterns of fairy basslet mortality were qualitatively DD (per capita loss increasing with higher prey densities) regardless of predator treatment, lionfish nevertheless reduced the likelihood of local persistence of fairy basslet populations by increasing the magnitude of mortality across a broad range of prey densities.

The observation that lionfish lower the probability of local prey persistence corroborates previous experimental research from the invaded range. Albins (2013) demonstrated that, over 8 wk, a single lionfish on a small patch reef can reduce prey richness by nearly 5 species compared to predator-free controls, an effect nearly twice as large as that caused by native piscivores. Similarly, Benkwitt (2015) observed increases in native species richness over the summer recruitment period only on lionfish-free control reefs; in the presence of the invader species richness remained unchanged. Additionally, on large patch reefs and over multiple recruitment periods, Albins (2015) showed that lionfish significantly reduced species richness and that the invader caused the greatest per capita effects on the rarest species. While the increased mortality rate of native prey driven by lionfish is not itself a novel result, this study demonstrates how an introduced generalist predator can cause extirpation of rare species (or a low-density population of a single species). By increasing loss rates even at the lowest prey densities — when prey populations are at their most vulnerable — predation by the invader heightens the likelihood that demographic stochasticity in local prey populations will result in local extinction.

The observation that lionfish remain effective predators at low prey density corroborates recent theory on the differential effects of a novel predator. Saul & Jeschke (2015) used mechanistic steady-state satiation equations (based on the predator functional response) to demonstrate that a novel predator with higher attack efficiency than natives and whose prey have low experience with the new predator will have higher consumption rates than natives across all prey densities. In such a scenario, the difference in consumption rate experienced by the prey will be most pronounced at low to intermediate prey densities (Saul & Jeschke 2015). Indeed, lionfish may have a lower threshold of prey density below which foraging becomes inefficient compared with native piscivores, a distinct possibility for a novel predator with no behavioral or morphological analogue in the western Atlantic (Albins & Lyons 2012). While native piscivores often cause strongly DD mortality through an aggregative effect, spatially congregating and increasing attack rates in the vicinity of high prey densities (reviewed by White et al. 2010), there is thus far no evidence that lionfish do the same, continuing to hunt even as prey densities decline and native predators move on to richer patches where foraging is more efficient. Alternatively, lionfish may be less likely than other generalist native piscivores to employ prey switching at low densities of the target species. In either case, per capita predation rates caused by natives would fall with decreasing prey density, but lionfish predation rates would remain high.

Another mechanism that could drive high predation rates at low prey density is naïveté, when prey fail to recognize and/or respond suboptimally to the threat of predation by a non-native predator (Banks & Dickman 2007, Sih et al. 2010, Anson & Dickman 2013). DD mortality caused by native predators often relies on intense competition for predator-free shelter at high prey densities. In contrast, when prey are rare, shelter is plentiful and predation risk low (Forrester & Steele 2004). However, if native prey are naïve to the risk of predation by this cryptic hunter with novel foraging behavior (Albins & Lyons 2012), lionfish would continue to consume prey at high rates even when shelter is abundant. Evidence of naïveté toward lionfish has been mixed in the invaded range. Recently, Anton et al. (2016) demonstrated that the Atlantic grunt, *Haemulon plumierii*, maintains a greater approach distance from native predators than from lionfish. Similarly, Kindinger (2015) showed that territorial 3-spot damselfish, *Stegastes planifrons*, that responded aggressively to all native fishes had reactions to captive lionfish that did not differ from the response toward empty controls. In contrast, Black et al. (2014) demonstrated, using another native Atlantic pomacentrid, *S. leucostictus*, that native prey can recognize and respond with anti-predator behavior in the presence of lionfish. However, this prey species did not modify their high-risk courtship behavior in the presence of the invader (Black et al. 2014), a result that suggests that other prey may similarly increase their risk predation when managing tradeoffs with foraging and/or reproductive demands.

Finally, differences in feeding behavior between lionfish and native predators could explain the observed mortality patterns in prey. Fairy basslet often occupy reef ledges where highest mortality rates occur toward the back of the ledges, the location at which native ambush hunters have the shortest pursuit distance (Webster & Hixon 2000). Larger, competitively dominant individuals that occupy the outer reaches of the ledge have access to passing plankton while remaining relatively near shelter, leading to lower predation risk. Thus, these individuals may represent a partial prey population refuge, such that native predators alone rarely cause complete extirpation of a population. Anecdotally, lionfish often hunt in the open along the outer margins of reef ledges and do not rely on a high-velocity pursuit from a hidden location. They may therefore have access to prey individuals unavailable to native predators. While both native predators and lionfish employ variants of a hybrid 'ram-suction' feeding behavior — combining a rapid burst of acceleration of the body (ram) with jaw protrusion and expansion of the buccal cavity to cause rapid flow of water into the mouth (suction) (Wainwright & Bellwood 2002) — common native predators of fairy basslet, such as serranids and aulostomids, employ considerably more ram movement than lionfish, which may approach prey quite closely before initiating a strike (Muller & Osse 1984). Speculatively, lionfish may therefore employ a more effective capture method for prey that are very near shelter or those that occupy primary feeding positions near the outer margin of a reef ledge.

While the pre- and post-invasion experimental design employed in previous work could not unequivocally attribute the altered patterns of prey mortality to lionfish (Ingeman & Webster 2015), here I provide evidence that the increase in prey mortality observed between predator treatments is caused by the invader. However, while the cumulative effect of lionfish and native predators (the invasion scenario) represents an increase in prey mortality rates compared to native predators alone, it is possible that interactions with lionfish alter consumption patterns by native predators. The experimental design employed here cannot distinguish the singular and interactive effects of native and invasive predators, and other studies of lionfish predatory effects have suggested non-additive effects of lionfish and native predators (Albins 2013). In all cases examined, the magnitude of the lionfish effect has been greater than that of native predators and the cumulative mortality rates have been higher than those caused by either predator alone. Yet, the marginal difference in loss rates observed between predator treatments may represent an underestimate of the lionfish effect if native predators' consumption rates are reduced in the presence of the invader (compensatory mortality).

Another limitation of the study is the use of per capita loss (or its converse, survival) in quantifying density dependence. First, as an aggregate demographic measure, survival does not distinguish between the presence of a prior resident individual and a new recruit that has replaced a prior resident that was consumed in the interval between studies. In the latter case, both the effective prey density over the interval and the true mortality rate would be underestimated in calculating per capita loss. It is possible that natural recruitment rates in this study were not systematically biased by predator treatment, especially as native post-larval settlers have been shown to selectively avoid reefs with caged native predators but not reefs with lionfish (C. Benkwitt unpubl. data). Alternatively, in this study, higher consumption of fairy basslet may have increased the level of conspecific, olfactory distress cues in the proximity of fairy basslet ledges on lionfish reefs, leading to reduced settlement and unreliable comparisons of basslet mortality. Notably, I observed that a natural recruitment pulse drove an increase in average fairy basslet population size (associated with a new moon soon after the third census) on native predator-only reefs. The absence of such an uptick in density on lionfish reefs is consistent with either reduced settlement or high post-settlement lionfish predation on uncensused fairy basslet recruits. In either case, measuring per capita loss as a function of previous population density may not capture the total effect of lionfish on DD dynamics.

In addition, if the underlying population dynamics follow a Beverton-Holt function, as is commonly observed in reef fishes (Osenberg et al. 2002, Shima & Osenberg 2003), fitting per capita loss as a linear function of prey density may not be appropriate for identifying changes in the intensity of density dependence caused by the invader (C. Osenberg pers. comm.). The mixed-effect model I employ here provides no evidence for lionfish-induced alteration of the intensity of density dependence (suggesting that lionfish could alter only the DI component of fairy basslet mortality). In contrast, an alternative analytical method assuming Beverton-Holt dynamics and using maximum likelihood parameter estimation for both recruitment and predator-specific mortality rates (K. Ingeman unpubl. data) suggests that lionfish in fact increase the DD component of mortality, albeit with wide confidence intervals around estimates of both DI and DD parameters. In the face of mixed evidence for changes in the intensity of density dependence and high variability in the data, I therefore refrain from making inference about lionfish changes to the DI or DD components of mortality based on these results. Future efforts should unambiguously measure demographic rates through tagging of prior resident fishes, and should adopt the appropriate dynamic model to infer effects of introduced predators on DD and DI mortality.

While I observed increased mortality rates and the local extinction of native prey populations, I do not conclude that fairy basslet is at high risk of global extinction as a result of this predator introduction; for this species, post-settlement demographics are largely disconnected from recruitment due to a pelagic larval phase, and local populations are regularly replenished by DI larval settlement (Webster 2003). Further, this common species is buffered from the risk of global extinction by high fecundity, large range size, and fairly broad habitat tolerances (Böhlke & Chaplin 1994). Of greater conservation concern are rare species, those with demographically isolated populations, and species whose range is completely encompassed by the lionfish invaded range, such as the fairy basslet congener, *G. dejongi* (Victor & Randall 2010). This recently described basslet has been observed only in Cuba and the nearby Cayman Islands (Lohr et al. 2014), and its entire geographic and habitat range (reef walls at 20–30 m depth) are inhabited by lionfish. Another endemic coral-reef fish with a restricted range, the critically endangered social wrasse, *Halichoeres socialis*, has recently been documented as a primary prey item in lionfish diet contents in Belize (Rocha et al. 2015). As this study demonstrates, such native populations are no longer protected from high predator consumption rates by low local prey densities, a result that mangers should consider when designing and evaluating conservation and mitigation efforts throughout the invaded rage.

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Appendix. Selection criteria for random effects and autocorrelation structure. Random effects—None: no random effects; Reef: separate random intercepts at the reef level; Ledge: separate random intercepts at the ledge level. LRT (likelihood ratio test) results display the likelihood ratio and associated p-value (corrected for testing-on-the-boundary) comparing the model with optimal random structure to a fixed-effects-only model. Optimum structure was chosen by AIC (Aikake's information criterion); the best fit model is indicated in **bold**

LRT results Likelihood ratio	 p	Random effect	Autocorrelation structure	AIC
		None	None	125.3
		Reef	None	120.1
		Ledge	None	118.1
		None	AR(1)	96.4
		Reef	AR(1)	126.9
9.28	**0.0010**	**Ledge**	**AR(1)**	**87.6**

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Contribution to the Theme Section 'Invasion of Atlantic coastal ecosystems by Pacific lionfish'

NOTE

Preferences of invasive lionfish and native grouper between congeneric prey fishes

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ABSTRACT: To gain insight about how an invasive predator may influence native prey, we performed a series of experiments in aquaria to characterize and compare the prey preferences of the invasive red lionfish *Pterois volitans* and an ecologically similar native mesopredator, the graysby grouper *Cephalopholis cruentata*. Preference for native congeneric fishes, the fairy basslet *Gramma loreto* and blackcap basslet *G. melacara*, were tested. We observed behavior of predators in response to 2 individual prey consisting of cross-factored combinations of species (fairy and blackcap basslets) and size (small and large). Upon initial exposure to prey, lionfish first hunted fairy basslet and graysby first hunted blackcap basslet, with both predators initially preferring large over small fish. Overall behavior (quantified from the entire duration of observation) indicated both predators lacked a preference between basslet species based on total number of strikes and hunting time. Despite essentially identical size ranges of predators studied, graysby overall preferred large basslet across all graysby sizes, whereas the overall preference of lionfish between prey size varied with lionfish size. Importantly, the initial preferences of predators were likely least affected by the unnatural setting in aquaria. By preferentially consuming the less-preferred prey species of native graysby or by increasing predation on larger basslets, invasive lionfish may enhance coexistence between basslet species or among basslet sizes within local populations structured according to a size hierarchy. Alternatively, increased consumption of basslets may deplete local basslet populations, especially if lionfish exhibit prey switching behavior.

KEY WORDS: Predation · Prey preference · Invasive species · Coral reefs · Lionfish · Grouper · Basslets

INTRODUCTION

Invasive predators typically have effects on native prey that are more severe than those of native predators (Salo et al. 2007) and can cause substantial declines in populations of native species (Pitt & Witmer 2007). These predators often have generalized diets and, in extreme cases, can drive native species to local or global extinction (Clavero & García-Berthou 2005). Therefore, accurately predicting the effects of invasive predators on native prey populations and communities is important for informing management and conservation strategies.

A key mechanism underlying predatory effects is prey preference. Predators may have a preferred prey which is disproportionately consumed, or they may exhibit prey switching behavior (sensu Murdoch 1969) where the predator switches to other available prey once the preferred prey becomes rare. Further, the combination of native and invasive predation may result in enhanced depletion of a single prey species if both predators have a preferred prey in

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common. If predators differ in prey preference, predation may also be enhanced as the invasive predator consumes the less-preferred prey of the native predator. Switching behavior exhibited by an invasive predator could ultimately lead to the extinction of native prey (e.g. Savidge 1987). Overall, understanding the prey preference of an invasive predator and comparing it to that of a native predator can reveal potential mechanisms underlying the overall effects of an invasion on native communities.

By performing a series of experiments, we characterized the prey preference of an invasive marine predator, the Pacific red lionfish *Pterois volitans*, and compared this preference to that of an ecologically similar mesopredator that is native throughout the Atlantic, the graysby grouper *Cephalopholis cruentata*. Invasive lionfish are commonly found on coral reefs throughout the tropical and subtropical Western Atlantic and greater Caribbean region (Schofield 2010) and, like the native graysby, are considered to be generalist predators (e.g. Morris & Akins 2009). As a voracious predator, invasive lionfish can cause large reductions in the abundance of small native fishes and declines in species richness at scales that range from smaller patch reefs (e.g. Albins & Hixon 2008) to large coral reefs (Albins 2015).

Marine piscivores often preferentially distinguish among prey by species (e.g. Almany et al. 2007) or by size (e.g. Floeter & Temming 2003). We hypothesized that native graysby and invasive lionfish have similar prey preferences, because they are both generalist mesopredators. We predicted that neither predator would display a strong preference between 2 congeneric prey species, and that both would exhibit shifts in preference from smaller- to larger-sized prey with increasing predator size, because both graysby and lionfish are gape-limited predators.

MATERIALS AND METHODS

Study area and fish collection

We conducted this study during August 2014 at the Cape Eleuthera Institute on Eleuthera, the Bahamas, where we investigated the preference of predators for 2 native coral-reef fishes, the fairy basslet *Gramma loreto* and blackcap basslet *G. melacara*. These congeners are popular aquarium fishes that differ in appearance primarily by coloration (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m558p247_supp.pdf) and are commonly found under ledges (rock overhangs) throughout Caribbean reefs (Böhlke & Randall 1963, Starck et al. 1978). SCUBA divers collected basslets from reefs in the Exuma Sound at maximum depths of 15 m with small aquarium hand nets and the fish anesthetic quinaldine. We collected graysby and lionfish from shallow patch reefs (<5 m deep) in Rock Sound using, respectively, hand fishing lines while snorkeling and hand nets on SCUBA. We collected 15 lionfish ranging in size from 10.2 to 20.9 cm total length (TL) and 15 graysby with a size range of 10.0 to 20.3 cm TL. All fish were maintained in outdoor tanks with continuous flow-through saltwater systems and fed daily; predators were fed live silverside fish and basslets were fed live brine shrimp (*Artemia* sp.).

Experimental design

We conducted all experimental trials in 50 gallon (ca. 190 l) acrylic aquarium tanks (91.5 × 38 × 51 cm) with continuous flow-through seawater systems. Food was withheld from predators for 24 h prior to observation to ensure predator response to the presence of prey. Tanks were divided in half with a removable central barrier of solid aluminum (Fig. 1). We released a single predator into one side of the tank and placed 2 basslets in the other side. Basslets were held in identical small glass containers (~500 ml) with mesh covers (1 basslet per container) positioned in each corner of the tank. These prey containers ensured that predators were able to receive both visual and chemical cues from basslets, but could neither make physical contact nor consume any basslets.

To determine whether the preference of predators for basslets was driven by basslet species (fairy and blackcap) or basslet size (small and large: 1.7–2.5 and 3.5–5.2 cm TL, respectively) we presented pairs of basslets in cross-factored combinations of the 2 variables, resulting in the following treatments: (1) small fairy and large fairy, (2) small blackcap and large blackcap, (3) small fairy and small blackcap, (4) large fairy and large blackcap, (5) small fairy and large blackcap, and (6) large fairy and small blackcap. In addition to randomizing the order of basslet treatments presented to each predator, we also randomized the corner of the tank basslets were placed in every time a treatment was presented.

Once the predator and basslets were in their respective sides of the tank, we allowed them to acclimate for 20 min, after which we removed the central barrier and observed the predator's behavior for 10 min. Observations were performed either in per-

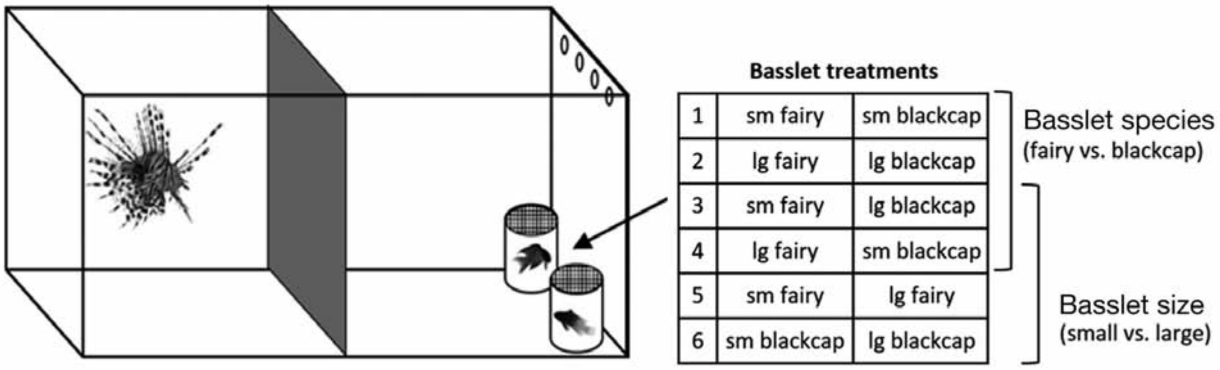

Fig. 1. Experimental tank setup (left), consisting of a 50 gallon (ca. 190 l) acrylic aquarium tank, divided by a removable aluminum central barrier separating basslets in ~500 ml glass containers with mesh covers from a predator (lionfish shown here). After a 20 min acclimation period, the central barrier was removed and predator behavior was observed in response to randomized combinations of individual basslets randomly placed in glass containers. Basslet treatments (right) consisted of basslet species (fairy and blackcap) cross-factored with basslet size (small: 1.7 to 2.5 cm TL; large: 3.5 to 5.2 cm TL) to determine whether the preference of predators was driven by either variable

son (74 lionfish trials; 73 graysby trials) or filmed with a digital video camera (16 lionfish trials; 17 graysby trials) positioned outside of the tank. During each 10 min trial, we recorded (1) which basslet the predator hunted first (initial hunting preference); (2) the number of times the predator's mouth made physical contact with each glass container (number of strikes); and (3) the amount of time the predator hunted each basslet (hunting time). We defined the hunting behavior of lionfish as occurring when an individual directly faced a basslet with flared pectoral fins and/or blew pulsed jets of water towards a basslet (Cure et al. 2012). We characterized graysby hunting behavior as occurring when an individual positioned itself near a basslet (<10 cm in this experiment) while directly facing the basslet (Webster 2004).

At the conclusion of the 10 min trial, we separated the predator from the basslets and placed the central barrier back in the tank. A new combination of basslets were placed in the glass containers, and all fish were allowed to acclimate for 20 min before removing the barrier and observing predator response for another 10 min. This procedure was repeated until all 6 basslet treatments had been presented to each predator in random order.

Statistical analyses

When testing for significant differences in predator response between fairy versus blackcap basslets, we analyzed only the 4 treatments where predators were presented with 2 different basslet species (lionfish: n = 11; graysby: n = 11). Similarly, we analyzed the 4 treatments where we presented predators with 2 basslets differing in size (small versus large) when comparing predator response between basslet sizes (lionfish: n = 13, graysby: n = 12). If a predator did not display any predatory behavior during any of the 4 treatments described in the treatment groupings above, then the individual was dropped from that respective group prior to analysis (resulting in the final sample sizes reported above).

To test whether initial hunting preferences between basslet species (fairy and blackcap) and basslet sizes (small and large) significantly differed between predators (lionfish and graysby) and/or among predator sizes (continuous variables), we fitted generalized estimation equations (GEEs) with binomial distributions and exchangeable correlation structures. GEEs are an extension to the generalized linear model approach that allow for correlations between observations from the same subject, thus allowing us to account for repeated measures. We fitted a full model with an interaction between predators and predator size, and then compared the model fit to that of the reduced additive model by calculating quasi-likelihood values under the independence model criterion (QIC; Pan 2001). If the initial hunting preference significantly varied between predators, we then performed a post-hoc McNemar test with a continuity correction for lionfish and graysby (separately) to test whether each predator had a significant initial preference.

We fitted full GEEs with Poisson distributions and exchangeable correlation structures to test whether the number of strikes and hunting time of predators significantly depended on a 3-way interaction among

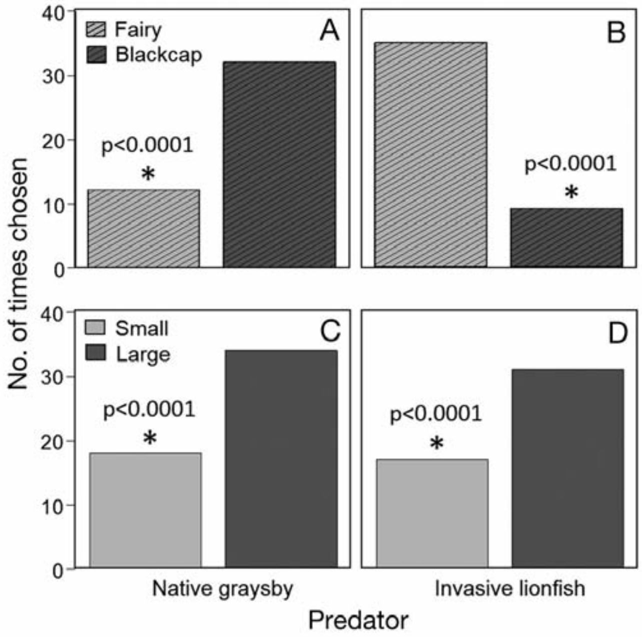

Fig. 2. Initial hunting preference of (A) native graysby and (B) invasive lionfish between fairy versus blackcap basslets (n = 11 graysby, n = 11 lionfish), and preference of (C) graysby and (D) lionfish between small versus large basslets (n = 12 graysby, n = 13 lionfish). Bars represent the total number of times that each predator initially hunted each basslet during treatments consisting of 2 different basslet species (n = 4 per individual predator) and 2 different basslet sizes (n = 4 per individual predator). Asterisks and p-values indicate significant differences in predator response between basslet species and size based on post-hoc McNemar tests

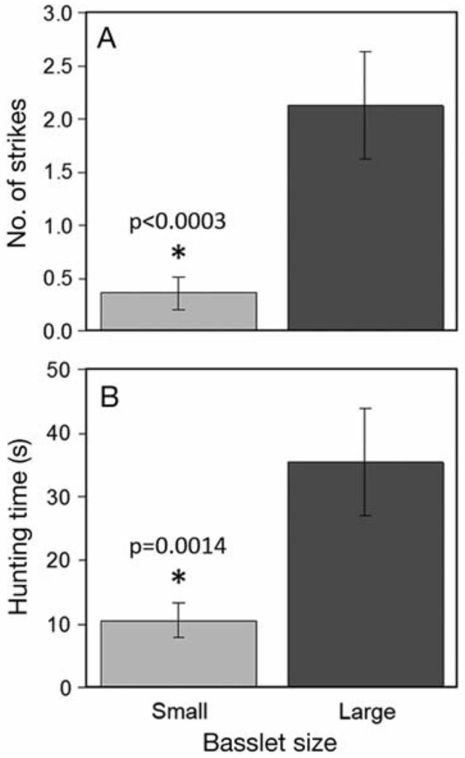

Fig. 3. Mean (±SE) (A) number of strikes and (B) amount of time spent hunting by native graysby (n = 12) in response to small versus large basslets during treatments consisting of 2 different basslet sizes (n = 4 per individual predator). Asterisks and p-values indicate significant differences in response between basslet sizes based on generalized estimation equations (GEEs)

the type of predator, predator size, and basslet species. We compared the full and reduced additive GEEs with QIC. If the 3-way interaction was significant, we fitted GEEs for lionfish and graysby separately to determine whether each predator's response significantly differed among predator size and/or basslet species (or an interaction between the 2). Again, final models (full versus reduced) were selected for each predator based on QIC values. We repeated this entire process, but with basslet size instead of basslet species as an explanatory variable in all the GEEs. All statistical analyses were conducted using R version 3.1.2 (R Core Team 2014) with the associated packages geepack (Halekoh et al. 2006) and MESS (Ekstrom 2014).

RESULTS

Invasive lionfish and native graysby exhibited clear initial hunting preferences for basslet species that significantly differed between predators (Fig. 2A,B; GEE, Wald $\chi^2 = 25.5$, $p < 0.0001$), yet did not significantly differ among predator sizes (GEE, Wald $\chi^2 = 1.49$, $p = 0.22$). Upon initial exposure to both basslet species, lionfish first hunted fairy basslet significantly more often than blackcap basslet (McNemar test $\chi^2 = 96.01$, $p < 0.0001$), whereas graysby initially hunted blackcap basslet (McNemar test; $\chi^2 = 62.02$, $p < 0.0001$). However, these initial preferences were not maintained for the remainder of the observational periods. Across all predator sizes observed, there was no significant difference in the number of strikes or hunting time directed at each basslet species exhibited by either predator (Table S2 in the Supplement at www.int-res.com/articles/suppl/m558p247_supp.pdf).

When testing the initial hunting preference between basslet sizes, we found that, despite the full GEE model having a lower QIC value than the reduced model (Table S1), the interaction between the predator species and predator size was not significant (GEE, Wald $\chi^2 = 2.60$, $p = 0.11$).

Initial preference between basslet sizes did not significantly differ between predator species (GEE,

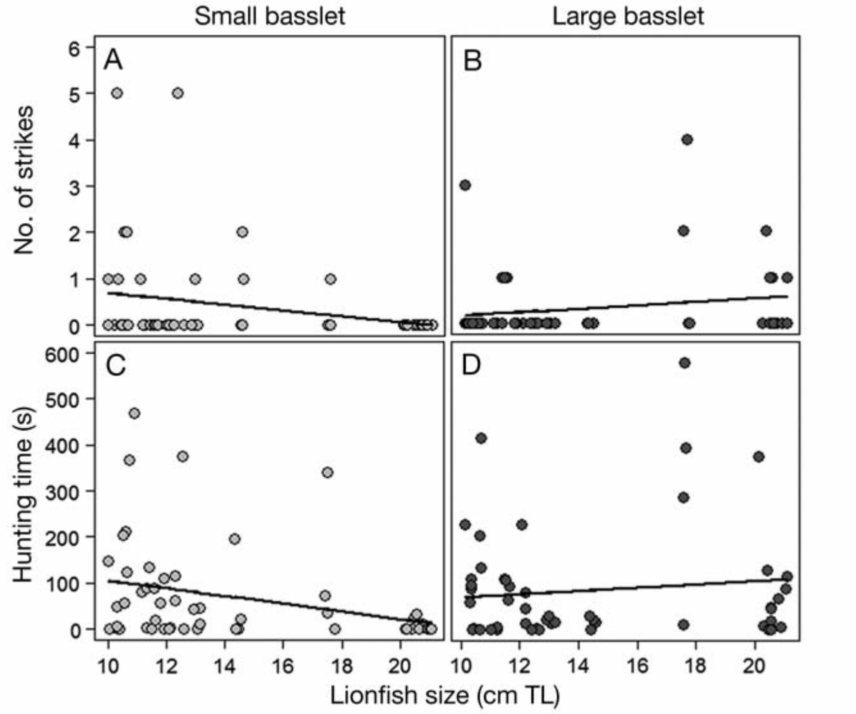

Fig. 4. Number of strikes by invasive lionfish (n = 13) throughout a range of lionfish sizes (cm TL) in response to (A) small and (B) large basslets, and amount of time spent hunting (C) small and (D) large basslets during treatments consisting of 2 different basslet sizes (n = 4 per individual predator). Regression lines were calculated from models with significant interactions between lionfish size and basslet size

Wald χ^2 = 2.57, p = 0.11) nor across predator sizes (GEE, Wald χ^2 = 1.01, p = 0.31). Both lionfish and graysby had a significant initial preference for large basslet (Fig. 2C,D; McNemar tests, χ^2 = 16.1 and 29.0, respectively; $p < 0.0001$ for both predators). This preference for large basslet remained consistent for graysby in terms of both the overall number of strikes (Fig. 3A; GEE, Wald χ^2 = 13.19, $p < 0.0003$) and hunting time (Fig. 3B, GEE, Wald χ^2 = 10.24, p = 0.0014). This preference was also maintained across all sizes of graysby tested (number of strikes: GEE, Wald χ^2 = 0.65, p = 0.4202; hunting time: GEE, Wald χ^2 = 0.01, p = 0.9433). In contrast, both the overall number of strikes and hunting time of lionfish depended on a significant interaction between the size of lionfish and basslet size (Fig. 4; number of strikes: GEE, Wald χ^2 = 8.42, p = 0.0037; hunting time: GEE, Wald χ^2 = 11.53, $p < 0.0007$). Predatory behavior directed at small basslet was greatest among smaller lionfish sizes, and gradually decreased with increasing lionfish size (Fig. 4A & C). We found the opposite trend in response to large basslet, with increasing levels of predatory response as lionfish size increased (Fig. 4B & D).

DISCUSSION

The distinctiveness hypothesis postulates that invasive predators are expected to have similar effects on prey species that are taxonomically and functionally similar (e.g. Ricciardi & Atkinson 2004). Contrary to this prediction, we have provided evidence of an invasive marine predator having strong prey preferences that depend on both the species and size of prey upon initial exposure to a pair of congeneric coral-reef fishes. Lionfish first hunted fairy basslet more often than blackcap basslet, and initially preferred large over small fishes. In contrast, native graysby first hunted blackcap basslet, yet were consistent with lionfish in exhibiting an initial preference for large fish. Following these initial preferences, overall predatory behavior quantified from the entire duration of observations revealed that both the invasive and native predators hunted and struck about equally at both basslet species. In terms of overall preference between prey size, only the preference of invasive lionfish varied with predator size. Native graysby preferred large fishes across all predator sizes, yet smaller lionfish preferred small basslets and larger lionfish preferred large basslets.

We also observed additional differences in behavior between predators in response to basslets. Graysby typically performed strikes at basslets in quick succession, striking the glass containers up to as many as 9 times in 3 s. In contrast, there was a minimum of 2 s between individual lionfish strikes. We also observed lionfish more often than graysby switching between the two prey basslets within a single trial. A review of the trials we recorded with a digital camera revealed that lionfish switched between basslets a total of 31 times, whereas graysby switched only 6 times. More than half of the observed switches by lionfish seemed associated with basslet movement. Typically, immediately following the movement of a basslet in the glass container, the lionfish turned its attention to that basslet. None of the switches between basslets by graysby were associated with basslet movement.

Both in the experimental setting of this study and on natural reefs, recognition of basslet species by lionfish and graysby likely involves the use of visual and/or olfactory cues from prey (or combinations of both). Most reef fish have acute color vision (McFarland 1991), so these predators may be able to interpret the differences in coloration between fairy and blackcap basslets. Preferences for a prey species could also be explained by varying activity levels between basslets. Anecdotally, fairy basslet appeared to be more active in the glass containers compared to blackcap basslet in this study, and our observations of lionfish often switching between basslets when hunting seemingly in response to basslet movement further supports this hypothesis. Kindinger (2016) revealed that on coral reefs, fairy basslet were more aggressive than blackcap basslet, which may indicate fairy basslet are also more conspicuous in a natural setting.

The behavior of predators observed in this study suggests that invasive lionfish may have a slightly broader range of effects on basslets than native graysby, given that lionfish are seemingly more likely to hunt both small and large basslets, and even may exhibit switching behavior. However, the initial preferences of predators are of particular importance, because these observations were least likely to reflect the unnatural setting used in this study. Predators in aquaria were unable to consume prey fishes, and the glass containers with basslets seemed to deter predators. Once a predator struck at the glass, there were often few subsequent strikes for the remainder of a trial, although predators did continue to display hunting behavior. Therefore, if the initial observations of behavior are indicative of the true preferences of these predators, then the addition of invasive lionfish on reefs may promote coexistence between basslets by consuming the less-preferred species of the native predator. In contrast, invasive lionfish may enhance overall predation of larger basslets.

The combination of invasive and native predation likely results in complex interactions with basslets. Basslets are found distributed among ledge positions in local populations based on a size hierarchy (Webster & Hixon 2000, Kindinger 2016). Under ledges, individuals compete both within and between species for feeding position, whereby larger individuals maintain coveted positions towards the fronts of ledges where the ability to obtain planktonic food is greatest. If both the invasive and native predators preferentially consume these larger fishes, the ability of smaller basslets to shift closer toward coveted feeding positions may increase. Interspecific competition between basslet species (Kindinger 2016) also may be altered by invasive lionfish via increased consumption of fairy basslet.

Alternatively, invasive lionfish may enhance predation of native basslets to the point where competition no longer exists within local populations. Indeed, previous field studies indicate that fairy basslet are faced with increased predation as a result of the addition of lionfish to native reefs (Ingeman & Webster 2015), and invasive lionfish can even drive local populations of fairy basslet to extinction (Ingeman 2016, this Theme Section). In addition to these effects on fairy basslet, invasive lionfish may substantially affect both basslet species via elevated consumption rates of larger individuals. Over time, this increased consumption of larger size classes of prey could cause shifts in the overall size distribution of basslets, or potentially even influence population growth rates via preferential targeting of adult basslets that are reproductively mature. Additionally, the enhanced depletion of prey fishes could also have potential indirect effects on native predators (including graysby) via competition for food.

Our study demonstrates aspects of prey preference that are different and similar between invasive and native predators. As a result, invasive lionfish may at one extreme enhance coexistence by preferentially consuming the less-preferred prey species of the native predator or by enhancing preferential predation on larger, competitively-dominant basslets. At the other extreme, increased consumption of basslets by invasive lionfish may deplete local basslet populations, especially if lionfish exhibit switching behavior following the reduced availability of preferred prey. Determining how the combination of invasive and native predation will ultimately affect native prey populations and communities is imperative for accurately predicting the extent of impact from an invasion, which can inform management and conservation initiatives.

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Invasive lionfish increase activity and foraging movements at greater local densities

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ABSTRACT: Density-dependent changes in predator foraging behavior due to intraspecific competition for food can have important implications for population dynamics of both the predator and its prey. The Indo-Pacific red lionfish *Pterois volitans* is an invasive predatory reef fish that has reached high population densities and can cause large reductions in small native fishes. To determine whether lionfish behavior or movement varies with local lionfish and/or prey densities, I conducted observations of lionfish on 16 coral patch reefs in The Bahamas. Lionfish foraging activity and movement varied significantly with lionfish density. At higher densities, lionfish exhibited greater activity levels, time away from shelter, and more short-term foraging movements between coral patch reefs and surrounding seagrass habitats. However, these changes were not uniform throughout the day, with differences in activity occurring only at dusk and differences in movement occurring at both dawn and dusk, but not midday. Although some lionfish foraging behaviors varied with prey density, overall lionfish density was more strongly related to differences in lionfish activity patterns. These temporal and spatial changes in lionfish foraging behaviors are consistent with the predicted effects of intraspecific competition and may have important consequences for lionfish removal efforts and native prey populations. Specifically, in areas with higher lionfish densities, prey fishes that are more active at dusk and/or inhabit seagrass beds near coral patch reefs may be more vulnerable to lionfish predation. By culling lionfish, managers may reduce the local foraging movements of lionfish and thus help maintain native fish communities in multiple habitats.

KEY WORDS: Behavior · Coral-reef fishes · Density dependence · Intraspecific competition · Movement · Non-native · Predator–prey interaction · *Pterois volitans*

INTRODUCTION

Local population density can strongly influence individual behavior and demographic rates through a variety of mechanisms. In some species there are benefits to living in groups, including increased access to mates, increased hunting efficiency, and reduced risk of predation (Packer & Ruttan 1988, Courchamp et al. 1999, Krause & Ruxton 2002, Gascoigne & Lipcius 2004). However, at high densities, intraspecific competition for food and shelter often causes reductions in individual growth and survival due to increased interference and/or exploitation of resources (Jones 1991, Keddy 2001, Krause & Ruxton 2002, Forrester 2015). Density-dependent behavioral and demographic changes are not only important to the population dynamics and regulation of a single species (Murdoch 1994, Hixon et al. 2002), but can also influence community-level dynamics and ecosystem processes (Micheli 1997, Clark et al. 2000).

If there is increased competition for food at higher conspecific densities, then predators may alter their foraging behavior in several ways, with subsequent consequences for prey populations. For example, if it takes longer to find and consume prey due to increased resource depletion or interference while foraging at higher densities, some individuals may increase the amount of time spent foraging (Clark & Mangel 1986,

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Shaw et al. 1995, Anholt & Werner 1995, Grand & Dill 1999, Bohlin & Johnsson 2004, White & Warner 2007), which may also be accompanied by an increase in conspecific aggressive encounters (Pintor et al. 2009, Kaspersson et al. 2010). Species that differentially forage over a diel cycle can expand their foraging time by hunting for longer during their typical hunting hours and/or by hunting at more periods of the day (Lawton 1987, Kronfeld-Schor & Dayan 2003, Wasserberg et al. 2006). Hunting at more periods of the day could in turn cause prey species with various diurnal patterns to be differentially susceptible to predation. In addition, predators at higher densities may expand their foraging range, which could enable them to exploit resources that have not yet been depleted and/or escape aggressive interactions in the areas with high densities of conspecifics (Micheli 1997, Forrester et al. 2006, Breed et al. 2013). If predators forage over broader distances, then prey species that inhabit the newly exploited habitats may be consumed.

Changes in foraging behavior as a result of increased local density may be particularly important to the population dynamics of both introduced predators and their native prey. Invasive species often reach higher abundances and individual body sizes in their invaded compared with their native ranges (Sakai et al. 2001, Grosholz & Ruiz 2003), and thus are likely to be strongly affected by intraspecific interactions. Furthermore, many invasive species are both competitively dominant and more abundant than ecologically similar native species in their new locations (Parker et al. 1999, Mack et al. 2000, Mooney & Cleland 2001, Sakai et al. 2001), suggesting that intraspecific rather than interspecific competition will have a larger influence on invasive species. Because invasive predators often cause larger reductions in native prey populations than do native predators (Salo et al. 2007), it is crucial to understand their foraging behavior at different densities and how this behavior in turn influences prey populations.

The Indo-Pacific red lionfish *Pterois volitans* is an invasive predatory reef fish that has reached extremely high abundances in parts of its invaded range (Côté et al. 2013, Albins & Hixon 2013). Since they were first sighted off the coast of Florida in the mid-1980s, lionfish have spread throughout the Caribbean, the Gulf of Mexico, and along the east coast of the Americas (Schofield 2010). Lionfish densities on reefs in their invaded range can be several orders of magnitude higher than in their native range (Green & Côté 2009, Darling et al. 2011, Kulbicki et al. 2012, McTee & Grubich 2014). At the same time, there is wide variation in lionfish densities within their invaded range, owing in part to differences in the length of time since establishment (Ruttenberg et al. 2012, Dahl & Patterson 2014), removal efforts (Frazer et al. 2012, de León et al. 2013), and abiotic site characteristics including depth and exposure to strong currents and wave surge (Whitfield et al. 2007, Anton et al. 2014). In contrast, invasive lionfish populations are likely unaffected by potential predators (Hackerott et al. 2013, Valdivia et al. 2014, but see Mumby et al. 2011) or interspecific competitors (Albins 2013), especially given that native predators are severely depleted throughout much of the Caribbean (Paddack et al. 2009, Stallings 2009).

Despite differences in lionfish densities both between and within their native and invaded ranges, how local lionfish density influences their hunting behavior has not yet been examined. Both native and invasive lionfish are primarily crepuscular hunters, with peaks in activity and stomach fullness occurring at dawn or at both dawn and dusk (Fishelson 1975, Morris & Akins 2009, Green et al. 2011, Cure et al. 2012, McTee & Grubich 2014). Invasive lionfish are extremely efficient predators, as they have high prey consumption rates (Albins & Hixon 2008, Côté & Maljkovic 2010) and cause large reductions in the abundance, biomass, and richness of native coral reef fishes (Albins & Hixon 2008, Albins 2013, 2015, Green et al. 2014, Benkwitt 2015). There is evidence that invasive lionfish experience intraspecific competition for food, as lionfish at higher densities on small patch reefs exhibit slower growth (Benkwitt 2013) and have diminishing per-capita effects on prey abundance and biomass (Benkwitt 2015). Given the effect of lionfish density on their individual growth rates, it seems likely that their foraging patterns also change at different local densities.

I conducted observations of lionfish to test the hypothesis that lionfish behavior changes at different lionfish and prey fish densities due to intraspecific competition for food. Specifically, I predicted that if there is intraspecific competition for food, then at higher lionfish densities and/or lower prey fish densities, lionfish will alter their foraging activity by (1) increasing the amount of time spent active and hunting at crepuscular periods and/or (2) increasing the number of periods per day during which they are active and hunting. In addition, I expected that lionfish would change the locations at which they hunt such that they would (3) spend less time sheltering within the reef and (4) increase the distances over which they travel while hunting at higher densities.

MATERIALS AND METHODS

Study sites

This study was conducted between June and August 2012 on coral patch reefs in Rock Sound near Cape Eleuthera, The Bahamas (24° 50′ 2.65″ N, 76° 16′ 6.78″ W). Lionfish first arrived at the study site in 2005 and there has been virtually no removal effort in the area, with the exception of isolated lionfish removals for field experiments (Green et al. 2014, Côté et al. 2014). I selected 16 reefs on which lionfish had not been previously manipulated and that were at least 300 m from any reef on which lionfish removals had occurred, which is greater than the maximum distance travelled by the majority of lionfish in the study area (Tamburello & Côté 2015). Reefs were selected to encompass a range of natural lionfish densities (1–16 lionfish reef^{-1}, 0.04–1.01 lionfish m^{-2}) and reef sizes (7.88–32.99 m^2 surface area), and there was no significant correlation between lionfish density and reef size (correlation = –0.11, t = 0.56, p = 0.58). Reefs were similar to each other in terms of rugosity, benthic community (algae-covered dead coral, live coral, and sponges), and surrounding habitat (sand and seagrass). Lionfish size on the reefs ranged from 6 to 30 cm total length (TL; mean = 18.2 cm), with the majority of lionfish (>90%) between 15 and 25 cm TL.

Lionfish behavior and movement

A pair of divers (observers) visited each reef at 3 times of day: within sunrise + 2 h ('dawn'), >3 h after sunrise and >3 h before sunset ('midday'), and within sunset – 2 h ('dusk'). During the study, sunrise varied between approximately 06:30 and 06:50 h and sunset varied between 19:30 and 20:00 h. Upon arriving at a reef, observers counted the number of lionfish present by conducting lionfish-focused searches, which involved first slowly circling reefs and then swimming over reefs until all areas had been covered. Divers paid particular attention to crevices and overhangs where lionfish are commonly found, and because of the small size of the reefs it was possible to thoroughly search the entire reef area. For each lionfish, observers recorded the size (TL visually estimated to the nearest cm), behavior, and location the moment it was sighted. Behaviors were categorized as resting (sitting on the substrate, not moving), hovering (in the water column oriented parallel to the bottom, but not moving), swimming (actively moving), or hunting (oriented head down with pectoral fins flared), with the latter 3 categories broadly grouped together as 'active' for some analyses. Similar classifications have been used in previous studies of lionfish behavior (Côté & Maljkovic 2010, Green et al. 2011, Cure et al. 2012). Location was categorized as the microhabitat on which lionfish were observed (e.g. under a ledge, on top of the reef, in the surrounding seagrass) and later divided into 2 major categories: sheltering (hidden under structure) or exposed (on top of reef or in surrounding area).

Then, 10-min focal observations were conducted on 2 lionfish selected using randomly generated numbers, or a single lionfish when there was only one individual present per reef. During focal observations, a trained observer recorded the behavior of lionfish at 30 s intervals for 10 min using the same categories as above. Simultaneously, a second observer videorecorded the focal lionfish to enable later analyses and confirmation of behaviors and allow divers to keep track of lionfish movement (see next paragraph). The observers also noted any strikes at prey, successful kills, and obviously aggressive interactions (chases, posturing) between lionfish or between lionfish and other species. However, there were very few observed strikes and aggressive interactions by focal lionfish, so those data were not analyzed. The observers maintained a distance of approximately 3 m from focal lionfish, a distance at which divers have no apparent influence on lionfish behavior (Côté & Maljkovic 2010, Green et al. 2011, Cure et al. 2012).

Throughout the entire visit to each reef, divers noted the time when any lionfish departed from or arrived at the reef and its behavior. A lionfish was defined as departing from the reef if it traveled at least 10 m from the reef. A lionfish was considered arriving at a reef if it swam in from the surrounding areas and had not been previously observed at that reef during that observation period. In only 3 instances were divers unsure whether an arriving lionfish was a new individual, as a lionfish was seen departing from the reef and traveled out of sight, and soon after another lionfish of the same size and coloration from the same direction returned. In these cases, it was assumed that these were the same lionfish rather than new individuals. Because the reefs were relatively small and divers had an unobstructed view of the surrounding area, the observers were reasonably confident that they counted all arrivals and departures of lionfish. However, because observers were most likely to miss arrivals and departures on reefs with the highest lionfish densities, if anything these results likely underestimate the effect of lionfish density on movement.

At the conclusion of the focal observations, the divers re-counted the number of lionfish present while conducting a survey of resident native fishes. Divers recorded the abundance and body size (TL) of all fish 1–15 cm TL, native mesopredators that are ecologically similar to lionfish (e.g. graysby grouper *Cephalopholis cruentata*), and top predators (e.g. Nassau grouper *Epinephelus striatus*) on and within 1 m of the reef. Surveys were conducted by slowly swimming in concentric circles gradually decreasing in size from the reef edge to the center of the reef until the entire reef area was surveyed. By slowly sweeping one hand just above the substrate, divers counted cryptic bottom-dwelling species such as gobies and blennies. Dive lights were used to search for cryptic species in crevices and under ledges.

Statistical analyses

Because multiple observations were conducted on the same reefs and there was evidence of heterogeneity in residuals based on reef, I conducted a series of generalized linear mixed effects models (GLMMs) fit using Gauss–Hermite quadrature with reef as a random effect (Zuur et al. 2009, Bolker et al. 2009). Fixed effects included lionfish density, prey fish density, and time of day. Because there were large fluxes in lionfish density on the reefs at dawn and dusk due to lionfish moving to and from the surrounding habitats (see 'Results'), I defined lionfish density as the density of lionfish on each reef at midday, which remained relatively constant during each observation period. This measure of lionfish density was significantly positively correlated with the maximum density of lionfish observed on each reef during each of the 3 observation periods (correlation = 0.83, $t = 13.3$, $p < 0.001$), and thus seemed to be an accurate representation of the relative density of lionfish that inhabited each reef throughout the study. Prey density was defined as the density of prey fishes at the time of each visit and was $\log(x + 1)$ transformed. I restricted prey fishes to those ≤5 cm TL, which are small enough to be vulnerable to a range of lionfish sizes and encompass the prey sizes most often consumed by all sizes of lionfish (Morris & Akins 2009, Muñoz et al. 2011). Prey density did not significantly vary with time of day (likelihood ratio test $\chi^2 = 2.75$, $p = 0.25$) and there was no significant correlation between lionfish density and prey density (correlation = −0.11, $t = 0.97$, $p = 0.33$). Because time of day had the largest influence on lionfish behavior in previous studies (Green et al. 2011, Cure et al. 2012) and to test whether the effect of lionfish or prey fish density on foraging behavior varies at different times of day, I also included interactions between time of day and each of the other explanatory variables as fixed effects. All reefs had similar densities of native mesopredators (0–0.05 fish m^{-2}) and top predators (0–0.30 fish m^{-2}), so I did not include these as explanatory variables in the analyses.

Response variables to test my predictions regarding lionfish behavior were based on observations of all lionfish on the reefs and on focal observations of individual lionfish. From all lionfish, the responses were the proportion of lionfish on each reef that were hunting upon arrival at the reef and the proportion of lionfish on each reef that were active upon arrival at the reef. From focal lionfish, the responses were the proportion of time individual focal lionfish spent hunting and the proportion of time individual focal lionfish spent active. Response variables to test predictions regarding lionfish position and movement were based on all lionfish on the reefs: the proportion of lionfish on each reef that were sheltering during initial observations, the proportion of lionfish arriving at each reef throughout observations, and the proportion of lionfish departing from each reef throughout observations. Because all responses were proportions, I modeled the data following binomial distributions with logit links. I conducted likelihood ratio tests (LRTs) to test for overall significance of fixed effects and Wald *Z*-tests to test for significance of single parameters (Zuur et al. 2009). All analyses were conducted in R version 3.0.2 (R Core Team 2013) with the associated package lme4 (Bates et al. 2014).

RESULTS

Lionfish behavior

A total of 95 lionfish were observed at dawn, 126 at midday, and 117 at dusk on and around the 16 coral patch reefs. A significantly higher proportion of lionfish hunted at dawn compared with at dusk and midday, and significantly more lionfish also hunted at dusk compared with at midday (all $z \geq 3.63$, all $p < 0.001$). This effect of time period on the proportion of lionfish hunting on each reef was not modified by lionfish or prey fish density (Lionfish × Time, Prey × Time: LRT $\chi^2 = 4.04, 2.31$, $p = 0.13, 0.32$, respectively; see Table S1, Figs. S1 & S2 in the Supplement at www.int-res.com/articles/suppl/m558p255_supp.pdf). However, the proportion of time that individual focal lionfish spent hunting varied with both time of day and prey density (Prey × Time LRT χ^2 = 16.91, $p < 0.001$; Fig. 1A, see Table S1 in the Supplement). At dawn and dusk, lionfish spent significantly more time hunting at higher prey densities (z = 3.09, 3.38, p = 0.002, 0.0007, respectively), with the odds of a lionfish hunting increasing by a factor of 2.43 at dawn and 2.58 at dusk for each doubling of prey density (95% CI = 1.60–8.12 and 1.76–8.28, respectively). Regardless of prey density, lionfish spent very little time hunting in the middle of the day. There was no significant effect of lionfish density on time spent hunting by focal lionfish at any time of day (all $z \leq$ 1.10, all $p > 0.27$; see Fig. S1 in the Supplement).

In contrast, the proportion of lionfish active (hovering, swimming, or hunting) varied with both time of day and lionfish density, but not prey density (Lion-Fish × Time: LRT χ^2 = 9.61, p = 0.008; Prey × Time: LRT χ^2 = 0.67, p = 0.71; Fig. 2A, see Table S1, Fig. S2 in the Supplement). Regardless of lionfish density, the majority of lionfish were active at dawn and few were active at midday. At dusk, however, a signifi-

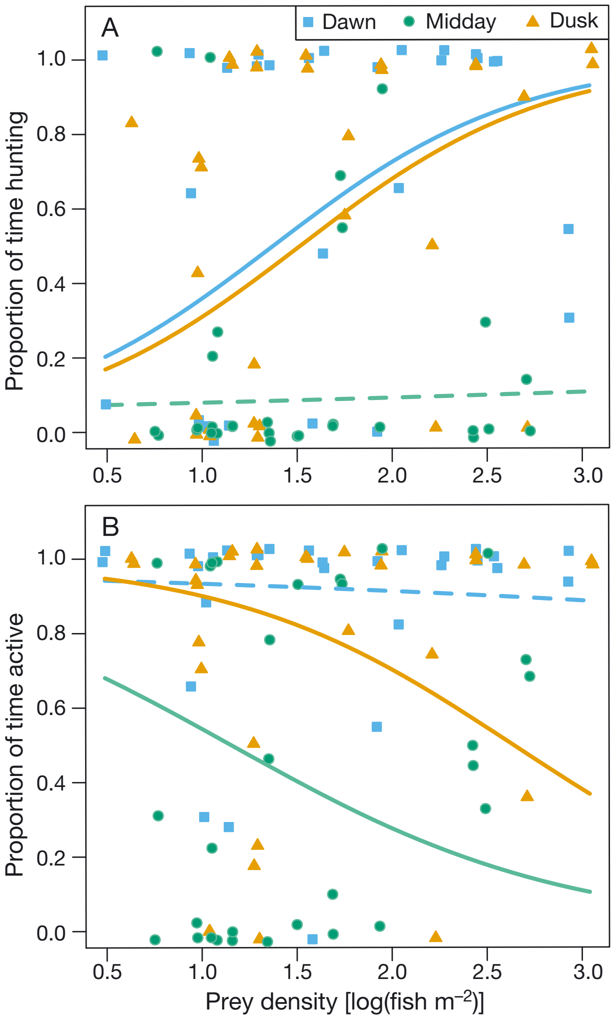

Fig. 1. *Pterois volitans*. Proportion of time individual focal lionfish spent (A) hunting and (B) active (hovering, swimming, or hunting) as a function of prey fish density at dawn, midday, and dusk. Lines are predicted probabilities based on generalized linear mixed effects models. Dashed lines represent slopes that are not significantly different than zero; solid lines represent slopes that are significantly different than zero ($p < 0.05$). Points represent individual lionfish and were randomly jittered to reduce overlap

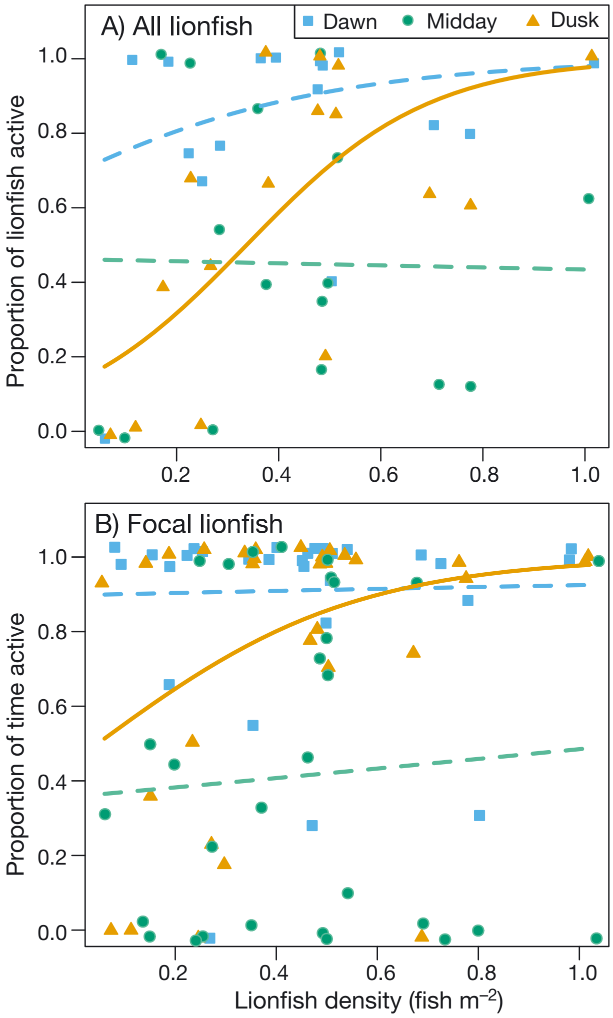

Fig. 2. *Pterois volitans*. (A) Proportion of lionfish that were active (hovering, swimming, or hunting) upon arrival at the reef and (B) proportion of time individual focal lionfish spent active as a function of lionfish density at dawn, midday, and dusk. Lines are predicted probabilities based on generalized linear mixed effects models. Dashed lines represent slopes that are not significantly different than zero; solid lines represent slopes that are significantly different than zero ($p < 0.05$). Points represent reefs (A) and individual lionfish (B) and were randomly jittered to reduce overlap

cantly higher proportion of lionfish were active at higher lionfish densities, with the odds of a lionfish being active increasing by a factor of 1.78 for each increase in lionfish density by 0.1 fish m^{-2} (95 % CI = 1.21–2.61; z = 2.93, p = 0.003). Similar to these reef-level patterns, individual focal lionfish spent significantly more time active at dusk at higher lionfish densities (z = 2.14, p = 0.032, 95 % CI = 1.03–2.07; Fig. 2B). The amount of time individual lionfish were active at dusk and midday also varied significantly with prey density (Fig. 1B), with the odds of being active decreasing by 37.8 % for each doubling of prey density on a reef at dusk (95 % CI = 19.3–73.7 %, z = 2.85, p = 0.004) and 42.3 % at midday (95 % CI = 19.7–90.5 %, z = 2.22, p = 0.027). These activity patterns were primarily driven by hunting and hovering behavior, as no lionfish were observed swimming upon arrival at the reef and focal lionfish spent an average of 1.6 % of their time swimming, compared with 46.3 % spent hunting and 21.1 % spent hovering.

Lionfish movement

The position of lionfish varied significantly with both lionfish density and time of day, but not with prey density (Lionfish × Time: LRT χ^2 = 14.59, p < 0.001; Prey × Time: LRT χ^2 = 0.88, p = 0.64; Fig. 3A, see Table S1, Fig. S3 in the Supplement). At dusk, for each increase in lionfish density by 0.1 lionfish m^{-2}, the odds of a lionfish sheltering decreased by a factor of 0.58 (95 % CI = 0.39–0.87, z = 2.63, p = 0.009). The majority of lionfish were exposed at dawn and the majority of lionfish sheltered at midday across all lionfish and prey densities.

Likewise, lionfish exhibited predictable movements between coral patch reefs and surrounding seagrass and sand habitats that varied with lionfish density and time of day, but not with prey density (Lionfish × Time: LRT $\chi^2 \geq 8.13$, p < 0.017; Prey × Time: LRT $\chi^2 \leq$ 4.15, p > 0.13; Fig. 3B,C, see Table S1, Fig. S3 in the Supplement). At dawn, a higher proportion of lionfish arrived from the surrounding areas at reefs with greater lionfish densities (z = 2.06, p = 0.039; Fig. 3B). For each increase in lionfish density by 0.1 fish m^{-2}, there was an increase in the odds of a lionfish arriving at the reefs by a factor of 1.42 (95 % CI = 1.02–1.99). At midday, there was very little lionfish movement, regardless of lionfish density. At dusk, a pattern opposite to that at dawn was observed, with a significantly higher proportion of lionfish departing from the reefs at higher lionfish densities (z = 2.96, p = 0.003; Fig. 3C). For each additional 0.1 lionfish m^{-2} on

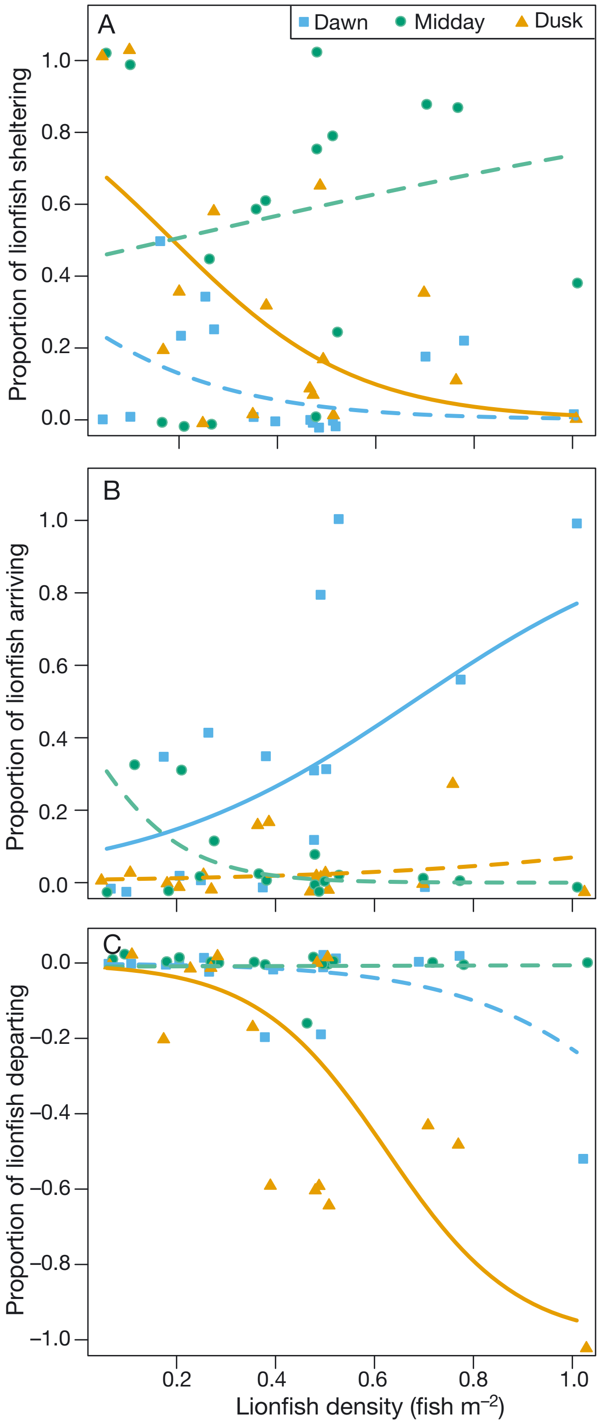

Fig. 3. *Pterois volitans*. (A) Proportion of lionfish that were sheltering upon arrival at the reef, (B) proportion of new lionfish that arrived at each reef, and (C) proportion of lionfish that departed from each reef as a function of lionfish density at dawn, midday, and dusk. Lines are predicted probabilities based on generalized linear mixed effects models. Dashed lines represent slopes that are not significantly different than zero; solid lines represent slopes that are significantly different than zero (p < 0.05). Points represent reefs and were randomly jittered to reduce overlap

a reef, the odds of a lionfish departing from the reef at dusk increased by a factor of 2.57 (95% CI = 1.75–4.79). Approximately 50% of the lionfish that departed from a reef returned to the same reef within the observation period. Larger lionfish exhibited more movements between coral patch reefs and the surrounding habitats, whereas no lionfish less than 15 cm TL was ever observed arriving at or departing from the reefs (Fig. 4).

DISCUSSION

There were significant differences in lionfish foraging activity and movement at different local densities that were consistent with the predicted effects of intraspecific competition. At higher densities, lionfish exhibited greater activity levels, time away from shelter, and more short-term movements between coral patch reefs and surrounding habitats. However, these changes with density were not consistent throughout the day, with the greatest differences in behavior occurring at dusk. Although prey density was associated with changes in some foraging behaviors by individual lionfish, overall it appears that lionfish density was more important in explaining activity patterns. These spatial and temporal changes in behavior may, in turn, change which prey individuals and species are most susceptible to lionfish predation.

Similar to prior studies in both their native and invaded ranges, I observed high levels of lionfish activity at dawn and dusk but very little activity during the middle of the day (Green et al. 2011, Cure et al. 2012, McTee & Grubich 2014). Despite this overall consistency in diel activity patterns, there is still considerable variation in lionfish activity levels among locations within both their native and invaded ranges due in part to local differences in habitat, current strength, and prey availability (Cure et al. 2012). The differences in behavior on reefs with varying lionfish densities in the present study, combined with the wide range of lionfish population density both within and between oceans (Green & Côté 2009, Darling et al. 2011, Kulbicki et al. 2012), suggest that local lionfish density may also help explain some of the site-specific variation in hunting behavior.

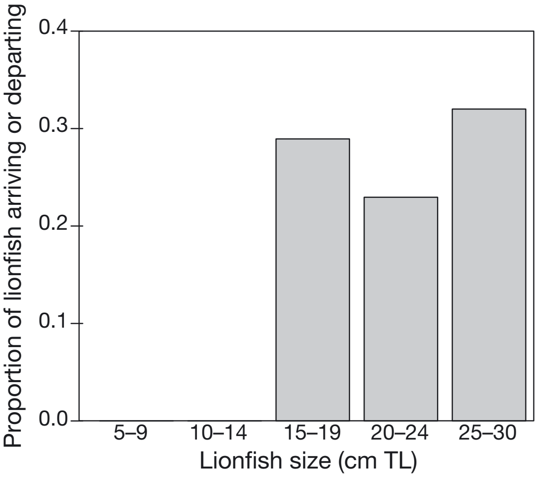

Fig. 4. *Pterois volitans*. Proportion of lionfish within each size class that arrived at, or departed from, the reefs. Sample sizes (number of lionfish) in each size class (from smallest to largest): 7, 10, 235, 61, and 25

Exploitative competition for food is the likely cause of reduced growth rates in juvenile lionfish at higher densities (Benkwitt 2013, 2015). However, if exploitative competition for food was the main driver of changes in lionfish foraging behavior, then lionfish should exhibit greater hunting, activity, and movement at lower prey densities regardless of lionfish density, which was not the case in this study. Instead, individual lionfish spent more time hunting but less time active at higher prey densities, which suggests that they may spend less time searching for prey at higher prey densities (Anholt & Werner 1995, Lubin & Henschel 1996, Harding et al. 2007). In contrast, the proportion of lionfish on each reef that were hunting, active, sheltering, and moving did not vary with prey density. A possible explanation is that the species composition of prey has a larger influence on lionfish foraging behavior than total prey density. Lionfish exhibit diet preferences for certain prey characteristics, with small, solitary, bottom-dwelling fishes most susceptible to lionfish predation (Green & Côté 2014). Therefore, teasing apart the influence of overall prey density compared with densities of preferred prey may further clarify the role of exploitative competition in affecting the foraging behavior of lionfish.

In contrast, many aspects of lionfish activity changed at increased lionfish densities, which suggests that interference competition is the main form of competition among lionfish and is driving the changes in lionfish foraging behavior. However, aggressive interactions between lionfish are rare (Cure et al. 2012), and I likewise observed few apparent aggressive encounters between lionfish at any density. Instead, lionfish may interfere with each other's foraging efficiency in ways other than direct aggression (Krause & Ruxton 2002). For example, in several instances I observed one lionfish strike at a prey fish, and immediately thereafter several others swam to the same location. Even without being aggressive, the presence of these other lionfish could potentially decrease the foraging efficiency of some individuals. In addition, perceived competition, in the form of mirrors or competitors in separate tanks, causes changes in the foraging activity and behavior of many other species (Barnard et al. 1983, Dill & Fraser 1984, Nonacs & Calabi 1992), further suggesting that even without direct aggressive encounters, conspecifics could have a significant effect on lionfish foraging behavior.

Escaping intraspecific competition is a major driver of density-dependent movement in many coral-reef fishes (Abesamis & Russ 2005, Grüss et al. 2011, Green et al. 2015), and a tagging study of lionfish conducted over the course of several months also found greater movement among patch reefs at higher lionfish densities (Tamburello & Côté 2015). The crepuscular movements observed in the present study, however, appeared to be short-term foraging excursions given that approximately half of the lionfish returned to the same reef within the observation period, and lionfish in this system cause reductions in prey fish populations in the areas surrounding coral patch reefs up to at least 30 m from the nearest reef (Benkwitt 2016). Many other families of coral-reef fishes, including grunts (Haemulidae) and snapper (Lutjanidae), exhibit similar crepuscular movements between coral patch reefs and seagrass beds. These fishes leave their daytime reef shelters and forage in the surrounding seagrass at night, with important consequences for prey populations as well as community structure and nutrient transfer (Nagelkerken 2009, Ogden et al. 2014). Several species of scorpionfish (Scorpaenidae), which are in the same family as lionfish, also leave their shelter in the evening, forage at night, and return to the same shelter in the morning (Harmelin-Vivien & Bouchon 1976).

The effects of intraspecific competition are often asymmetric, such that smaller, subordinate individuals in a population are most affected (Jones 1991, Peckarsky & Cowan 1991, Davey et al. 2005, Nilsson 2006, Ward et al. 2006, Samhouri et al. 2009). If this is the case, then only certain individuals may change their foraging locations and behavior at higher levels of competition (Webster 2004, Breed et al. 2013). In the present study, the behaviors of randomly selected individual lionfish were fairly consistent with the initial behaviors of all lionfish on the reefs, which suggests that the effects of competition may be equally experienced by all individuals. However, no focal observations were conducted on lionfish less than 13 cm TL, so there may be differences in the behavior of the smallest individuals that were not captured in the present study. In terms of movement, larger lionfish were more likely to travel between patch reefs and the surrounding habitats, with smaller lionfish (<15 cm TL) never departing from their home reefs. Similarly, previous studies of lionfish movement have found high site fidelity of small lionfish over the course of several months (Jud & Layman 2012, Benkwitt 2013, Tamburello & Côté 2015), although some larger lionfish also have high site fidelity (Akins et al. 2014). This apparent discrepancy may be caused by factors besides competition. Smaller individuals may remain closer to shelter as an adaptive response to predation (Lima & Dill 1990), although there are few documented predators of lionfish in either their native or invaded range (Bernadsky & Goulet 1991, Maljković et al. 2008). Smaller lionfish may also be more affected by strong ocean currents, and therefore remain sheltered on coral patch reefs because they are unable to swim into open areas. Finally, smaller lionfish consume different prey than larger lionfish, as there is an ontogenetic shift from a primarily invertebrate-based to a fish-based diet by the time lionfish reach approximately 20 cm TL (Morris & Akins 2009, Muñoz et al. 2011). Consequently, smaller individuals may not need to forage over broader distances because their preferred food may not be depleted near reefs. This seems likely given that the majority of lionfish were between 15 and 25 cm TL, with only a few smaller individuals on each reef. Therefore, the majority of competition for food likely occurred between individuals in larger size classes.

Although intraspecific competition is a likely explanation for increased activity and movement of lionfish at higher densities, there are several other non-mutually exclusive possibilities. First, at higher densities there may be more opportunities to engage in group hunting behavior, which has been shown to increase per-capita consumption rates of lionfish in the Indo-Pacific (Kendall 1990, Lönnstedt et al. 2014). If invasive lionfish also have increased hunting success when in groups, then there may be an advantage to being more active and increasing their foraging movements at higher densities. Lionfish may also spend less time sheltering and undergo more foraging movements at higher densities due to differences in the perceived risk of being attacked by larger predators. Even when a prey species is not subject to direct predation, it may still modify its behavior in response to predators, so departing from the relatively safe shelter of the reefs in groups may provide ‘safety in numbers’ for lionfish (Creel &

Christianson 2008). Finally, some activity and movement into the surrounding seagrass may be related to spawning behavior, which likely occurs at night. On one occasion, I observed apparent courtship behavior similar to that described by Fishelson (1975) and Green et al. (2011). Three lionfish (between 19 and 28 cm TL) rapidly departed the reef, and once 20 m away in the open sand and seagrass they swam in circles around each other for approximately 2 min. Two lionfish then returned to the reef while the remaining lionfish continued swimming rapidly away from the reef for at least 200 m. The fact that lionfish can spawn up to every 4 d (Morris 2009) suggests another explanation for why larger lionfish departed the reefs on a regular basis, especially when there were higher local densities of mature adults.

Overall, the observed behavioral differences among invasive lionfish at different densities may have implications for native prey populations. There was a peak in feeding and activity at dawn regardless of lionfish density, but at dusk lionfish were more active only at higher densities. This observation suggests that native prey species that are primarily active at dusk will be affected only in areas with high lionfish densities. At both dawn and dusk, there was increased movement to and from habitats surrounding patch reefs at higher lionfish densities. Given that lionfish deplete prey populations in the habitats surrounding coral patch reefs (Benkwitt 2016), lionfish at higher densities may be causing greater reductions in a wider range of prey species, potentially including juveniles of commercially and ecologically important fishes that use seagrass beds as nursery grounds (Nagelkerken 2009, Ogden et al. 2014). In contrast, lionfish were almost always resting during the middle of the day regardless of lionfish density, which suggests that strictly diurnal fishes may be relatively safe from lionfish predation when these prey fishes are most active. In addition to increasing their time spent foraging and their foraging movements, many fishes shift to feeding on less preferred prey at higher densities (Coates 1980, Dill 1983, Holbrook & Schmitt 1992, Schindler et al. 1997, Svanbäck & Persson 2004, Agashe & Bolnick 2010). There is currently no evidence that lionfish have different isotopic diet signatures at sites with high compared with low lionfish densities (J. Curtis unpubl. data), but if they do alter their diets at higher densities, then there may be even more consequences for prey populations.

Future studies should be conducted to determine the extent to which these results apply to other locations with different lionfish densities and habitat characteristics. Densities of lionfish observed here (mean = 3763 lionfish per hectare) are comparable to the maximum reported densities from small artificial structures in the Gulf of Mexico (3850 lionfish per hectare; Dahl & Patterson 2014), but higher than those observed on larger patch and continuous reefs in many parts of the invaded range (mean ~3–640 lionfish per hectare; McTee & Grubich 2014). Based on these differences, the effects of lionfish density on patch reefs where lionfish are concentrated within small, isolated areas may be diluted when scaled up to continuous reefs. However, even on larger reefs, lionfish often aggregate in small groups around structures during the day, and there is some evidence that on large patch reefs (100–1200 m^2) lionfish similarly move over a broader area including the surrounding sand while foraging during crepuscular times (A. C. D. Davis unpubl. obs.).

The main method of managing lionfish populations is through manual removal by divers, which is a time- and labor-intensive process. However, removals can significantly reduce local lionfish abundances (Frazer et al. 2012, de León et al. 2013) and even partial removals can help maintain native prey populations on coral patch reefs (Green et al. 2014). Given that at higher densities lionfish also have increased activity levels at dusk and movements at both dawn and dusk, removals on patch reefs may be most efficient during midday when lionfish are aggregated on the reefs. Furthermore, removals may have more benefits than previously documented. By keeping lionfish densities low, managers may reduce the local foraging movements of lionfish and thus help maintain native fish communities in multiple habitats.

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Contribution to the Theme Section 'Invasion of Atlantic coastal ecosystems by Pacific lionfish'

Native grouper indirectly ameliorates the negative effects of invasive lionfish

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ABSTRACT: Non-trophic interactions between Indo-Pacific lionfish *Pterois volitans* and *P. miles* and Atlantic and Caribbean reef fishes are not yet well understood. To determine the effects of potential competitive and behavioral interactions between native predators and invasive lionfish, we experimentally altered the presence of lionfish and red grouper *Epinephelus morio* in karst solution holes in Florida Bay, USA, and then tracked subsequent changes in the juvenile reef fish and motile macroinvertebrate communities for 6 wk. Relative to solution holes where we excluded both predators, mean juvenile reef fish abundance declined 83.7% in solution holes with a lionfish but increased by 154% in solution holes with a red grouper. There was no difference in juvenile reef fish abundance in solution holes with both lionfish and red grouper compared to holes where we excluded both predators. The composition of lionfish stomach contents shifted from mostly teleost fishes when lionfish were present in solution holes alone, to mostly crustaceans when in the presence of a red grouper. Concurrently, the abundance of 2 species of cleaner shrimp (*Ancylomenes pedersoni* and *Periclimenes yucatanicus*) decreased by 14.7% when lionfish were present but increased by 56.2% at holes where lionfish were excluded. We suggest that these results are due to altered lionfish predatory behavior in the presence of red grouper and highlight the importance of maintaining intact native predator communities for ameliorating the negative effects of the lionfish invasion.

KEY WORDS: Lionfish · Red grouper · Interspecific interactions · Community ecology · Reef fish · Invasive species · Ecological impacts

INTRODUCTION

A number of classic studies in ecology have shown the importance of predators in shaping the structure of prey communities across diverse marine ecosystems. More specifically, substantial evidence supports the ability of resident fish predators to affect the size and structure of reef fish communities via piscivory on post-settlement fish recruits (Hixon & Beets 1993, Stallings 2009). These studies have largely focused on the direct interactions between predators and prey fish; however, predators can also affect the size and structure of prey communities via indirect interactions. For example, predators can shape prey communities when they alter the traits or behaviors of intermediate species, a phenomenon commonly known as behaviorally mediated indirect interactions (BMIIs; Strauss 1991). Interaction chains driven by changes in the behavior or traits of intermediate species are often at least as strong as density-driven effects and may in fact account for the majority of predator effects on food chains (see reviews by Werner & Peacor 2003 and Preisser et al. 2005).

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Among the studies to explicitly test piscivory-driven BMIIs, Stallings (2008) found a strong, positive effect of Nassau grouper *Epinephelus striatus* on the abundance of juvenile coral reef fishes in Bahamian patch reefs that was driven by changes in the foraging behavior of small-bodied groupers. Pusack (2013) investigated a similar interaction in which he found evidence for a BMII between Nassau grouper and juvenile coral reef fishes mediated by the behaviors of the lionfish *Pterois volitans* and *P. miles*, piscivores native to the Indo-Pacific and invasive in the western Atlantic and Caribbean since at least 1985 (Schofield 2009). Since about 2004, lionfish have experienced a rapid range expansion throughout the Caribbean, Gulf of Mexico, and southeastern US Atlantic waters and have been present on reefs in the Florida Keys, USA, since at least 2009 (Ruttenberg et al. 2012). As generalist predators, lionfish consume a diverse array of fishes and invertebrates (Valdez-Moreno et al. 2012, Côté et al. 2013a), and some evidence suggests an ontogenetic shift in diet where larger lionfish consume mostly fish (Morris & Akins 2009). On invaded coral reefs, lionfish predation can reduce the abundance of native fishes by 80 to 94% and the biomass by up to 65% (Albins & Hixon 2008, Green et al. 2012a, Albins 2013). By 2010 lionfish had invaded the hard bottom habitats of Florida Bay (see Fig. 1), which serves as an important nursery habitat for many fishes and invertebrates that move to nearby coral reefs as adults (Fourqurean & Robblee 1999). Consequentially, predation by invasive lionfish in Florida Bay on juvenile coral reef fishes may have cascading consequences for the health of nearby coral reefs.

A common mesopredator resident in the hard bottom habitats of Florida Bay that will interact with invasive lionfish is the red grouper *Epinephelus morio*. In Florida Bay, red grouper are primarily associated with karst hard bottom features called solution holes — pockmarked pits in the limestone formed by past freshwater incursion — which they excavate by removing sediment and detritus (Coleman et al. 2010). Previous experiments conducted on the faunal communities associated with Florida Bay solution holes showed that red grouper presence positively affected the abundance and diversity of these communities and that the community-level effects were driven by strong interactions with only a small number of individual species from the total species pool (Ellis 2015). This group included some juvenile coral-reef fishes, primarily small juvenile grunts *Haemulon* spp., which were consistently among the most numerous fauna encountered in solution holes. Red grouper consume primarily crustaceans and some demersal fishes (Moe 1969, Weaver 1996) and are territorial, making aggressive displays that include low frequency sound production and rapid direct approaches to conspecifics and other resident solution-hole fishes (Ellis 2015). These behaviors, combined with the fact that the red grouper is usually the largest individual animal encountered in solution holes, may displace or disrupt predation by resident and transient predators around solution holes. Over time, such behavioral interactions between red grouper and other piscivores could result in differential survival of post-settlement juvenile reef fish when compared to their survival in habitats without red grouper. This hypothetical BMII could be important in altering the predatory effects of lionfish that invade Florida Bay solution holes.

Several species of shrimp commonly found in Florida Bay solution holes, including *Periclimenes yucatanicus*, *Ancylomenes pedersoni*, and *Stenopus hispidus*, have been found in lionfish stomach contents (Morris et al. 2009, Faletti & Ellis 2014). At least one of these species, *A. pedersoni*, is an experimentally verified cleaner that removes ectoparasites from reef fishes (Bunkley-Williams & Williams 1998, McCammon et al. 2010). It is yet unknown how the presence of invasive lionfish may affect crustacean behaviors. Some shrimp species will change their behavior in the presence of finfish predators, often relying on habitat features for protection (Ory & Thiel 2013). Two common species of anemones in Florida Bay, *Condylactis gigantea* and *Bartholomea annulata*, have symbiotic relationships with cleaner shrimp and may offer protection from predation for these species (Silbiger & Childress 2008, Briones-Fourzán et al. 2012). Given that ectoparasites removed by cleaner shrimp can have negative and even lethal consequences for parasitized fish (Artim et al. 2015), lionfish predation on these species could represent another indirect negative effect on the native reef fishes in Florida Bay.

While other large-bodied groupers, including Nassau and tiger grouper *Mycteroperca tigris*, reportedly prey on lionfish (Maljković et al. 2008), red grouper apparently do not (Morris 2009). Mesocosm experiments have shown little effect of native grouper presence on lionfish behaviors (Morris 2009, Raymond et al. 2015). Furthermore, it has been widely debated whether mesopredators such as groupers actually function as biocontrol for invasive lionfish (see Mumby et al. 2011 and subsequent responses by Hackerott et al. 2013 and Valdivia et al. 2014). However, Pusack (2013) reported that Nassau grouper appeared to reduce the effect of lionfish predation on native reef fish abundance. As congeners, Nassau and red groupers are extremely similar in appearance and size. Given these similarities, we were motivated to investigate if a similar behavioral interaction may occur between lionfish and red grouper. To do this, we set up an experiment to test the potential BMII between red grouper and juvenile reef fish mediated through lionfish in Florida Bay solution holes. Here we present the results of an experiment designed to quantify (1) the effects of red grouper on solution hole-associated juvenile reef fish abundance and diversity; (2) the effects of lionfish on solution hole-associated juvenile reef fish abundance and diversity; and (3) the modification in lionfish effects on juvenile reef fish abundance and diversity in the presence of red grouper.

MATERIALS AND METHODS

Study site

Florida Bay is a large open embayment in south Florida bordered by the Florida Keys, the Everglades, and the Gulf of Mexico (Fig. 1). The benthic habitat of Florida Bay is primarily seagrass, interspersed with areas of karst hard bottom that is usually covered in a thin veneer of sediment and pockmarked with solution holes (Fourqurean et al. 2002). For this study, we used a set of 16 solution holes located in outer Florida Bay that were similar in terms of size and location (see Table S1 in the Supplement at www.int-res.com/articles/suppl/m558p267_supp.pdf). Solution hole area, defined here as the product of the 2 longest perpendicular measurements, ranged from 1.69 to 6.99 m^2 (mean ± SE: 4.20 ± 0.371 m^2). The maximum excavated depth of solution holes, defined here as the deepest single measurement taken within the excavated area of the solution hole, ranged from 26 to 77 cm (45.3 ± 3.01 cm).

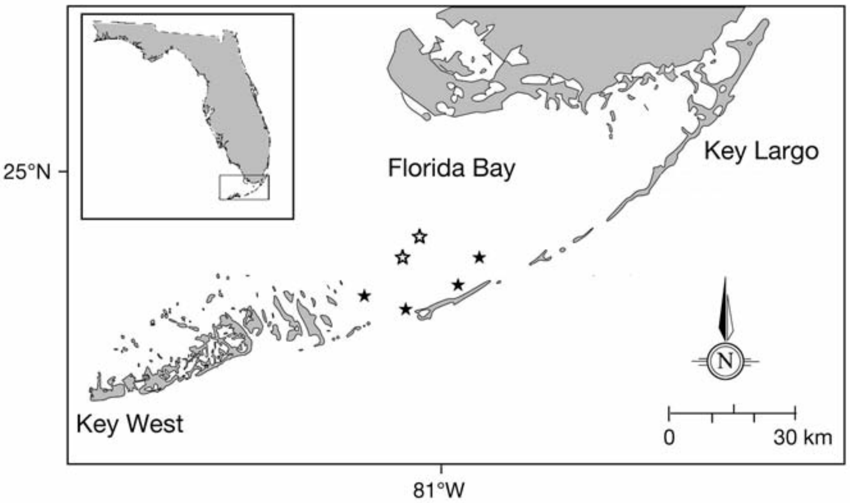

Fig. 1. Approximate locations of solution hole sites in southwest Florida Bay, USA, used in this study. Open stars: sites where we conducted experimental manipulations; closed stars: sites where we collected lionfish

Experimental design

To test the effects of lionfish on the abundance, diversity, and community structure of juvenile reef fish associated with solution holes in the presence and absence of red grouper, we conducted a 6 wk field experiment where we manipulated the presence of both red grouper and lionfish in solution holes. We first surveyed solution holes in early June 2013 to assess red grouper and lionfish presence. All solution holes were occupied by a red grouper, but no lionfish were present at any of the solution holes at the start of the experiment. We randomly assigned the 16 red grouper-occupied solution holes to one of 4 treatment groups (n = 4): (1) no predators; (2) lionfish alone; (3) red grouper alone; and (4) both lionfish and red grouper. Here we use the term 'no predators' to refer only to the absence of red grouper and lionfish in this treatment; we did not manipulate the abundance of any other native predators during the experiment. We used the 'no predator' treatment to estimate the effect of all other native predators (e.g. black grouper, toadfish, etc.) on solution hole communities. For the predator treatments, we used only a single lionfish or single red grouper to match the typical densities of these predators as observed in Florida Bay solution holes. Red grouper are generally solitary and often displayed territorial aggressive displays towards conspecifics (Ellis 2015). Lionfish densities in Florida Bay from 2010 to 2012 were similarly low and, despite an increase in occurrence over time, it was rare to encounter more than a single individual lionfish in a solution hole. We assumed that red grouper were unlikely to prey on lionfish but that red grouper presence would disrupt lionfish predation. This assumption allowed us to ignore any density-mediated indirect interactions (DMIIs) and just test for BMIIs between red grouper and the suite of solution hole-associated juvenile coral reef fishes via lionfish. For the purposes of estimating the BMII between red grouper and juvenile reef fish via lionfish, the no predator treatment represents the baseline 'control' condition, the red grouper alone and lionfish alone treatments estimate the total effect of each predator separately on juvenile reef fish abundance, and the both predator treatment represents the 'threat' condition (sensu Okuyama & Bolker 2007).

Red grouper present at the no predator and lionfish alone treatment holes were captured using hook-and-line, measured for total length (TL), tagged with a dart tag, and released at a vacant solution hole located at least 5 km from the experimental study area. Previous experiments conducted in this system showed that relocating red grouper in this method was sufficient to prevent recolonization during the experiment (Ellis 2015). We collected 8 lionfish from hard bottom habitats located elsewhere in Florida Bay using monofilament hand nets, and then immediately transported them to the study area. Lionfish were measured and then individually released at each of the appropriate assigned lionfish alone or both predator solution holes. Lionfish are known to exhibit high site fidelity (Jud & Layman 2012), so we did not expect them to migrate between solution holes during the study. The average size of lionfish used during the 2013 experiments was 17.4 ± 0.89 (SE) cm TL, which corresponds to an age of ~4–5 mo (Potts et al. 2011).

Prior to predator manipulations, a team of 2 divers on SCUBA conducted a visual census of all reef fishes associated with all 16 solution holes, following the methods described by Hixon & Beets (1993). After slowly approaching the solution hole to a distance of ~1 m from the edge, each diver slowly circled the hole while recording the number and identity of each fish species. Divers first focused on the active planktivores hovering above the hole, then enumerated and recorded any demersal and cryptic fishes and macroinvertebrates found inside the hole using flashlights to aid identification. Divers then summed their recorded abundances for each species and determined the average number per species. Total species richness was determined as the sum of all distinct species observed by both divers. Divers visually estimated the size of each fish to the nearest 1 cm (below 10 cm TL) or to the nearest 5 cm (above 10 cm TL). Each survey lasted until all individuals were counted, or for a minimum of 5 min (mean census duration was about 12 min). The grunt species complex in Florida Bay contains at least 6 different species that are visually indistinguishable at sizes <5 cm TL, so grunts were identified to species when possible and individuals <5 cm were grouped together as 'grunt recruits.' Divers also noted the identity and habitat association of cleaner shrimp. Specifically, divers noted if shrimp were found within 10 cm of either of the 2 anemones found in Florida Bay known to host cleaner shrimp (*C. gigantea* and *B. annulata*), or were found elsewhere in the solution hole not near anemones. This protocol was repeated weekly for 6 wk (7 total surveys at each of the 16 solution holes). On average, it took 2 d to survey all 16 experimental solution holes.

In addition to conducting weekly diver censuses, we checked the no predator and lionfish alone treatment holes once every 48 h for the duration of the experiment to ensure that no new red grouper or lionfish had moved onto these sites. New individuals encountered were captured, measured, tagged, and released at unoccupied sites as described above. At the end of the experiment all lionfish were collected with hand nets and euthanized with an overdose of MS-222. We followed the methods described by Green et al. (2012b) for all lionfish dissections: first, we recorded lionfish total length and dry blotted weight, then removed and weighed the stomach, entire alimentary canal, and all stomach contents. We recorded the length and dry-blotted weight of all prey items before identifying prey to the lowest possible taxonomic group using guides from Humann & Deloach (2002) for fishes and Abele & Kim (1986) for crustaceans. Unrecognizable prey items were identified using undigested hard parts (e.g. otoliths, skeletons) whenever possible. Finally, we calculated the proportion of each prey group in the diet by number, size, weight, and frequency of occurrence.

Statistical analysis

We calculated a variety of community response variables to measure the effects of red grouper and lionfish separately and in concert on the native juvenile reef fish populations associated with solution holes: total abundance (N), Hill's diversity numbers H_0, H_1, and H_2, and Hill's evenness (E). Hill's numbers provide a means of calculating commonly used diversity indices using the single equation:

$$H_a = \left(\sum_{i=1}^{S} p_i^a \right)^{\frac{1}{1-a}} \qquad (1)$$

where p_i is the relative proportion of the community made up by species i (Hill 1973). When evaluated for integer values for a of 0, 1, and 2, H_a reduces to species richness, the antilog of the Shannon-Wiener index, and the reciprocal of the Simpson's index, respectfully. Generally, as *a* increases, the index gives greater weight to more abundant species. Hill's evenness, *E*, was calculated using the equation:

$$E_{2,1} = H_2/H_1 \quad (2)$$

that Hill initially proposed because it does not include species richness (H_0), and therefore is relatively insensitive to sample size (Hill 1997). Hill's evenness converges to 1 when all species are equally abundant, so smaller values indicate more uneven communities.

We used linear mixed-effects models (LMM) to draw inferences about the effects of predator treatment on the 5 community metrics (N, H_0, H_1, H_2, and E), following the methods described by Albins (2013). The models included 2 categorical explanatory variables, *predator treatment* and *week*, and a random intercept for each solution hole. Time (*week*) was included as a categorical variable to eliminate any assumptions about the relationship between response variables and time. Solution hole identity was included as a random variable due to the repeated measures design of the experiment that required multiple observations of the same hole across time. Model selection was based on likelihood-ratio tests (LRT) performed on nested models to determine if including a treatment by the week interaction term improved the model fit. Visual examination of the model residuals suggested violations of the assumptions of both homogeneity of variance and independence, so we fit alternative models for each community response metric: one that incorporated heteroscedasticity among treatments, one that incorporated temporal autocorrelation among observations within solution holes using the $AR(1)$ autoregression model, and one that incorporated both variance and autocorrelation structures. Because the addition of the variance and autocorrelation structures caused the models to be non-nested, the resulting model fits were compared with Akaike's information criterion (AIC) to determine the optimal model for hypothesis testing. *t*-tests were used to evaluate differences between all treatment contrasts for each of the 5 community response metrics at the end of the experiment, when the best-fit model from the LMM analysis indicated that predator treatment had a significant effect.

To evaluate the effects of red grouper and lionfish on the structure of the juvenile reef fish communities, we performed ordinations with non-metric multidimensional scaling (NMDS; McCune & Grace 2002). For all ordinations, recruit abundances were square-root transformed and standardized using the Wisconsin double standardization, where abundances were first standardized by species maxima and then by the sample total. We calculated Bray-Curtis distances for the ordinations and for hypothesis testing of recruit community structure and then tested for differences in the composition of recruit communities with permutational multivariate analysis of variance (PERMANOVA; Anderson 2001). Recruit abundances were not transformed or standardized, and all analyses were run with 1000 unconstrained permutations. Statistical analyses were conducted in the R software environment (R Core Team 2014) using the 'lme4' package for the LMMs (Bates et al. 2015), and the 'MASS' and 'vegan' packages for the NMDS and PERMANOVA analysis (Venables & Ripley 2002, Oksanen et al. 2011).

We estimated effect sizes for the direct effects of lionfish, the total indirect effects of red grouper, and the effects of both predators together on juvenile reef fish recruit abundance using a ratio-based approach (Trussell et al. 2006, Hughes et al. 2012). The direct effect (DE) of lionfish (LF) on recruit abundance, *R*, was calculated with the ratio of recruit abundance in the lionfish alone treatment to the mean recruit abundance in the no predator (NP) treatment:

$$\mathrm{DE}_{\mathrm{LF}} = (R_{\mathrm{LF}}/R_{\mathrm{NP}}) - 1 \quad (3)$$

Similarly, the indirect effect (IE) of red grouper on recruit abundance was calculated with the ratio of recruit abundance in the red grouper alone treatment (RG) to the mean recruit abundance in the no predator treatment:

$$\mathrm{IE}_{\mathrm{RG}} = (R_{\mathrm{RG}}/R_{\mathrm{NP}}) - 1 \quad (4)$$

Finally, the BMII of red grouper on recruit abundance via lionfish was calculated with the ratio of the recruit abundance with both predators (BP) (i.e. the effect of both predators or the lionfish effect in the presence of predator cues) to the mean recruit abundance in the lionfish alone treatment (following Okuyama & Bolker 2007):

$$\mathrm{BMII} = (R_{\mathrm{BP}}/R_{\mathrm{LF}}) - 1 \quad (5)$$

The numerators for all ratios were provided by all replicates of the given treatment, whereas the denominator was the mean recruit abundance at the end of the experiment for the given treatment. We estimated means and 95% confidence intervals for all effect sizes by bootstrapping 1000 times with replacement to account for the low number of experimental replicates available for each of the treatments. This approach is similar to the methods used by Paine (1992) to quantify interaction strength.

In an earlier analysis, we found a relatively high

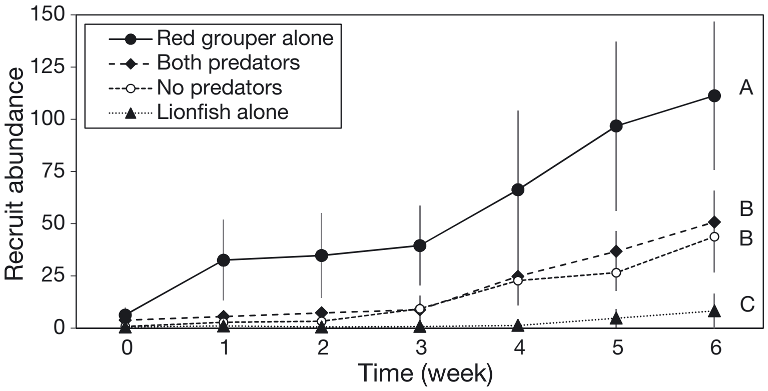

Fig. 2. Abundance (mean ± SE) of juvenile reef fish recruits at solution holes in Florida Bay from 4 experimental predator treatments (n = 4 per predator treatment). Letters at the far right: results of pairwise comparisons among treatments at the final census; matching letters indicate $p > 0.05$ based on pairwise *t*-tests performed on the best-fit linear mixed-effects model

Table 1. Estimated mean ± SE of juvenile reef fish recruit community abundance, species richness, diversity, and evenness. p-values from pairwise *t*-tests performed on each of the 6 *a priori* contrasts based on the 5 community response variables at the end of the 6 wk experiment; n = 4 per predator treatment; *$p \le 0.1$, **$p \le 0.05$. NP: no predator; LF: lionfish alone; RG: red grouper alone; BP: both predators

Response	Treatment	Estimate	Contrast	p
Abundance			NP vs. LF**	0.037
	NP	43.8 ± 17.2	NP vs. RG**	0.050
	LF	8.25 ± 8.25	NP vs. BP	0.413
	RG	111 ± 35.4	LF vs. RG**	0.023
	BP	50.8 ± 15.2	LF vs. BP**	0.027
			RG vs. BP*	0.059
Species richness (H_0)			NP vs. LF**	0.010
	NP	4.50 ± 0.87	NP vs. RG	0.148
	LF	1.00 ± 1.00	NP vs. BP	0.296
	RG	3.25 ± 0.63	LF vs. RG**	0.014
	BP	5.75 ± 0.63	LF vs. BP**	<0.001
			RG vs. BP**	0.031
Shannon diversity (H_1)			NP vs. LF*	0.059
	NP	2.78 ± 0.17	NP vs. RG	0.171
	LF	0.86 ± 0.86	NP vs. BP	0.448
	RG	2.30 ± 0.41	LF vs. RG	0.104
	BP	3.24 ± 0.71	LF vs. BP**	0.033
			RG vs. BP	0.208
Simpson diversity (H_2)			NP vs. LF*	0.077
	NP	2.25 ± 0.08	NP vs. RG	0.722
	LF	0.78 ± 0.78	NP vs. BP	0.470
	RG	2.09 ± 0.41	LF vs. RG*	0.097
	BP	2.55 ± 0.35	LF vs. BP*	0.053
			RG vs. BP	0.427
Evenness (H_2/H_1)			NP vs. LF*	0.080
	NP	0.82 ± 0.03	NP vs. RG*	0.083
	LF	0.22 ± 0.22	NP vs. BP	0.663
	RG	0.90 ± 0.02	LF vs. RG*	0.058
	BP	0.80 ± 0.03	LF vs. BP*	0.086
			RG vs. BP**	0.042

proportion of decapod crustaceans in the stomach contents of lionfish collected from Florida Bay including some species known to provide cleaning services (Faletti & Ellis 2014), so we were motivated to test for predator treatment effects on shrimp abundance using the LMM framework described above. When specific treatment effects were not found, we grouped treatments by lionfish presence — lionfish present (either alone or with a red grouper) and lionfish absent (no predators or red grouper alone) — and tested for differences in shrimp abundance between the start and end of the experiment using paired-sample *t*-tests. We tested for differences in cleaner shrimp habitat association between the start and end of the experiment and for differences in the fish to invertebrate ratio in lionfish stomach contents in the presence and absence of red grouper using a 2-tailed Fisher's exact test. We set the level of statistical significance for all tests at $\alpha = 0.05$ and considered results to be marginally significant when $0.05 < p \le 0.10$. Except when noted otherwise, all results are presented as means ± SE.

RESULTS

Predator effects

After 6 wk the abundance of juvenile reef fish recruits was greatest at solution holes with red grouper alone (111 ± 35.4), followed by holes with both predators (50.8 ± 15.2), and holes with neither predator (43.8 ± 17.2; Fig. 2). Of the 4 solution holes of the lionfish alone treatment, 3 had zero recruits present after 6 wk, while at the fourth we counted 33 recruits (8.25 ± 8.25). On average, the abundance of juvenile reef fish recruits at solution holes with both predators was significantly greater compared to the lionfish alone treatment and less than at the red grouper alone holes, but was not different from the no predator holes (Table 1). The results of LMM analysis supported including predator treatment either as a main effect or as an interaction with time for all metrics (see Table S2 in the Supplement). The preferred variance

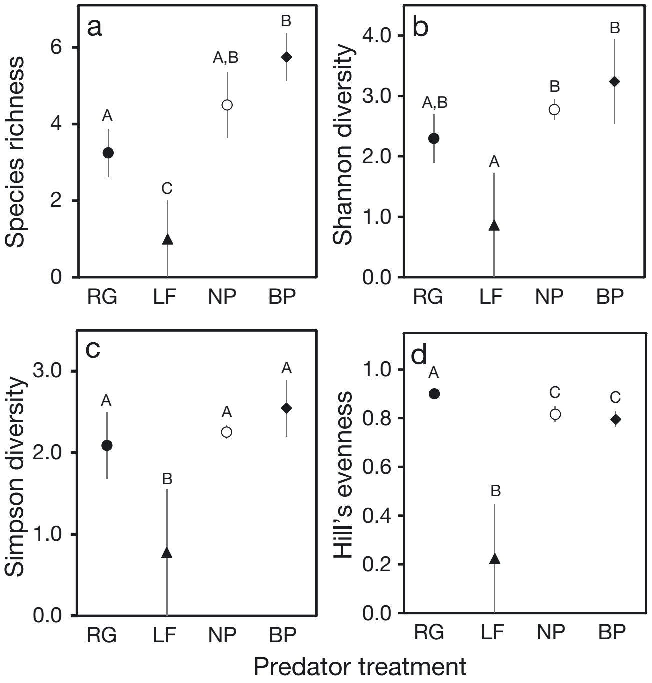

Fig. 3. (a) Species richness, (b) Shannon and (c) Simpson diversity, and (d) Hill's evenness of juvenile reef fish recruit communities after 6 wk with red grouper alone (RG; ●), lionfish alone (LF; ▲), no predator (NP; ○), or both predators (BP; ◆); n = 4 per predator treatment. Different letters indicate significant differences between groups after 6 wk based on pairwise *t*-tests performed on the best-fit linear mixed model

structure and inclusion of temporal autocorrelation differed among the metrics tested, i.e. allowing variance to vary by treatment improved the models for abundance and evenness while including temporal autocorrelation improved the models for abundance and species richness.

Red grouper and lionfish had varying effects on the other community metrics when compared to the no predator holes. Red grouper alone had a marginally significant positive effect on the evenness of juvenile reef fish recruits compared to the no predator treatment (red grouper alone = 0.900 ± 0.021; no predators = 0.816 ± 0.032; p = 0.083), but no other comparisons were significant (see Table 1). However, lionfish alone had a significant negative effect on all 5 of the community metrics tested compared to the no predator holes (Fig. 3). The species richness, Simpson's diversity, and evenness of juvenile reef fish communities were all significantly greater with red grouper compared to holes with lionfish alone. In general, communities were more species rich, diverse, and even with both predators compared to holes with lionfish alone (Fig. 3).

Compared to the no predator treatment, communities with both predators were not significantly different in terms of any of the community response metrics analyzed, including recruit abundance. However, we found a marginally significant difference between communities in the presence of both predators (5.75 ± 0.63 recruit species) compared to those with red grouper alone (3.25 ± 0.63 recruit species; p = 0.059), but these communities were significantly less even with both predators (0.80 ± 0.03) than they were with red grouper alone (0.90 ± 0.02; p = 0.042).

The structure of recruit communities varied greatly among predator treatments. During the 6 wk experiment, we encountered 14 unique species of juvenile reef fishes. However, we counted only 9 of these species during the final survey at the end of the experiment (Table 2). Of this group, the grunts, specifically white and French grunts (*Haemulon plumerii* and *H.*

Table 2. Treatment effects, in terms of the relative difference in mean abundance of each of the 9 species encountered during the final (Week 6) survey and the full juvenile reef fish recruit community, for red grouper alone (RG), lionfish alone (LF), both predators together (BP), and the BMII estimate. Control values presented are the mean abundance of each species in the no predator (NP) treatment

Species	Common name	Control N_{NP}	RG effect (N_{RG}–N_{NP})	LF effect (N_{LF}–N_{NP})	Combined effect (N_{BP}–N_{NP})	BMII (N_{BP}–N_{LF})
Haemulon plumierii	White grunt	17.3	47.8	–13.5	8.5	22.0
Haemulon flavolineatum	French grunt	11.3	14.5	–9.0	2.75	11.8
Haemulon spp.	Juvenile grunts	11	7.75	–9.5	–4.5	5
Anisotremus virginicus	Porkfish	0.25	0.25	–0.25	0.5	0.75
Pomacanthus arcuatus	Gray angelfish	0.0	0.0	0.0	0.75	0.75
Holacanthus ciliaris	Queen angelfish	0.25	–0.25	–0.25	0.0	0.25
Haemulon parra	Sailors choice	1.0	–0.5	–1.0	0.0	1.0
Lutjanus synagris	Lane snapper	0.5	–0.5	–0.5	–0.5	0.0
Pareques acuminatus	Highhat	2	–1.25	–1.25	–1.0	0.25
Total		43.5	67.8	–35.3	6.5	41.8
% change		–	156	–81.1	14.9	96.1

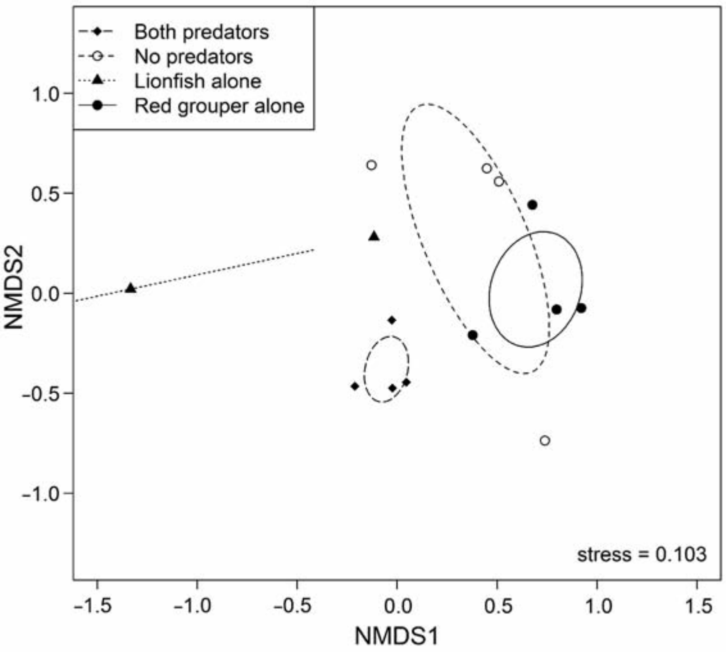

Fig. 4. Non-metric multidimensional scaling (NMDS) ordination of juvenile reef fish communities (abundance by species) associated with experimental solution holes at the end of the 6 wk experiment conducted in Florida Bay during June–July, 2013. Ellipses show the SD of all points for each predator treatment group; n = 4 per predator treatment. No ellipse for the lionfish alone treatment as 3 of the 4 juvenile fish communities were non-existent (n = 0) at the end of the experiment; these sites are represented by the single point located at (−1.33, 0.0217)

flavolineatum, respectively) were the most numerous. NMDS ordination of recruit communities showed clear separation of communities with lionfish (Fig. 4). However, because 3 of the 4 lionfish alone solution holes had zero recruits at the end of the experiment, the 3 zero-abundance points overlaid on top of each other (see point at [−1.33, 0.02] in Fig. 4), so the ellipse representing the standard deviation of the lionfish alone treatment communities collapsed to a line. There was significant overlap in the red grouper alone and no predator communities, while communities with both predators appeared to be separate from all other groups. PERMANOVA results indicated that predator treatment had a significant effect on community structure (pseudo-$F_{3,15}$ = 2.27, p = 0.029), supporting the separation of communities by treatment visualized in the NMDS.

Overall lionfish alone reduced juvenile reef fish recruit abundance by 81.1% compared to the no predator treatment (Table 2). The bootstrapped estimate of the direct effect of lionfish on recruit abundance, calculated as the ratio of recruit abundance with lionfish to recruit abundance with no predators, was −0.802 (range: −1.00 to −0.434). The estimated indirect effect of red grouper alone on recruit abundance was 1.546 (range: 0.206–2.77), in line with the 156% increase in recruit abundance observed during the experiment. The estimated effect size of the BMII between red grouper and juvenile reef fish recruits via lionfish according to the ratio-based method was estimated as 5.18 (range: 2.15–8.73) or about 5 times the expected recruit abundance with lionfish alone.

After 6 wk, we found no effect of predator treatment on cleaner shrimp abundance. The LRT did not support including predator treatment in the final model for shrimp abundance (likelihood-ratio$_{9,8}$ = 0.77; p = 0.38). However, the mean abundance of cleaner shrimp declined by 14.7% in solution holes with lionfish between the start and end of the experiment, irrespective of the presence or absence of red grouper. Conversely, mean cleaner shrimp abundance in solution holes without lionfish increased by 56.2%, a marginally significant effect (t = −2.23; p = 0.052; Fig. 5). We also observed a significant shift in cleaner shrimp association with anemones in solution holes with lionfish, where 42.3% of cleaner shrimp were found within 10 cm of an anemone at the start of the experiment and 61.2% were within 10 cm of an anemone at the end of the experiment (p = 0.011). In solution holes without lionfish, cleaner shrimp association with anemones did not significantly change (p = 0.89; Fig. 6).

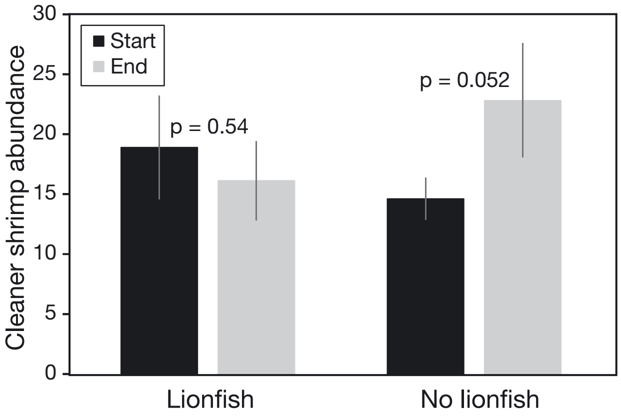

Fig. 5. Comparison between cleaner shrimp abundance (mean ± SE) at the start and end of the experiment at solution holes with (lionfish alone + both predator treatments) and without (no predator + red grouper alone treatments) lionfish. p-values reported above bars were based on paired t-tests comparing shrimp abundance at the start and end of the experiment for each group

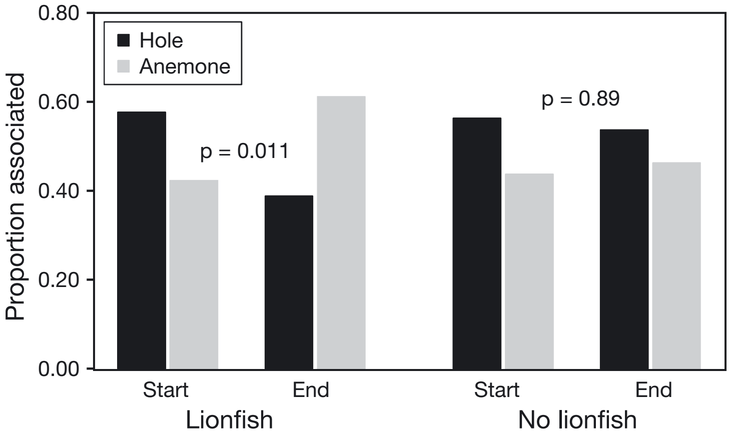

Fig. 6. Proportion of cleaner shrimp encountered on or within 10 cm of an anemone ('Anemone') or more than 10 cm from an anemone ('Hole') at the start versus the end of the experiment. Predator treatments were grouped by lionfish presence (lionfish alone + both predators treatments) or absence (no predator + red grouper alone treatments) in solution holes; n = 8 for each group. p-values reported above bars are based on Fisher's exact test results comparing shrimp–anemone association at the start and end of the experiment for each group

Lionfish diet analysis

Between June and August 2013 we analyzed the stomach contents of 29 lionfish captured at solution holes with (n = 13) and without (n = 16) red grouper. Overall, fish made up 51 % of stomach contents by number and crustaceans made up 35.4 %, with the remaining 13.5 % of prey items unidentifiable (see Table S3 in the Supplement). Mean gut content weight was 1.92 ± 0.56 g; only one stomach was empty. Palaemonid shrimps were the largest consumed family by number (19.8 %), while collectively cleaner shrimps (e.g. *Lysmata* spp., *Ancylomenes pedersoni* and *Periclimenes yucatanicus*) made up 30.1 % of lionfish stomach contents. By weight, fish prey comprised 87.1 % of lionfish stomach contents and crustaceans comprised 9.68 %; 3.23 % of prey items by weight were unidentifiable. Within teleost prey groups, Lutjanids made up the largest percentage by weight (34.2 %), followed by unidentifiable teleosts (22.3 %), grunts (genus *Haemulon*; 16.4 %) and gobies (family Gobiidae; 9.43 %). We documented a shift in lionfish diet in the presence of red grouper from a primarily piscivorous diet when the lionfish were in solution holes alone to a crustacean-based one in holes when both lionfish and red grouper were present together (p = 0.028; Fig. 7). When alone, lionfish consumed 78.4 % fish by number but fish prey made up just 43.4 % of lionfish stomach contents when a red grouper was also present at the time of capture.

DISCUSSION

The results of our experiment and diet analysis suggest that red grouper enhance the abundance of juvenile reef fishes that recruit to solution holes in Florida Bay and support the hypothesis that this effect occurs via changes in piscivore behavior in solution holes. Juvenile reef fishes benefited from the presence of the relatively large, territorial, habitat manipulating red grouper, while, conversely, recruit abundance was significantly depleted with lionfish. Lionfish are extremely efficient predators on Caribbean reef fishes, and reef fish populations on coral reefs and hard bottom habitats invaded by lionfish have suffered significant declines shortly following invasion (Albins & Hixon 2008, Green et al. 2012a). The results presented here confirm that the negative effects of lionfish on native reef fish populations observed elsewhere in the invaded range

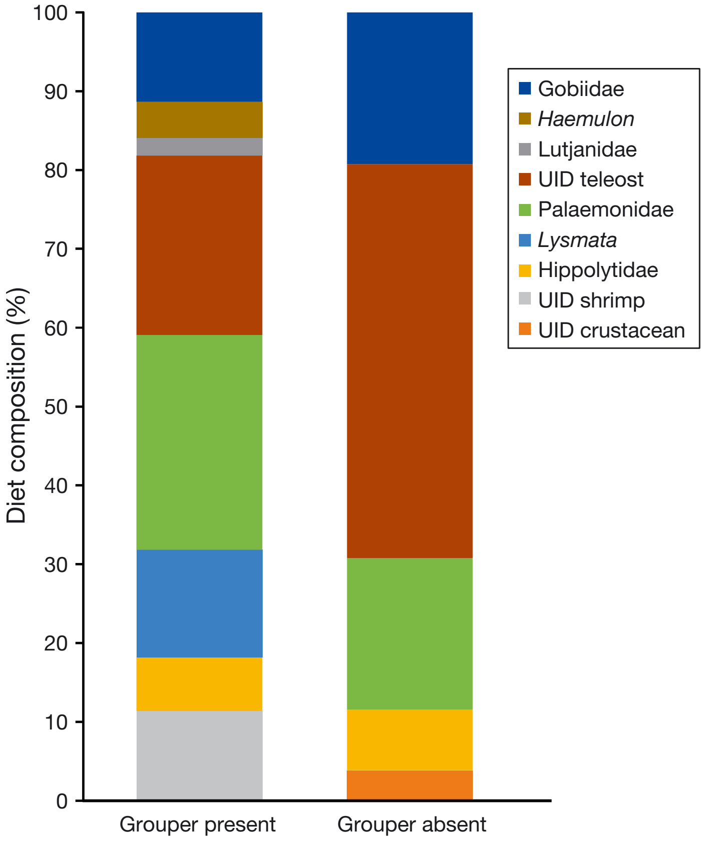

Fig. 7. Diet composition (% by number) of lionfish collected from solution holes in Florida Bay with ('Grouper present') and without ('Grouper absent') a red grouper at the time of collection. UID: unidentifiable

also hold true for Florida Bay hard bottom fish communities.

Lionfish and red grouper had very different effects on the diversity of juvenile reef fishes in Florida Bay solution holes. In general, solution holes with red grouper alone were similar to those with neither predator in terms of species richness and diversity. Meanwhile, similar comparisons are difficult to make for lionfish as they reduced recruitment to zero at 3 of the 4 lionfish-only treatment holes. While all 4 of the community diversity metrics were depressed in the lionfish alone treatment compared to the others, making specific conclusions about predator-driven effects on diversity are problematic when the predator leaves no prey at all. Certainly, this is a strong qualitative result if not a quantitative one: lionfish reduce the abundance and diversity of native Caribbean reef fishes, apparently through indiscriminate piscivory. Our results parallel those found in other parts of the invaded range including reductions in native reef fish recruitment (Albins & Hixon 2008), reef fish abundance (Green et al. 2012a, Côté et al. 2013b), and species richness (Albins 2015).

We found no differences in recruit abundance in solution holes with both predators compared to the other treatments, and solution holes with both predators had comparable species richness and diversity compared to the red grouper alone and no predator treatments (see Table 1). These results generally support the BMII hypothesis: when red grouper were present in solution holes with lionfish, the associated communities of juvenile reef fishes, although depressed in abundance, were still similar to those in solution holes without red grouper or without either predator. Native fish communities appeared to benefit from the presence of red grouper compared to when the exotic piscivorous lionfish was present alone.

Our reevaluation of the diet composition of lionfish found in Florida Bay solution holes revealed that teleosts made up a smaller portion of the diet by number in Florida Bay compared to other diet studies done in the Bahamas and North Carolina (Morris et al. 2009, Munoz et al. 2011). We also found that gobies had the highest index of relative importance (IRI) of all prey groups, supporting reports elsewhere that gobies may be especially vulnerable to lionfish predation (Morris & Akins 2009, Albins & Lyons 2012). The shift we observed in diet from teleost-dominated to crustacean-dominated appeared to be driven by the presence of red grouper. This result further supports our BMII hypothesis that the disruptive presence of red grouper cause lionfish to alter their predation behavior to consume more benthic crustaceans that may be easier prey to consume compared to juvenile reef fish. Further investigation is needed to decipher the specific mechanisms driving this interaction. However, if lionfish alter their diets to target certain species such as cleaner shrimp, this could ultimately lead to a loss of the ecosystem services provided by those species. Larval settlement patterns for these 2 species in Florida Bay are unknown, though some other species of shrimps exhibit peaks of post larval settlement in the Middle Keys range of Florida Bay during the summer months (Criales et al. 2006). A summer influx of post larval shrimps could explain the increase in shrimp abundance we observed in the absence of lionfish throughout the study period.

Red grouper do not compete with lionfish for prey; although both will consume crustaceans and demersal fishes, the diets of lionfish collected from Florida Bay do not overlap with red grouper diets. However, we suspect that lionfish and red grouper may compete for space in solution holes. The exact nature of the lionfish response to red grouper, via modification of some specific behaviors by the lionfish or a more generally disruptive effect of red grouper presence, remains untested. The few studies that have explicitly investigated behavioral interactions between native Atlantic reef fishes and lionfish have found that lionfish generally ignore potential predators while native fish actively avoid the lionfish. One study of competitive shelter use between lionfish and Nassau grouper in experimental mesocosms found that Nassau grouper avoided lionfish even when they were much larger than the lionfish, but lionfish did not change their use of shelter even when the grouper was much larger (Raymond et al. 2015). During an experimental feeding trial, Morris (2009) reported that red grouper moved away from lionfish when approached. In the present study, we did not observe avoidance of lionfish by red grouper, or vice versa. However, the duration of such observations was limited to the time we spent conducting community censuses, and we did not quantify avoidance behaviors by either fish. Nevertheless, our results and those reported by Pusack (2013) suggest that invasive lionfish alter their feeding behaviors in the presence of larger native groupers.

The link between recruitment, post-settlement mortality, and adult population size for reef fish is complex. However, Shulman & Ogden (1987) found that changes in immediate post-settlement survival of French grunts was a more important factor in regulating the ultimate abundance of adult grunts on coral reefs than to changes in recruitment. We tested the hypothesis that enhanced reef fish abundance at red grouper occupied solution holes results in part from disruptive behavioral interactions with piscivores that lead to enhanced survival of juvenile reef fishes. Importantly, we did not observe any predation on the transplanted lionfish during the experiment, despite reports elsewhere that groupers may act as predators of invasive lionfish (Maljković et al. 2008, Mumby et al. 2011). There are increasing control efforts across the invaded range, including attempts by spearfishers to 'teach' native groupers and sharks to feed on lionfish that have been largely unsuccessful and dangerous for divers (Diller et al. 2014). Ultimately, it may be that intact native fish communities that include native mesopredators are the best way to ameliorate the worst-case effects of lionfish by competing with and altering their behavior (Albins & Hixon 2013). Some recent evidence suggests that Caribbean reefs with relatively high native predator density can maintain unchanged prey populations despite being invaded by lionfish (Elise et al. 2015). Other studies have found that lionfish-induced reductions in the density of native fishes <10 cm TL did not translate to larger prey (10–20 cm TL; Albins 2015). Lionfish, then, may represent an enhanced gauntlet that juvenile reef fish must pass through, but not necessarily an impenetrable one.

The successful invasion of exotic species is thought to be more likely in human-altered ecosystems (Sax & Brown 2000). As humans have reduced the biomass of native mesopredators on coral reefs and hard bottom habitats throughout the Caribbean, there are fewer competitive and behavioral interactions that limit both the population size and predatory effects of lionfish in its native range. The experiment presented here supports the mesopredator release hypothesis by showing how the presence of a native predator, the red grouper, can ameliorate the negative effects of lionfish predation on native reef fish communities. This study sheds some light on the community-level effects of both native and invasive predators, an integral part of expanding fisheries management from single-species stock assessments to ecosystem-based fisheries management (Pikitch et al. 2004). Here we show how an important fishery species, the red grouper, has complex direct and indirect interactions with the other species that colonize grouper-excavated solution holes in Florida Bay. Some of these interactions may have population-level effects on species that support fisheries and provide important ecosystem services, services that are lost or reduced by the presence of lionfish (Johnston et al. 2015). Albins & Hixon's (2013) description of a 'worst-case scenario' for lionfish in the western Atlantic highlights the need for intact predator communities to ameliorate the effects of the lionfish invasion. Our study provides some of the first experimental evidence of this effect and begins to shed light on the mechanisms by which native predators may lessen the negative effects of this exotic invader.

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