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Diet richness of invasive Indo-Pacific lionfish revealed by DNA barcoding

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ABSTRACT: Indo-Pacific lionfish *Pterois* spp. have recently invaded marine habitats throughout the western Atlantic, Gulf of Mexico, and Caribbean Sea. Their unusual hunting behaviour suggests that they could prey on most fish species within their gape size limits. However, few prey species have been identified so far due to the challenges of identifying partly digested prey. Moreover, it is not clear how well the identifiable diet reflects the unidentified portion. To address these issues, we DNA-barcoded unidentifiable fish items from the stomachs of 130 lionfish captured on Bahamian coral reefs. We identified 37 fish prey species, nearly half of which had not previously been recorded in this region. The total richness of lionfish prey fish recorded so far may represent up to ~54% of potential prey species on the study reefs. The relative importance of prey species in the visually identifiable diet portion, which was limited to 25% of prey items, differed from that in the 'unidentifiable' portion, which was largely resolved here with barcoding, weakening extrapolations from visual identification. The high diet resolution afforded by barcoding can increase our ability to predict the impacts of invasive predators on recipient communities.

KEY WORDS: Barcoding · Diet composition · Lionfish · Invasion biology · Predator–prey interactions · Stomach content analysis

INTRODUCTION

A rapid marine invasion of potentially far-reaching ecological impact is occurring in the western Atlantic. Lionfish *Pterois volitans*, released in Florida in the mid-1980s, were first reported on Bahamian coral reefs in 2004 (Whitfield et al. 2002, Freshwater et al. 2009, Schofield 2009). Since then, they have become established in >4 million km^2 of the western Atlantic, Caribbean, and Gulf of Mexico (Schofield 2009). Invasive lionfish reach higher densities and larger sizes than in their native range (Darling et al. 2011), hunt using a prey-herding strategy unlike that of any Atlantic predator (Côté & Maljković 2010, Green et al. 2011), ingest whole prey that can be half their own body size (S. J. Green & J. L. Akins pers. obs.), and reduce significantly the recruitment and biomass of native reef fishes (Albins & Hixon 2008, Green et al. 2012). An in-depth understanding of diet is essential to predict the extent to which these invaders will affect the structure of the communities of which they are now part.

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Given their hunting mode, lionfish could prey on most fish species within their gape size limits. To date, only 42 fish species have been identified in the stomachs of >1200 lionfish from across the Bahamas (Morris & Akins 2009, Layman & Allgeier 2012). The richness of lionfish prey is likely higher, because a large proportion of prey in fish stomachs (e.g. ~70% in Morris & Akins 2009) is usually unidentifiable to species, owing to degradation by digestion. This shortcoming of visual identification methods, particularly at low sample sizes, could bias predictions of ecological impact, because the prey identified may not represent the unidentified portion.

Here, we use DNA barcoding to document, at high taxonomic resolution, the diet of lionfish on invaded reefs. Barcoding relies on the matching of short sequences of DNA from unidentified samples to known sequences held in global databases (e.g. GenBank or the Barcode of Life Database; Ratnasingham & Hebert 2007). This technique has been successfully used to identify dietary items of species that are challenging to study owing to their rarity (e.g. deep-water sharks; Dunn et al. 2010), small body size (e.g. larval fish; Victor et al. 2009, Riemann et al. 2010), or species-rich generalist diets (e.g. coral reef fish; Leray et al. 2012). We specifically ask (1) how many native fish species are preyed upon by lionfish inhabiting a circumscribed reef area, (2) how this prey richness compares to the local richness of potential prey, and (3) whether conclusions about prey species dietary importance agree when a small proportion of the diet is identified (by conventional visual identification) and when a higher proportion is resolved (by barcoding). It has been suggested that barcoding could be valuable in diet studies of invasive species (Armstrong & Ball 2005, Ball & Armstrong 2006). Our study offers an application of the method in this context (see also Valdez-Moreno et al. 2012).

MATERIALS AND METHODS

Study sites and lionfish collection

We collected 130 lionfish *Pterois volitans* from 9 reef sites off southwest New Providence, Bahamas, between April and September 2008. All sites were located along a 15 km stretch of reef wall, at 3 to 20 m depths. Lionfish were captured using hand nets and euthanized in saltwater ice baths. Each prey item ingested was identified to the lowest taxonomic level possible, relying on field guides and personal experience, labelled and frozen individually.

Surveys of species richness of potential prey

To assess overall richness of potential fish prey across sites, we conducted 6 to 12 transects (30 m long × 2 m wide) at each site where we collected lionfish. Transects were laid parallel to the reef crest and stratified by depth and zone, with 2 to 4 transects at each of 3 depths: 20 m (reef wall), 15 m (reef crest), and 10 m (reef flat). On each transect, we conducted detailed searches for all fish species, focussing particularly on small and cryptic species, which entailed a thorough examination of the substratum and of all holes, crevices, and overhangs within each transect area. We recorded the species identity and size (total length to the nearest 1 cm) of each fish encountered. We considered as potential prey fishes of <13 cm total length (TL), which is the maximum prey size recorded for lionfish in the Bahamas (S. J. Green unpubl. data). We tallied the total number of potential prey species across all sites. Although our surveys specifically targeted small and cryptic species, visual surveys always overlook some of these species (e.g. Smith-Vaniz et al. 2006). Our estimate of species richness of potential prey should therefore be seen as conservative.

Barcoding sample preparation and analysis

A small sample of muscle tissue (2 to 3 mm^3) was taken from each frozen prey item that was identified as a fish but could not be identified to species. Whenever possible, the sample was taken from the dorsal musculature. To reduce the likelihood of sample contamination by lionfish cells, we first removed a thin top layer of muscle tissue (~1 mm), which would have been exposed to stomach fluids, before taking a sample for barcoding. Between each sample, all residual tissue was removed from the scalpel and forceps, the tools were sterilized with 95% ethanol, and finally heat-sterilized using a Bunsen burner flame.

Barcoding was conducted at the Canadian Centre for DNA Barcoding at the University of Guelph, Ontario. We followed the protocol outlined in Steinke & Hanner (2011). DNA from each specimen was extracted using an automated Glass Fiber protocol (Ivanova et al. 2006). The 650 bp barcode region of cytochrome C oxidase Subunit I (COI) was subsequently amplified under the following thermal conditions: 2 min at 95°C; 35 cycles of 0.5 min at 94°C, 0.5 min at 52°C, and 1 min at 72°C; 10 min at 72°C; and held at 4°C. The 12.5 µl polymerase chain reaction (PCR) mixes included 6.25 µl of 10% trehalose,

2.00 µl of ultrapure water, 1.25 µl 10× PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 0.625 µl $MgCl_2$ (50 mM), 0.125 µl of each primer cocktail (0.01 mM, using primer cocktails C_FishF1t1 and C_FishR1t1 (Ivanova et al. 2007), 0.062 µl of each dNTP (10 mM), 0.060 µl of Platinum® Taq Polymerase (Invitrogen), and 2.0 µl of DNA template.

PCR amplicons were visualized on a 1.2% agarose gel E-Gel® (Invitrogen) and bidirectionally sequenced using sequencing primers M13F or M13R (Ivanova et al. 2007) and the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI 3730 capillary sequencer following manufacturer's instructions. Sequence data are available from both GenBank (Accession Numbers JQ843098 to JQ843296) and the Barcode of Life Data System (BOLD, www.boldsystems.org; Ratnasingham & Hebert 2007). Specimen and collection data, sequences, and trace files are listed in the same project folder as collection data (lionfish stomach contents analysis — LION) in BOLD.

We analysed the DNA barcode sequences derived from unknown samples with the 'species'-level identification function of the BOLD ID Engine (Version November 2011). A top species match was identified with a sequence similarity of at least 99% to avoid false positives. At the time of the analysis, the BOLD database held sequences for 85 of the 90 potential prey species recorded at our study sites. The 5 missing species were not reported as prey in the largest study of lionfish diet (using visual identification) (Morris & Akins 2009).

To verify the accuracy of barcoding, 22 of the unidentified prey items were sent in duplicate. All duplicate samples returned matching species identification.

Prey species accumulation and richness assessment

To establish the rate at which new prey species identified by barcoding accumulated with the number of lionfish examined, we generated a sample-based rarefaction curve with EstimateS (Version 8.2), using 100 sample randomisations without replacement (Colwell 2009). We assessed the extent to which our sample characterised lionfish diet richness by examining the trends in numbers of unique (i.e. species occurring in a single stomach) and duplicate prey occurrences (i.e. occurring in only 2 stomachs), and estimating asymptotic prey species richness using 2 established non-parametric richness estimators (Chao2, Jacknife1) (Chao 2005).

Metrics of prey species importance to diet

To assess the agreement between the portion of the diet identified visually and the unidentified portion, which was well described by barcoding (see 'Results'), 2 metrics of prey species importance in the diet were calculated for each fish prey. The percentage by number (%N) of each prey species was calculated as the total number of individuals of a given prey species divided by the total number of individual prey recorded across all lionfish stomachs (Hyslop 1980). The percentage frequency of occurrence (%F) was calculated as the total number of lionfish in which a given prey species was found divided by the total number of lionfish with identified prey in their stomachs (Hyslop 1980). Both metrics were calculated separately for diet derived from visual identification only ($\%N_{vis}$, $\%F_{vis}$), and for diet derived from barcoding identification only ($\%N_{bar}$, $\%F_{bar}$), using the relevant denominator for each ratio (e.g. total prey number from either visual or barcoding identification). We assumed that $\%N_{bar} = 0$ and $\%F_{bar} = 0$ for those prey species recorded visually but undetected by barcoding. This assumption is valid because the BOLD database held sequences for all species that were visually identified; hence, their presence would definitely have been recorded by barcoding if they had been among the samples analysed. The relationships between $\%N_{vis}$ and $\%N_{bar}$ and between $\%F_{vis}$ and $\%F_{bar}$ were examined using linear regressions.

RESULTS

A total of 397 fish prey items were found in the 130 lionfish *Pterois volitans* stomachs examined (range: 1 to 31 items per stomach; lionfish length range: 122 to 372 mm). Of all items, 102 (25.7%) were identified visually to species. By comparison, of 283 barcoded prey items, 199 yielded a sequence, and of these, 181 (64% of total items analysed) were identified to species, based on our criterion of a sequence similarity of 99% or higher to sequences held in the BOLD database. The length (mean ± SD) of identified COI barcodes was 625 ± 53 bp.

Using visual identification, we recorded 17 fish prey species in 8 families (Table 1). With barcoding, we identified 34 prey species in 16 families (Table 1). Three species were detected only by visual identification, while 20 species were detected only by barcoding (Table 1). We recorded 90 potential prey fish species on the reefs where lionfish were collected.

Table 1. Diet of *Pterois volitans*. Prey fishes identified visually (vis) and by barcoding (bar) in the stomachs of lionfish from Bahamian coral reefs. Families are listed alphabetically for convenience. %N: percentage by number of each prey species (total number of individuals of a given prey species / total number of individual prey recorded across all lionfish stomachs); %F: percentage frequency of occurrence (total number of lionfish in which a given prey species was found / total number of lionfish with identified prey in their stomachs)

Prey fish species identified	Visually		Barcoding	
	$\%N_{vis}$	$\%F_{vis}$	$\%N_{bar}$	$\%F_{bar}$
n:	102	130	168	130
Apogonidae (cardinalfishes)				
Apogon binotatus	0	0	1.2	1.1
A. phenax[a]	0	0	0.6	2.2
A. townsendi	0	0	3.0	4.4
Phaeoptyx pigmentaria[a]	0	0	1.2	1.1
Aulostomidae (trumpetfishes)				
Aulostomus maculatus	1.0	3.3	0.6	1.1
Bothidae (flounders)				
Bothus ocellatus[a]	0	0	1.2	1.1
Chaenopsidae (blennies)				
Acanthemblemaria aspera[a]	0	0	0.6	1.1
Gobiidae (gobies)				
Coryphopterus bol[a,b]	0	0	3.0	3.3
C. eidolon	3.9	3.3	10.7	14.1
C. glaucofraenum	6.9	3.3	6.0	7.6
C. hyalinus	0	0	1.2	2.2
C. personatus	33.3	46.7	13.1	16.3
Gnatholepis thompsoni[a]	0	0	1.2	2.2
Lythrypnus spilus[a]	0	0	0.6	1.1
Priolepis hipoliti	1.0	3.3	0	0
Grammatidae (basslets)				
Gramma loreto	5.0	6.7	0.6	1.1
Holocentridae (squirrelfishes)				
Holocentrus rufus[a]	0	0	0.6	1.1
Sargocentron coruscum[a]	0	0	0.6	1.1
Inermiidae (bogas)				
Inermia vittata[a]	0	0	1.8	3.3
Labridae (wrasses)				
Clepticus parrae	4.9	6.7	3.6	5.4
Halichoeres bivittatus	2.9	3.3	3.0	2.2
H. garnoti	7.8	3.3	1.2	2.2
H. maculipinna	7.8	3.3	0	0
Thalassoma bifasciatum	12.8	13.3	8.9	14.1
Labrisomidae (blennies)				
Labrisomus haitiensis[a]	0	0	0.6	1.1
Malacoctenus boehlkei	0	0	2.4	4.4
Monacanthidae (filefishes)				
Monacanthus tuckeri	2.0	3.3	1.2	2.2
Pomacentridae (damselfishes)				
Chromis cyanea	2.9	6.7	9.5	10.9
C. multilineata	2.9	3.3	2.4	3.3
Stegastes partitus	0	0	6.6	8.7
S. variabilis	1.0	3.3	1.2	2.2
Scaridae (parrotfishes)				
Sparisoma aurofrenatum[a]	1.0	3.3	0.6	1.1
Scorpaenidae (scorpionfishes)				
Pterois volitans[a]	0	0	3.0	4.4
Serranidae (groupers)				
Cephalopholis cruentata[a]	0	0	0.6	1.1
Liopropoma rubre	2.9	3.3	0	0
Serranus tigrinus	0	0	3.0	5.4
Synodontidae (lizardfishes)				
Synodus synodus[a]	0	0	4.8	6.5

[a]species not recorded in the lionfish study by Morris & Akins (2009).
[b]species described in 2008 so could not have been identified visually by us or by Morris & Akins (2009) at the time of sampling

Not surprisingly, the number of observed prey species increased with the number of lionfish examined, but the relationship did not reach a clear asymptote (Fig. 1). The numbers of species found in 1 (unique) or 2 (duplicate) lionfish stomachs were either stable or continued to increase as the maximum number of samples was approached, with no indication of decline (Fig. 1). While the Chao2 richness estimator appeared to plateau beyond a sample of ~60 lionfish, the first-order jackknife estimator continued to increase (Fig. 1). At maximum sample size, both nonparametric estimates remained significantly higher than the observed prey richness.

When dietary importance was measured in terms of percentage of prey items (%N), there was a significant positive relationship between species-specific prey importance in the visually identified portion of the diet ($\%N_{visual}$) and in the 'unidentified' portion, which was largely identified by barcoding ($\%N_{barcoded}$) ($r^2 = 0.41$, $F_{1,35} = 24.03$, $p < 0.001$; Fig. 2). However, the slope ($\beta = 0.35$, 95% confidence interval [CI] = 0.21 to 0.48) was significantly shallower than 1. The removal of the outlying data point pertaining to *Coryphopterus personatus* did not alter the results ($r^2 = 0.16$, $F_{1,34} = 6.33$, $p = 0.02$; $\beta = 0.36$, 95% CI = 0.08 to 0.64). The same patterns were observed when dietary importance was measured in terms of percentage frequency of occurrence (%F) (Fig. 3).

DISCUSSION

Insights into lionfish diet breadth

Barcoding provided great taxonomic resolution to the identification of fish prey of invasive lionfish *Pterois volitans*. We were able to visually identify to species only 25% of ingested prey items, owing to degradation of key features such as colouration and fin ray shape. This proportion is similar to the identification success of other fish stomach

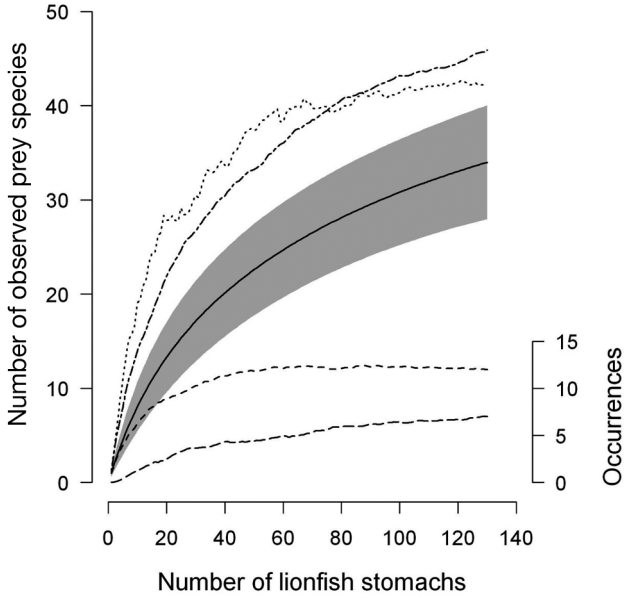

Fig. 1. Diet of *Pterois volitans*. Rarefaction curve of observed prey species (solid line, shaded area = 95% CI) and corresponding estimators (dotted line: Chao2; dot-dash line: first-order jackknife) of total prey richness found in the stomach contents of invasive lionfish collected from Bahamian coral reefs. The numbers of occurrences of unique (short dashed line) and duplicate prey species (long dashed line) are shown on the right-hand axis

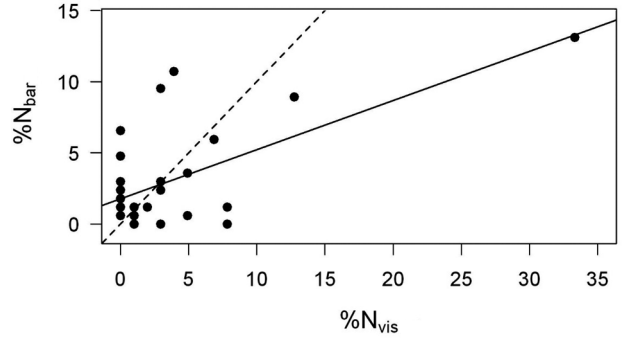

Fig. 2. Diet of *Pterois volitans*. Relationship between the dietary importance, in percentage by number, of lionfish prey when prey are identified visually ($\%N_{vis}$; 25% of prey identified) and when visually unidentifiable prey are identified by barcoding ($\%N_{bar}$; 64% of prey identified). ●: prey fish species. Solid line: line of best-fit of a linear regression ($y = 0.35x + 1.77$). Dashed line = 1:1 slope

contents studies (e.g. Arnal & Côté 2000, Morris & Akins 2009, Woodland et al. 2011). In contrast, we were able to identify to species 64% of prey items submitted for barcoding.

Barcoding revealed that the diet of invasive lionfish in the Bahamas is broader than previously documented. In total, we recorded 37 fish prey species in

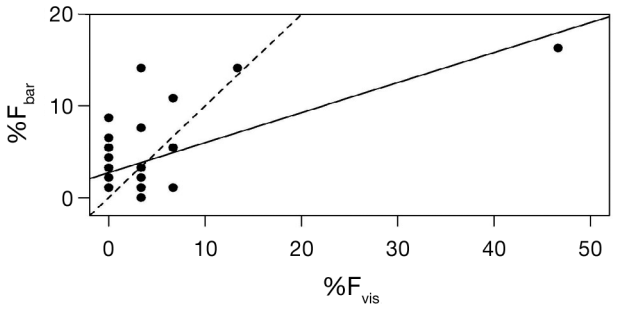

Fig. 3. Diet of *Pterois volitans*. Relationship between the frequency occurrence of various prey fish species (as a proportion of number of lionfish stomachs examined) identified visually ($\%F_{vis}$) and by barcoding ($\%F_{bar}$). ●: prey fish species. Solid line = line of best fit of a linear regression ($y = 0.33x + 2.70$; $r^2 = 0.38$, $F_{1,35} = 21.73$, $p < 0.001$). Dashed line = 1:1 slope. The observed slope was significantly shallower than 1 (95% CI of slope = 0.19 to 0.46). Exclusion of the right-most data point (*Coryphopterus personatus*) yielded similar results ($y = 0.58x + 2.26$; $r^2 = 0.22$, $F_{1,34} = 9.35$, $p = 0.004$; 95% CI of slope = 0.21 to 0.95)

the stomachs of just 130 lionfish, which were collected from a limited area (i.e. a 15 km stretch of reef). This is very similar to the 34 prey fish species identified by barcoding from the stomach contents of 144 lionfish from Cozumel, Mexico (Valdez-Moreno et al. 2012). Our prey tally included 15 species and 4 families, which were recorded for the first time as lionfish prey in the Bahamas (Table 1). By comparison, a large lionfish diet study (>1000 ind.) based on visual identification yielded 42 positively identified fish prey species (Morris & Akins 2009). All of our sampling sites were included in this larger study; thus, the new prey species uncovered here are not the result of geographic differences in sampling.

Of particular interest as a new prey species is lionfish itself (Table 1). It is unlikely that this result stems from contamination because the lionfish DNA sequences retrieved matched different records on BOLD which originate from different regions of the Pacific. Contamination would have more likely yielded similar sequences across lionfish samples given the strong founder effect documented in the Bahamas (Betancur-R et al. 2011). Large *Pterois volitans* do consume smaller conspecifics in captivity (Fishelson 1997). Our observations confirm cannibalism by lionfish in the wild, which has recently been reported for lionfish in the Mexican Caribbean (Valdez-Moreno et al. 2012). The low frequency of cannibalism (3% of lionfish examined) makes it unlikely that intraspecific predation can regulate lionfish populations on invaded reefs in the Bahamas.

Despite the high proportion of prey identified with barcoding, a sample of 130 lionfish was insufficient to

characterise diet breadth completely. An asymptote in observed prey richness was not reached (Fig. 1). Moreover, the trends in numbers of unique and duplicate prey occurrences did not decline with sample size, as would be expected if sampling were adequate (Longino et al. 2002). Finally, at least one of the asymptotic richness estimators (first-order jackknife) rose continuously with sample size, and both estimators remained well above observed prey species richness. In fact, at maximum sample size, estimated prey richness remained 30 to 35% higher than observed prey richness. Since non-parametric richness estimators provide lower bounds (Chao 2005), a considerable number of lionfish prey remains to be identified.

Insights into the importance of specific prey

A near-complete accounting of prey richness is important because extrapolating conclusions about the importance of individual prey species from a poorly identified diet may not be robust. With both metrics of dietary importance (%N and %F), prey species that were important in the visually identified portion of the diet were also important in the 'unidentified' portion, which was largely identified by barcoding. However, in both cases, species-specific dietary importance on the basis of visual identification usually exceeded the matching value obtained with barcoding. Thus, extrapolating from a poorly described diet (i.e. usually through visual identification) might often result in overestimating the importance of many prey species: a diet may thus appear much less specialised (i.e. focussed on only one or a few prey items) with more complete prey enumeration.

Only 2 of the 5 prey species identified as most important on the basis of $\%N_{vis}$ — the goby *Coryphopterus personatus* and the wrasse *Thalassoma bifasciatum* — also ranked in the top 5 on the basis of $\%N_{bar}$. The apparently great importance of *C. personatus* in the lionfish diet based on visual prey identification ($\%N_{vis}$ = 33%; $\%F_{vis}$ = 46%) was much reduced when a greater proportion of the diet was identified ($\%N_{bar}$ = 13%; $\%F_{bar}$ = 16%). This discrepancy could be due to the fact that many individuals of the prey species that are easily recognised visually are removed from the unidentified portion of the diet; thus, species with high values for $\%N_{vis}$ may be expected to have lower values for $\%N_{bar}$. In addition, differences in prey importance between $\%N_{vis}$ and $\%N_{bar}$ (and between $\%F_{vis}$ and $\%F_{bar}$) will arise from the high prey identification rate of barcoding, which generates a large denominator for the barcoded ratios. In general, the discrepancy between the identified and unidentified portions of a consumer's diet probably attenuates as sampling improves, but it remains unclear at what sample size conclusions about the importance of individual prey items based on visual assessment become robust.

Implications of diet breadth for ecological impacts of lionfish

Invasive species are often generalists (Olden et al. 2004). This trophic label was suggested for lionfish at the population level on the basis of stable isotope analyses, which suggested that lionfish feed across multiple trophic levels and on prey that rely on various carbon sources (Layman & Allgeier 2012). Our study confirms this conclusion by revealing the actual species richness of the lionfish diet.

Consumer diet breadth is rarely placed in the context of potential prey availability. Our surveys of potential prey fish allow us to do this. We encountered 90 prey-sized fish species on the reefs where lionfish were collected. According to barcoding identification alone, lionfish consumed nearly 40% of species potentially available as prey. When all known prey species from the Bahamas are considered (i.e. Morris & Akins 2009, Layman & Allgeier 2012, present study), this figure rises to as high as 54%, if our estimate of richness of potential prey species is reasonably accurate. Given that estimated prey richness may be at least 30% higher than observed (Fig. 1), the true proportion of potential prey species exploited by lionfish may be even higher. The lack of similar comparisons for other generalist consumers makes it difficult to judge whether this proportion is unusually high. Nevertheless, these estimates highlight the fact that a large number of native Caribbean reef fishes now share a new enemy.

The ecological implications of our findings are numerous and potentially profound. Evidence for a generalist diet does not mean that individual lionfish lack prey preferences (Layman & Allgeier 2012). Estimates of prey abundance are needed to establish preferences, which, in turn, should allow predictions of the direct impacts of lionfish on specific prey species. Such predictions may, however, become complicated by the fact that by targeting multiple species, lionfish are generating novel, indirect interactions among species that were interacting weakly prior to

the invasion. Thus, in the short term, the presence of alternative prey can reduce lionfish predation pressure on preferred prey species since lionfish can readily switch prey. In the longer term, however, lionfish densities may be sustained at higher levels because many prey species are available and used, which may be particularly detrimental to preferred prey (Holt & Lawton 1994). In extreme cases, this could lead to the extirpation of preferred prey, as has been shown in terrestrial and freshwater invasions by generalist predators (Pimm 1987, Savidge 1987, Miller 1989). The trophic flexibility of lionfish, which may be even greater than suggested by our results since they are known also to prey on invertebrates and their prey use may change ontogenetically (Morris & Akins 2009), means that these invasive predators should be able to substantially reduce the populations of many of their prey before a numerical response in lionfish numbers is elicited.

The impacts of invading trophic generalists on food web structure can be extensive because of the large number of interspecific interactions they can create or disrupt, particularly in species-rich ecosystems like coral reefs (Leray et al. 2012). A taxonomically accurate characterisation of diet can provide a better understanding of these interactions and a fuller accounting of the potential impacts of invasive species on biodiversity. We found that DNA barcoding can help achieve this while it simultaneously reduces sampling effort and the need for identification expertise. Taxonomically well-resolved diet information can be combined with prey availability data to identify the species most at risk from lionfish predation. Beyond lionfish, this method can also be applied to any situation where the rapid acquisition of reliable diet information is crucial, whether to aid in the prevention of invader impacts or in the protection of rare species.

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