

# The Management and Feeding Ecology of the Invasive Lionfish (*Pterois volitans*) in Puerto Rico.

By

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## Abstract

Since the mid-1980s, two Indo-Pacific lionfishes, *Pterois volitans* and *P. miles*, have established a significant presence in the Western North Atlantic, including the Caribbean Sea and the Gulf of Mexico. The first documented report of lionfish (*Pterois volitans*) in Puerto Rico occurred in 2008, and these predatory fish were first observed in La Parguera a year later. In general, lionfish are professed to significantly affect ecosystem function and associated ecosystem services. This threat and the perceived negative impact of invasive lionfish on the marine ecosystems in the Western North Atlantic and Caribbean accentuate the need for sustained research on their biological and ecological dynamics. Removal efforts are an effective means of reducing lionfish densities, yet most studies fail to address the reaction of the native fish communities in response to these removals. Furthermore, assessing impacts should also involve investigating the feeding ecology to characterize the dietary profile of lionfish as their diets have been shown to be site specific. The main purpose of this five year study was to investigate the perceived impacts of lionfish by determining the real impacts on native fish communities in Puerto Rico by addressing the following objectives: 1) characterize the dietary profile of the lionfish using next generation sequencing, 2) determine the digestion rate of fish and shrimp prey items, with recommendations for future feeding ecology studies and 3) assess the effectiveness of a small-scale dedicated lionfish removal and the response of the native predators and prey.

The major disadvantage of morphological identification and DNA barcoding is the inability to utilize the digested material. However, next generation sequencing (i.e., DNA metabarcoding) can 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 and stomach contents were separated into two sample regimes – a liquid (i.e., digested) and a tissue. A 313bp region of the cytochrome oxidase subunit I (COI) gene was amplified from extracted DNA and samples were sequenced with Illumina MiSeq. The resulting sequences were compared to known databases to identify specimens to the lowest taxon. 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. Utilizing the digested material proved efficient in detecting prey species, especially those that would have been missed with traditional methods. Furthermore, prey species lists and frequencies of

occurrence could be used to assess prey selectivity overall and diet differences among areas or of fish of different size.

Identifying a partially digested prey item via morphological identification is highly dependent on the digestive level of the prey item. This study analyzed the digestion rate of known teleost and invertebrate prey items in lionfish stomachs, over set time intervals, to provide an estimate of time to digestion for these prey types. Two size classes of lionfish were examined and digestion of prey items were ranked. Approximately 4 hours were required before a fish prey item was unrecognizable at the species level, and 5 hours for shrimp. Predator size, prey type and time since ingestion were observed to predict digestion rank of the prey items. Temperature was not correlated with digestion, by effect of the experimental design. Furthermore, a detailed prey digestion scale was developed for shrimp and recommendations were provided to maximize prey identification obtained using morphological gut content analyses.

Pelotas reef, a inshore linear coral reef in La Parguera, was an ideal location for the small-scale removal experiment as the size mimicked that of the near-shore, culled marine protected area in Rincón (Tres Palmas). Pelotas had a small lionfish population that was not targeted by the dive industry or local fishermen prior to the study. Three removal events over a one month period reduced lionfish densities in this 0.6 km area. Nine months were required for densities to return, although lionfish biomass was still reduced. The absence of tagged lionfish and the observed small sizes in post-removal months suggests re-colonization occurred via ontogenetic migration rather than lateral immigration by adults from nearby reefs. Contrary to what was anticipated given the perceived impacts, no detectable effects of the removal were observed on native prey or piscivore fish. This project demonstrated that while removals are an effective means of controlling lionfish, native fish abundance is not impacted when lionfish densities are relatively low. Removal efforts with the goal of increasing native biodiversity will need to consider these factors before committing resources to this strategy.

## Resumen

Desde mediados de la década de 1980, dos peces león del Indo-Pacífico, *Pterois volitans* y *P. miles*, han establecido una presencia significativa en el Atlántico Norte occidental, incluyendo el Mar Caribe y el Golfo de México. El primer informe documentado del pez león (*Pterois volitans*) en Puerto Rico se produjo en 2008, y estos peces depredadores se observaron por primera vez en La Parguera un año más tarde. En general, el pez león se profesaban afectar significativamente la función de los ecosistemas y servicios de los ecosistemas asociados. Esta amenaza y el impacto negativo de las invasiva pez león en los ecosistemas marinos en el Atlántico Norte occidental y el Caribe acentúan la necesidad de una investigación sostenida en sus dinámicas biológicas y ecológicas. Esfuerzos de remoción son un medio eficaz de reducir las densidades de peces león, sin embargo, la mayoría de los estudios no tienen en cuenta la reacción de las comunidades de peces nativos en respuesta a estas extracciones. Además, la evaluación de impactos también deben incluir la investigación de la ecología de alimentación para caracterizar el perfil dietético de pez león como sus dietas han demostrado ser sitio específico. El propósito principal de este estudio de cinco años fue investigar los efectos percibidos del pez león mediante la determinación de los impactos reales en las comunidades de peces nativos en Puerto Rico, abordando los siguientes objetivos: 1) caracterizar el perfil de la dieta del pez león mediante la secuenciación de próxima generación, 2) determinar la tasa de digestión de los peces y camarones presa artículos, con recomendaciones para futuros estudios de ecología de alimentación y 3) evaluar la eficacia de un pez león extracción a pequeña escala dedicada y la respuesta de los depredadores nativos y presas.

La principal desventaja de identificación morfológica y los códigos de barras de DNA es la incapacidad para utilizar el material digerido. Sin embargo, la secuenciación de próxima generación (es decir, metabarcoding DNA) puede analizar todos los componentes de los contenidos intestinales, incluyendo la porción previamente no identificable. Sesenta y tres peces león fueron capturados a partir de los contenidos de bajura y arrecifes de la costa de La Parguera y el estómago se separaron en dos regímenes de muestra - un líquido (es decir, digerido) y un pañuelo de papel. Una región 313bp de la I gen (COI) de la subunidad de la citocromo oxidasa se amplificó a partir de DNA extraído y las muestras fueron secuenciados con Illumina MiSeq. Las secuencias resultantes se compararon con bases de datos conocidos para identificar los especímenes al taxón más bajo. Se identificaron treinta y nueve especies de peces de 16 familias

(35 cada uno en las fracciones digeridas y tejidos), incluidos los miembros de Pomacentridae, Acanthuridae, Gobiidae, Apogonidae y Scaridae. Utilizando el material digerido resultó eficaz en la detección de especies de presa, especialmente aquellos que se habrían perdido con los métodos tradicionales. Además, las listas y frecuencias de ocurrencia de especies de presa podrían utilizarse para evaluar la selectividad de presas en general y las diferencias entre las áreas de la dieta o de los peces de diferentes tamaños.

La identificación de un artículo de la presa parcialmente digerido a través de la identificación morfológica es dependiente del nivel digestivo de la presa altamente. Este estudio analizó la tasa de digestión de los teleósteos conocido y presas de invertebrados en el estómago del pez león, en intervalos de tiempo determinados, para proporcionar una estimación de tiempo a la digestión de estos tipos de presas. Dos clases de tamaño de pez león se examinaron y la digestión de las presas se clasificaron. Se requieren aproximadamente 4 horas antes de una presa de pescado era irreconocible a nivel de especie, y 5 horas para el camarón. Predator tamaño, tipo de presa y el tiempo transcurrido desde la ingestión se observaron para predecir el rango digestión de las presas. La temperatura no se correlacionó con la digestión, por efecto del diseño experimental. Por otra parte, una escala detallada digestión presa fue desarrollado para camarones y se proporcionan recomendaciones para maximizar la identificación presas obtenidas utilizando análisis de contenido estomacal morfológica.

Pelotas, un arrecife de coral cerca de la costa lineal en La Parguera, fue una ubicación ideal para el experimento de extracción a pequeña escala como el tamaño imitaba la de la cerca de la costa, sacrificadas área marina protegida en Rincón (Tres Palmas). Pelotas tenía una pequeña población de peces león que no fue objeto de la industria del buceo o pescadores locales antes del estudio. Tres eventos de eliminación de más de un período de un mes reducen las densidades de peces león en esta zona 0.6 km. Se requieren nueve meses para las densidades de volver, aunque la biomasa de peces león todavía se redujo. La ausencia de pez león etiquetado y los pequeños tamaños observados en los meses posteriores a la eliminación sugiere recolonización ocurrió a través de la migración ontogénica en lugar de la inmigración laterales por los adultos a partir de los arrecifes cercanos. Al contrario de lo que se preveía dados los impactos percibidos, no se observaron efectos detectables de la eliminación de presas nativo o ictiófagos. Este proyecto demostró que mientras que las extracciones son un medio eficaz de

controlar el pez león, la abundancia de peces nativos no se ve afectada cuando las densidades de peces león son relativamente bajas. Esfuerzos de remoción con el objetivo de aumentar la biodiversidad nativa tendrán que considerar estos factores antes de comprometer recursos para esta estrategia.

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**The Lionfish Limericks:  
A Story of Invasion**

*A fish of Pacific origin  
made its way to Atlantic Borikén  
It would have been nice  
if that fish had thought twice  
before coming to reefs Puerto Rican.*

*That fish called lion is voracious  
and its impact from feeding hellacious  
plus with venomous spines  
to protect their behinds  
they're defended from fishes predacious*

*Although pez león is outlandish  
we may see them slowly to vanish  
As we eat them to beat them  
and locally defeat them  
Their beauty we quickly will banish*

**In Honor of Dr. Appeldoorn**  
by Chelsea A. Harms-Tuohy



## **Dedication**

To my husband, Evan Tuohy, for his ceaseless encouragement and motivation from day one of this degree and his eagerness to pursue a life of marine science with me thereafter. To my parents, Steven and Linda Harms, and my brother Cameron Harms, for their unconditional love and support of this dream despite its relentless determination to keep me from returning to Texas.

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## Chapter One: General Introduction

In the mid-1980s, two Indo-Pacific lionfishes, *Pterois volitans* and *Pterois miles*, were released into the Western Atlantic around Dania Beach, Florida likely as a result of unwanted pets purchased in the aquarium trade (Hare and Whitfield 2003; Semmens et al. 2004; Ruiz-Carus et al. 2006; Schofield 2009). Although two species were originally introduced, only one species (*Pterois volitans*) has been documented in the entire Caribbean, Gulf of Mexico and most of the Western Atlantic up past North Carolina, where thermal tolerance seems to be a factor in their spread further north (Kimball et al. 2004). Lionfish integration into the non-native environment could be a factor of various traits often identified in successful invasive species, including rapid growth, continuous reproduction, broad habitat preference and generalist feeding strategy, among others (Rahel and Olden 2008; Rahel et al. 2008). The lionfish is sexually mature within one year (Ahrenholz and Morris 2010) and grows at 0.1-0.6 mm/day (Akins et al. 2014). They spawn approximately every four days and in all seasons, releasing anywhere from 10,000 to 30,000 eggs per spawning bout (Morris 2009). The eggs float in a gelatinous mass and the larval duration is approximately 26 days (Morris et al. 2009; Ahrenholz and Morris 2010), likely contributing to their dispersal and ability to cross geographical barriers; however ballast transport cannot be ruled out as a facilitating factor. Almost a decade after the abundance of sightings in the early 2000s, researchers eventually identified lionfish densities in the invaded range to be an order of magnitude or greater than the native range (Green and Côté 2009; Grubich et al. 2009).

However, their spread is not without consequence to themselves as well as the native fish communities. Lionfish have undergone at least two genetic bottlenecks (Hamner et al. 2007) resulting in low diversity. The devil firefish (*Pterois miles*) has yet to actually be reported at

latitudes below Puerto Rico, and several haplotypes of *Pterois volitans* have been identified throughout the invaded range (Freshwater et al. 2009). Their impacts – both real and perceived – on native fish communities have been documented extensively (Albins and Hixon 2008; Morris and Akins 2009; Côté and Maljkovic 2010; Lesser and Slattery 2011). They are characterized as habitat generalists (Cure et al. 2014), as well as feeding generalists consuming both invertebrates and fish (Morris and Akins 2009) and do not seem to be prey-limited (Bejarano et al. 2015). They are found in all marine habitats, including estuaries (Jud et al. 2011), mangroves (Barbour et al. 2010), artificial structures and coral reefs. As marine fish invasions are typically quite rare in their documentation and success (Semmens et al. 2004), the lionfish invasion has resulted in an understandable, albeit retroactive, response from biologists and ecologists seeking to understand the mechanisms of the invasion success, the impacts and the future management of this predatory fish.

Lionfish (*Pterois volitans*) were first reported in Puerto Rico via an undocumented sighting in 2007 from Vieques and a northwestern location. Later in 2008, the first documented sighting was recorded in Dorado and Vieques (Schofield 2009) and La Parguera in 2009. Since then, their densities were observed to be the greatest on the northwestern and southwestern coasts, but more recently along the south coast (Jannette Ramos, pers. comm.) while the eastern coast appears to be the scarcest (Toledo-Hernandez et al. 2015). The local response was not immediate. However both recreational and commercial fishermen have taken an interest in lionfish, while local restaurants are promoting the fish on their menus. In recent years, the Department of Natural and Environmental Resources (DNER) developed a strategic plan for controlling lionfish in Puerto Rico, which involved requirements for all spearfishermen to register with the DNER in order to remove lionfish from marine protected areas, or areas usually

off-limits to fishing. However, despite the lionfish fishing regulations, there is still little management of this invasive species on a large scale around the island. The tournaments and derbies of South Florida and the Bahamas are not a popular method for control in Puerto Rico, but more importantly, the lack of dedicated management or assessment of any current management strategies renders such removal strategies untested if the response of the native fish communities is not addressed or considered. Puerto Rico's source of protein is largely supplemented by fishing, and the fishing community is comprised of both artisanal and commercial fishers. Unfortunately, the fisheries of Puerto Rico are largely unsustainable despite the presence of regulations, resulting in few large piscivorous predators like groupers or sharks, and leaving behind an overabundance of small territorial fish like damselfish, or small ecologically important fish like parrotfish (Pittman et al. 2010). Thus, the perceived pressure imposed by lionfish on the current fishery in Puerto Rico was a motivation to investigate their feeding ecology and future management potential. This dissertation sought to address the paucity of information available for lionfish diet and management efforts in Puerto Rico, with the specific objectives of 1) seeking to identify prey frequency of occurrence and assess presence of commercially and ecologically important fish, 2) determine the actual digestion rate of certain prey items with recommendations for future studies and 3) address the effectiveness of dedicated removals for the potential restoration of ecological function by investigating the impacts of small lionfish densities on the native fish population on a reef in La Parguera. The study is divided into three independent chapters (Chapters 2, 3 and 4), titled:

- 1.) The use of DNA metabarcoding for stomach content analysis in the invasive lionfish (*Pterois volitans*) in Puerto Rico

- 2.) Determining the digestion rate of known prey items in lionfish (*Pterois volitans*) stomachs with recommendations for future feeding ecology studies
- 3.) The effectiveness of small-scale lionfish removals in Puerto Rico and the response of native prey and piscivores

Two independent studies addressed concepts of lionfish feeding ecology (Chapters 2 and 3). Feeding ecology studies provide insight to predator/prey interactions and how the predator can alter the assemblages of native prey (Gerking 1994). Oftentimes, feeding ecology is assessed through gut content analysis, which requires an understanding of the digestion of prey items within fish stomachs (Hyslop 1980). Digestion of prey ultimately determines the accuracy of these tests, as well as the observer's ability to identify partially digested specimens (Baker et al. 2014). Within lionfish, feeding ecology has been addressed predominantly using gut content analysis with morphological identification (Morris and Akins 2009; Alexander and Haynes 2011; Jud et al. 2011; Green et al. 2012; Frazer et al. 2012; Layman and Allgeier 2012; Green and Côté 2014) or DNA barcoding (Barbour et al. 2010, Muñoz et al. 2011, Valdez-Moreno et al. 2012, Côté et al. 2013). This dissertation introduced a new method of gut content analysis through the use of next generation sequencing (i.e., DNA metabarcoding). Metabarcoding provided a more robust and accurate method of characterizing the entire dietary profile of the lionfish in Puerto Rico with the ability to utilize all portions of the stomach contents, including the digested prey. The outcome presented a more cost-effective and precise method of gut content analysis that reduced effort and maximized taxonomic resolution. However, not every feeding ecology study has the financial resources available to perform a molecular experiment or lack of laboratory equipment may exclude this method of gut content analysis. Thus, in order to maximize the amount of information obtained through traditional morphological identification, one must

consider digestion of prey items, time of sampling, characteristics of the fish feeding habits and local abundance and diversity of native “prey” options. Chapter Three investigated the role of digestion of prey items, which no study has yet addressed in lionfish feeding ecology. This study analyzed the digestion of shrimp and fish prey items (i.e., time to unidentifiable) in lionfish to provide recommendations to future studies. The results identified the time at which a prey item cannot be recognized to the species level, a factor of significant importance as taxonomic resolution decreases beyond this point, while difficulty in identification increases. These results assist with determining the most opportune sampling times, as well as what to expect for prey digestion given the estimated time since ingestion. Factors such as temperature, prey type and predator size can be used to predict digestion.

Chapter Four addressed dedicated lionfish removals on a small-scale as an effective means of controlling lionfish densities. The small-scale removal would be a beneficial strategy for management agencies lacking both the finances and manpower required to organize a large scale removal effort around the island. Small scale removals also allow these agencies or organizations to focus efforts on particular areas of interest, such as marine protected areas (MPAs) like Isla Desecheo or Reserva Marina Tres Palmas. Although removals have demonstrated to be an effective means of reducing lionfish densities (Frazer et al. 2012; de Leon et al. 2013; Côté et al. 2014), only one manipulative and predictive modeling study has addressed the response of the native fish to these removals (Green et al. 2014). However, as evidenced by Benkwitt (2015), the effects of lionfish scale non-linearly with increased densities and a universal rule to management cannot be applied to all areas of the invasion simply based on scaling up or down the impacts observed in high density areas. Thus, the need for addressing removal effectiveness and responses of the native community in the presence of low lionfish

densities underlined the objective of this study. The outcome provided evidence to suggest that native fish communities are not heavily impacted by the presence of lionfish in low densities, a result in agreement with recent research in Belize (Hackerott 2014) and Venezuela (Elise et al. 2014).

The final chapter (Chapter Five) provides a summary of the overall conclusions drawn from this dissertation. The overall results of each study are presented which include recommendations for future research and management of the lionfish in Puerto Rico.

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**Chapter Two:**  
**The use of DNA metabarcoding for stomach content analysis in the invasive  
lionfish (*Pterois volitans*) in Puerto Rico**

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Running title: DNA metabarcoding of lionfish stomach contents

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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 two 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 two sample components – a liquid (i.e., digested) and undigested tissue. A 313bp 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 the GenBank and BOLD databases 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.

## **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 others (Mooney & Cleland 2001). In particular, predation in the marine environment is a driving force structuring the fish communities 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 proved useful in biodiversity applications (Hebert et al. 2003), and has recently been promoted as an ecological tool for addressing issues of a species' invasion-potential, trophic interactions, and food webs among others (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. 2014). 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 of 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 prove to 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). Next generation sequencing 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 & Maljkovic 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 two 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, Layman & Allgeier 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 whose 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, metabarcoding analysis of all lionfish stomach contents, regardless of their digestive stage, is used 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 & Maljkovic 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 Rico 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 & 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 (Figure 2.1). La Parguera is a natural reserve in 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 on scuba at depths equal to or less than 30m. 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 (Table 2.1). The stomachs were removed in less than two hours after lionfish capture, and preserved whole in a -80°C freezer until further processing.

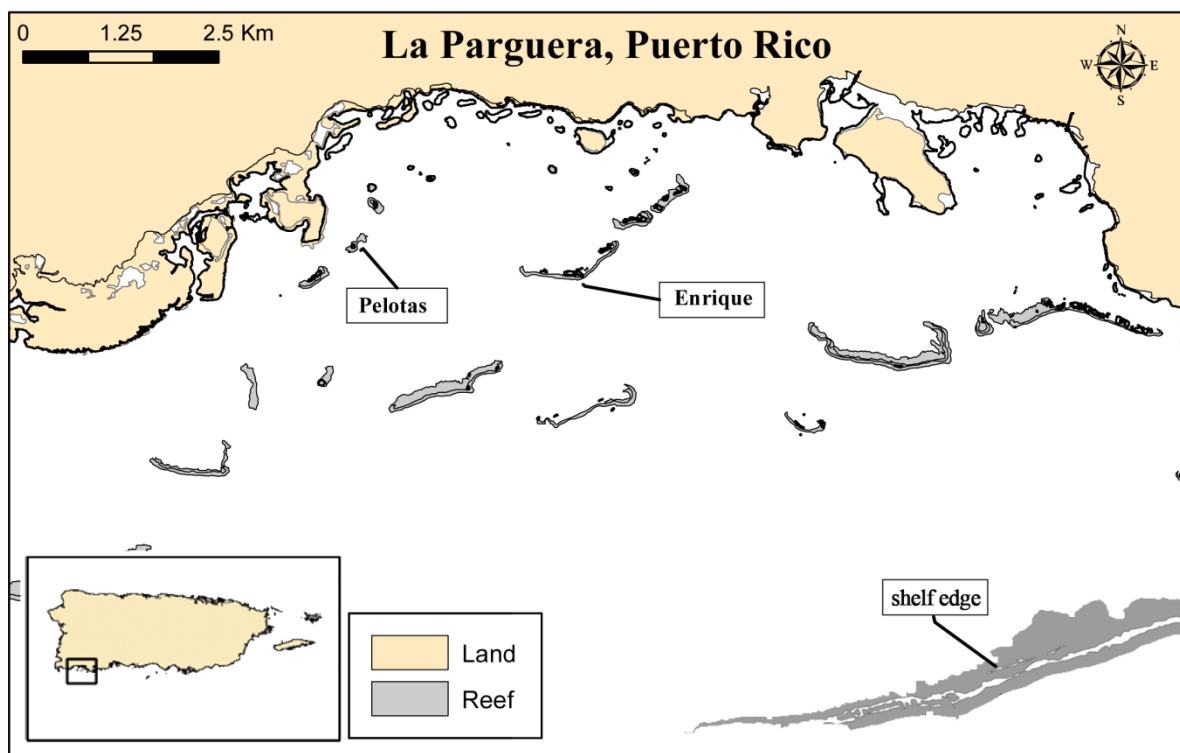


Figure 2.1 Map of La Parguera, Puerto Rico and the insular shelf, with study sites identified. Pelotas and Enrique reefs were the inshore collection sites, while the shelf edge was the offshore location. All sampling was performed at or above 30m

Table 2.1 Metrics reported for all lionfish. Gonadal stages are IA =inactive, ED = early development, SM = sexually mature and Gravid = females containing eggs. For sex, F= female,

M = male, and IM = immature where visual inspection could not identify sex. Weight is recorded with the stomach but without venomous spines.

<b>Lionfish ID</b>	<b>DATE COLLECTED</b>	<b>LOCATION</b>	<b>TL (mm)</b>	<b>SL (mm)</b>	<b>Weight (g)</b>	<b>Sex</b>	<b>Gonad Stage</b>
PL001	1/JUN/2013	INSHORE	252	190	179	IM	IA
PL003	1/JUN/2013	INSHORE	222	170	115	IM	IA
PL004	1/JUN/2013	INSHORE	240	175	171	IM	IA
PL005	1/JUN/2013	INSHORE	255	193	201	IM	IA
PL006	1/JUN/2013	INSHORE	274	207	209	IM	IA
PL007	1/JUN/2013	INSHORE	290	221	302	IM	IA
PL008	1/JUN/2013	INSHORE	225	171	112	IM	IA
PL009	1/JUN/2013	INSHORE	165	122	41	IM	IA
PL010	1/JUN/2013	INSHORE	228	171	145	IM	IA
PL011	15/JUN/2013	INSHORE	237	179	131	IM	IA
PL012	15/JUN/2013	INSHORE	267	204	176	IM	IA
PL013	15/JUN/2013	INSHORE	255	196	199	IM	IA
PL014	15/JUN/2013	INSHORE	219	168	117	IM	IA
PL015	15/JUN/2013	INSHORE	172	134	57	IM	IA
PL016	15/JUN/2013	INSHORE	246	186	139	IM	IA
PL017	15/JUN/2013	INSHORE	256	196	163	IM	IA
PL018	15/JUN/2013	INSHORE	262	204	179	IM	IA
PL020	15/JUN/2013	INSHORE	232	179	148	IM	IA
PL021	29/JUN/2013	INSHORE	99	74	9	IM	IA
PL022	29/JUN/2013	INSHORE	134	95	25	IM	IA
PL-N-01	30/JUN/2013	INSHORE	N/A	71	8	IM	IA
PL-N-02	30/JUN/2013	INSHORE	N/A	60	5	IM	IA
PL-N-03	9/JUL/2013	INSHORE	275	193	239	IM	IA
PL-N-04	9/JUL/2013	INSHORE	221	162	111	IM	IA
PL-N-05	9/JUL/2013	INSHORE	237	174	153	F	ED
PL-N-06	9/JUL/2013	INSHORE	252	190	218	IM	IA
PL-N-07	9/JUL/2013	INSHORE	244	174	154	IM	IA
PL-N-08	9/JUL/2013	INSHORE	230	165	137	IM	IA
PL-N-09	9/JUL/2013	INSHORE	320	239	438	IM	IA
PL-N-10	19/JUL/2013	OFFSHORE	270	214	251	F	GRAVID
PL-N-11	19/JUL/2013	OFFSHORE	242	180	154	F	GRAVID
PL-N-12	19/JUL/2013	OFFSHORE	255	189	184	M	ED
PL-N-13	19/JUL/2013	OFFSHORE	240	181	168	F	GRAVID
PL-N-14	19/JUL/2013	OFFSHORE	241	180	158	F	GRAVID
PL-N-15	19/JUL/2013	OFFSHORE	238	178	143	F	GRAVID
PL-N-16	19/JUL/2013	OFFSHORE	230	174	152	F	GRAVID
PL-N-17	30/JAN/2014	OFFSHORE	250	190	170	F	GRAVID
PL-N-18	30/JAN/2014	OFFSHORE	273	204	224	F	GRAVID
PL-N-19	30/JAN/2014	OFFSHORE	280	215	255	M	SM



PL-N-20	30/JAN/2014	OFFSHORE	265	195	224	F	GRAVID
PL-N-21	30/JAN/2014	OFFSHORE	370	284	541	M	SM
PL-N-22	30/JAN/2014	OFFSHORE	310	245	383	M	SM
PL-N-23	30/JAN/2014	OFFSHORE	250	192	166	F	GRAVID
PL-N-24	30/JAN/2014	OFFSHORE	230	175	146	F	GRAVID
PL-N-25	30/JAN/2014	OFFSHORE	335	260	428	M	SM
PL-N-26	30/JAN/2014	OFFSHORE	305	235	324	M	SM
PL-N-27	30/JAN/2014	OFFSHORE	255	195	186	F	GRAVID
PL-N-28	30/JAN/2014	OFFSHORE	290	230	309	M	SM
PL-N-29	30/JAN/2014	OFFSHORE	275	215	252	F	GRAVID
PL-N-30	30/JAN/2014	OFFSHORE	270	205	195	F	GRAVID
PL-N-31	30/JAN/2014	OFFSHORE	265	198	191	M	SM
PL-N-32	30/JAN/2014	OFFSHORE	235	173	139	F	GRAVID
PL-N-33	30/JAN/2014	OFFSHORE	240	183	175	F	GRAVID
PL-N-34	30/JAN/2014	OFFSHORE	245	185	177	F	GRAVID
PL-N-35	30/JAN/2014	OFFSHORE	250	195	171	F	GRAVID
PL-N-36	30/JAN/2014	OFFSHORE	275	205	256	F	GRAVID
PL-N-37	30/JAN/2014	OFFSHORE	243	175	153	F	GRAVID
PL-N-39	30/JAN/2014	OFFSHORE	295	224	278	M	SM
PL-N-40	30/JAN/2014	OFFSHORE	260	197	200	F	GRAVID
PL-N-41	30/JAN/2014	OFFSHORE	265	205	235	F	GRAVID
PL-N-42	30/JAN/2014	OFFSHORE	220	170	117	F	GRAVID
PL-N-43	30/JAN/2014	OFFSHORE	255	195	196	F	GRAVID
PL-N-44	30/JAN/2014	OFFSHORE	210	163	105	F	GRAVID

### 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 two 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 in 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, Inc.). Samples were stored in a -20°C freezer for downstream analysis.

A 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 (Herbert et al. 2003). Taxon-specific primers (for fish and invertebrates in coral reef fish guts) were utilized; the mlCOIintF forward primer (5' GGWACWGGWTGAACWGTWTAYCCYCC) in conjunction with the jgHCO2198 reverse primer (5'TAIACYTCIGGRTGICCRAARAAYCA) (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 20µl on the MyCycler (Bio-Rad Laboratories, Inc). The PCR recipe contained 0.6µl of 10µM of each forward and reverse primers, 10µl of MyTaq DNA polymerase mix (Bioline) and 0.5µl of genomic DNA. This recipe varied slightly depending on the success of the PCR, in which the concentration of DNA was increased up to 1.5µl and all other ingredients varied accordingly to maintain a 20µl reaction. We adopted the PCR profile from Leray et al. (2013) and conducted 16 initial cycles: denaturation for 10s at 95°C, annealing for 30s at 62°C and extension for 60s 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 minutes. 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 three

base identifier (ATG), followed by a specific six 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 Supplementary Material). One-hundred-twenty-six unique combinations of barcodes were produced 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 minutes. 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 1.5ml centrifuge tubes. In total, 108 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 one Illumina MiSeq lane (Scripps Research Institute, CA). Resulting reads were cleaned in the FASTQ filing and extended using FLASH pair software (Magoč & 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 de-multiplexed to identify reads back to their original stomach sample.

A total of 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 less than 200bp 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 greater 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 (BINs) 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 1-2 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 downstream analysis. Species that were represented by only one 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 on GenBank (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 two phyla, five orders, 19 families, 23 genera and 40 species. We assume that all prey DNA recovered from the gut was prey of lionfish. All fish sequences matched to a reference in BOLD and GenBank databases. 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 two insertions while eight had one deletion and seven sequences had an additional amino acid. Thirty-seven sequences could not be de-multiplexed 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 one 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 two sequences less than 200bp. Lastly, 99 sequences could not be matched at 98% or higher to either database. At similarity match of 80-100% in GenBank and BOLD, 22 sequences corresponded to invertebrates, with five 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, one Penaeidae, two Portunidae and one 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 2.2). Pomacentridae had the greatest frequency of occurrence (35%), which was dominated by three species: *Chromis multilineata* (71%), *Chromis cyanea* (63%), and *Stegastes partitus* (58%) (Table 2.3).

Table 2.2 Fish families represented in the lionfish (*Pterois volitans*) diet. Number of species corresponds to those identified to species level. Lutjanidae was identified only to family level. 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	0	0.55
Pomacentridae	4	34.69
Priacanthidae	1	0.55
Scaridae	5	10.65
Serranidae	3	9.29
Synodontidae	1	2.18

Table 2.3 Number of stomachs in which fish species were found, by location 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 (%)
<i>Acanthurus tractus</i>	3	5	4	5	0	12.31
<i>Apogon maculatus</i>	7	11	9	10	2	30.77
<i>Apogon pillionatus</i>	3	10	9	5	0	20.00
<i>Apogon townsendi</i>	0	4	4	1	1	7.69
<i>Bodianus rufus</i>	1	2	1	2	0	4.62
<i>Chaetodon capistratus</i>	2	2	0	4	0	6.15
<i>Chromis cyanea</i>	7	27	16	24	7	63.08
<i>Chromis multilineata</i>	15	28	7	9	3	70.77
<i>Clepticus parrae</i>	0	2	1	1	0	3.08
<i>Coryphopterus glaucofraenum</i>	9	5	8	10	1	23.08
<i>Coryphopterus hyalinus</i>	1	1	1	1	0	3.08
<i>Coryphopterus lipernes</i>	3	8	3	8	2	20.00
<i>Coryphopterus personatus</i>	2	0	1	2	0	3.08
<i>Coryphopterus tortugae</i>	2	0	1	1	0	3.08
<i>Emblemariopsis arawak</i>	0	2	0	2	0	3.08
<i>Emblemariopsis</i> spp.	4	4	5	4	1	13.85
<i>Gnatholepsis thompsoni</i>	0	1	1	0	0	1.54
<i>Gramma loreto</i>	2	0	0	2	0	3.08

<i>Haemulon flavolineatum</i>	2	2	2	3	0	6.15
<i>Halichoeres garnoti</i>	0	6	3	3	0	9.23
<i>Heteropriacanthus cruentatus</i>	1	1	0	2	0	3.08
<i>Hypoplectrus</i> spp.	6	10	8	10	1	26.15
<i>Hypoplectrus aberrans</i>	0	1	1	0	0	1.54
<i>Hypoplectrus nigricans</i>	4	3	3	5	3	15.38
<i>Hypoplectrus puella</i>	2	2	2	2	2	9.23
Lutjanidae sp.	0	1	1	0	0	1.54
<i>Malacoctenus macropus</i>	2	2	1	3	0	6.15
<i>Phaeoptyx conklini</i>	8	11	13	10	1	30.77
<i>Phaeoptyx pigmentaria</i>	2	5	3	5	3	15.38
<i>Sargocentron coruscum</i>	2	1	0	3	1	6.15
<i>Scarus iseri</i>	14	10	14	14	3	41.54
<i>Scarus taeniopterus</i>	0	2	1	1	0	3.08
<i>Scarus vetula</i>	1	1	1	1	0	3.08
<i>Sparisoma radians</i>	3	0	0	3	1	6.15
<i>Sparisoma viride</i>	1	3	2	2	0	6.15
<i>Starksia williamsi</i>	0	2	2	0	1	4.62
<i>Stegastes partitus</i>	8	26	17	15	4	58.46
<i>Stegastes variabilis</i>	1	1	1	1	0	3.08
<i>Synodus intermedius</i>	1	6	4	3	1	12.31

Four species were observed only in the inshore lionfish stomach contents, while eight species and one family were unique to offshore diets. Furthermore, three taxa were detected only in the liquid portion of the diet including the first account of the labrisomid *Starksia williamsi* in Puerto Rico (Table 2.4).

Table 2.4 Species that were only observed in one of the following habitat or type of stomach content categories.

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

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 taxa (76%) (Table 2.5). 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*.

Table 2.5 Number of stomachs in which invertebrate taxa were found, by location and gut fraction. Stomachs could have taxa represented in both liquid and tissue fractions. Frequency is the frequency of occurrence for each taxa from all stomachs. The percent similarity refers to the match to a reference in BOLD.

<b>Taxon</b>	<b>Inshore</b>	<b>Offshore</b>	<b>Liquid</b>	<b>Tissue</b>	<b>Frequency</b>	<b>% Sim.</b>
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
<i>Metapenaeopsis gerardoi</i>	0	1	0	1	5.88	97.6

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 to 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 due to high fishing intensity. Fishermen in the shallow water reef systems of La Parguera



typically 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 *Sparisoma viride*, *Scarus vetula*, *S. taeniopterus*, and *S. iseri*, 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 (Clark et al. 2015) provide field-occurrence data for a comparison to observed prey frequencies within the guts (Table 2.6). 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 & Maljkovic 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 2.6), represented by the absence of *Thalassoma bifasciatum* 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 prey traits for lionfish prey (Green et al. 2014).

Table 2.6 Percent frequency of occurrence of key species within lionfish gut contents and on the insular shelf of La Parguera (Pittman et al. 2010, Nemeth 2013) and Puerto Rico wide (Clark et

al. 2015). Nemeth's (2013) frequencies refer to abundance in terms of percent mean density of individuals/100m<sup>2</sup>.

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

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, Clark et al. 2015) and were not largely represented in the lionfish diet overall. In contrast, *Stegastes partitus* was one of 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 eight 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 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 100m<sup>2</sup> 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 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 three species are known to be inshore. Additionally, two 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 (Cervený 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 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 SL) and adult lionfish (n=7, 190-239 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 two databases would 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 on both BOLD and GenBank. The crustacean Order Decapoda contributed to the greatest resolution and highest frequency. The diet included both shrimps and crabs, 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 (Harms-Tuohy, personal observation), 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 fore reef habitat of the same depth and characteristics (Harms-Tuohy 2016). Considering 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 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 (0800 – 1400), 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. 2013). Regardless, this method has been used widely in lionfish feeding ecology. Visual assessment of lionfish 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 2.7).

Table 2.7 Yield of new species identified per sampling method. Comparison of visual identification, DNA barcoding and DNA metabarcoding methods.

<b>No. Stomachs</b>	<b>No. Fish Species</b>	<b>Method</b>	<b>Yield</b>	<b>Reference</b>
1,069	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	Current study

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 and true prey (e.g., items that were consumed by a prey fish that the lionfish subsequently ate). Although, our lionfish diet was mostly comprised of herbivores and planktivores with few piscivores, therefore this scenario is unlikely affecting 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 largely 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 holds particularly 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.

There are limitations to the metabarcoding approach (as with other molecular methods) that involve biases in each step of the process. In extraction, DNA may be differentially degraded which results in varying levels of expression of specific prey DNA. The gene amplification step introduces bias through PCR, which may selectively amplify COI from certain species to a greater degree than others. This selectivity is often an effect of the primers, where it is unlikely that they amplify COI from all possible prey items. In addition, there is also bias in the sequencing specific to certain next generation platforms. Illumina MiSeq is known to produce high error rates at the 5' end of the sequence (Thomas et al. 2012). A limitation of the metabarcoding approach and other molecular methods is the availability of reference information in both BOLD and GenBank. These databases may contain errors, which is known among GenBank users (Harris 2003). Lastly, a limitation of this study in particular is the comparison of metabarcoding to other lionfish gut content analyses (Table 2.7). Comparisons of species identified will depend on the species richness of the location and not solely on the method used to assess diet.

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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### **Chapter Three:**

#### **Determining the digestion rate of known prey items in lionfish (*Pterois volitans*) stomachs with recommendations for future feeding ecology studies.**

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## **Abstract**

Feeding ecology studies have provided valuable insight for addressing the impacts of lionfish on the native reef fish communities of the Western Atlantic and Caribbean. However, the majority of these studies have been based on morphological identification, usually of partially digested specimens. Morphological identification can be biased and prone to human error where these factors are highly dependent on the digestive level of the prey item. Additionally, lionfish predominantly feed during crepuscular hours, while many lionfish collections occur at opportune times for investigators, which usually do not correlate with feeding. Quantifying digestion rates of the dominant prey items in lionfish stomachs will provide insight to direct feeding ecology studies and assist in understanding existing biases. This study analyzed the digestion rate of known fish and shrimp prey items in lionfish stomachs, over set time intervals, to provide an estimate of time to digestion for these prey types. Two size classes of lionfish were examined and digestion of prey items were ranked. Approximately 4 hours were required before a fish prey item was unrecognizable at the species level, and 5 hours for shrimp. However, variation in level of identification (i.e., species, family) was evident at 2 hours and 30 minutes post-ingestion. Predator size, prey type and time since ingestion were observed to significantly influence digestion rank of the prey items. Furthermore, a detailed prey digestion scale was developed for shrimp and recommendations were provided to maximize high resolution dietary profiles obtained using morphological gut content analyses.

**Keywords:** gut content analysis, morphological identification, Caribbean, invasive species

## **Introduction**

Feeding ecology studies are valuable for estimating potential impacts of predators on fish communities. Such studies are fundamental to understanding predator-prey interactions, addressing topics of food habits, foraging habits and foraging habitats (Gerking 1994). The mechanics and factors associated with foraging habits (i.e., prey capture and handling) in fish is well defined. For example, the ability of fish to modulate prey-capture kinematics has been found to directly affect their trophic ecology, since these feeding mechanisms often limit the diet of the fish predator (Wainwright and Lauder 1986). Furthermore, the gape and mouth aperture of fish is correlated with the size of prey that a fish predator is capable of consuming (Werner 1977; Liem 1990; Nemeth 1997). Optimal foraging theory predicts that prey choice is shaped by natural selection such that a predator will utilize a foraging strategy that maximizes fitness (Pyke 1984; but see Pierce and Ollason 1987). Thus, theory would suggest that a predator will select a prey of maximum energy input requiring the least amount of prey handling. However, since optimal foraging conditions are often nonexistent in nature (Pierce and Ollason 1987), predators will often select prey of smaller size than predicted (Hoyle and Keast 1987), a choice regulated by availability of different prey types and abundance of prey (Werner and Hall 1974; Fossette et al. 2011), intraspecific competition (Milinski 1982), and habitat influences (MacArthur and Pianka 1966; Charnov 1976). Yet, while these variables contribute to understanding predator-prey relationships in a functional and ecological sense, other variables associated with feeding, such as prey digestion, are also important. Foraging, and a predator's functional response (relationship between prey density and predation rate, Solomon 1949), is influenced by two processes: prey handling and prey digestion, where digestion has been identified as a background process that determines hunger level and willingness to forage (Jeschke et al. 2002). In reviewing the physiological (digestion) versus ecological (foraging) processes associated with predation

rate and diet selection, Whelan and Brown (2005) concluded that digestion plays a critical role in willingness to feed, wherein some predators are constrained by this factor (Jeschke et al. 2002). Thus, digestion is directly related to the functional response of the predator, and predicting digestion rate of prey items is an important factor in enhancing the ability to characterize prey selection and investigate the role of optimal foraging.

Digestion and gastric evacuation, as the last phase of feeding, presents the only real evidence of the diet aside from observer observations of prey capture. In some cases, the remnants of digestion are the sole means of addressing a question, such as assessing the distribution of cephalopods by investigating the diet of pelagic fish predators (Romeo et al. 2012) or examining the contribution of plastic debris in large pelagic fish (Romeo et al. 2015). The rate at which items digest (or do not digest, i.e., plastics) in fish stomachs ultimately determines what can be detected by observers. Differential prey digestion can lead to a biased representation of certain prey in the diet (Sheffield et al. 2001; Braley et al. 2010), especially when visual inspection of dietary components is the sole method for assessing diet composition. The most widely applied method of gut content analysis involves the use of morphological identification of partially digested prey items. Unfortunately this method is prone to observer bias and the steep learning curve of identifying specimens based on taxonomic characters that may not be represented beyond a certain stage of digestion (Baker et al. 2014). Given these disadvantages, studies that use morphological identification should structure their experimental design such that prey digestion, and identification bias, is minimized.

The Western Atlantic and Caribbean lionfish (*Pterois volitans*) invasion has been well documented and researched for nearly a decade. The lionfish is known to consume over 70 fish species (Morris and Akins 2009; Côté and Maljković 2010; Valdez-Moreno et al. 2012) and a poorly documented diversity of invertebrates, whose relative abundance as lionfish prey is



strongly site specific (Côté and Maljkovic 2010; Muñoz et al. 2011; Layman and Allgeier 2012). The versatility in prey consumed may be attributed to the ability to utilize various feeding strategies to bring prey closer to the mouth, although the actual prey-capture kinematics of lionfish are not shown to modulate based on prey type (Pfeiffenberger 2012). Lionfish are suction feeders, and their versatility in prey capture behavior, such as corralling (Fishelson 1997), cooperative hunting (Lönnstedt et al. 2014) and blowing jet streams to potentially re-orient prey towards the mouth (Albins and Lyons 2012), supports their trophic position as a generalist predator. The generalist feeding strategy is common to invasive species (Rahel and Olden 2008; Rahel et al. 2008; Doupe and Knott 2010), but presents a hindrance to assessing impacts to native prey assemblages as prey preference is often indeterminable (Braley et al. 2010). In the case of the lionfish, prey preference has been correlated with particular prey species abundance in certain locations (Green et al. 2012a; Dahl and Patterson 2014), or traits of prey (Green and Côté 2014, Rocha et al. 2015). These conclusions have been drawn from feeding ecology studies that utilize stomach content analysis (i.e., visual inspection or DNA barcoding), stable isotope analysis or a combination of the two methods. However, the majority of these studies have been based on gut content analysis through morphological identification (Albins and Hixon 2008; Morris and Akins 2009; Alexander and Haynes 2011; Jud et al. 2011; Muñoz et al. 2011; Green et al. 2012a; Frazer et al. 2012; Layman and Allgeier 2012; Green et al. 2013; Green and Côté 2014), which may be underestimating diet composition or unintentionally introducing observer bias (Baker et al. 2014). Additionally, many lionfish collections occur at opportune times for researchers (e.g., between the work day hours of 8:00-15:00), which usually do not correlate with lionfish feeding.

The predation of lionfish on native reef fish has been studied extensively (Morris and Akins 2009; Côté and Maljkovic 2010; Green et al. 2011), and such studies suggest that lionfish

alter the dynamics of reef systems by reducing recruitment and biomass of native fauna (Albins and Hixon, 2008; Green et al 2012a). The same level of investigation has not been conducted on invertebrates, where only one study focused solely on their contribution to lionfish diet (Layman et al. 2014). The paucity of such studies could be an effect of the difficulty in identifying partially digested invertebrates. Lionfish feed predominantly during crepuscular hours (Morris and Akins 2009; Green et al 2011; Cure et al. 2012), but may also “snack” periodically throughout the day (Côté and Maljkovic 2010) and are hypothesized to not satiate at just one crepuscular period (Green et al. 2011), although this trend may be site specific. Feeding to satiation in piscivores affects estimation of predation rates because the short term storage in the gut is exceeded (Essington et al. 2000) and motivation to feed may be reduced (Gill 2003). Although, the contrast has also been demonstrated, where stomach fullness does not necessarily constrain willingness to eat when prey are available (Gill and Hart 1994). While lionfish likely feed to satiation, observations of predation attempts would suggest that stomach fullness may not deter motivation to feed when prey are in abundance. When satiated, prey items within the stomach are exposed to the digestive enzymes at varying levels, which affect prey detection in stomach content analysis. Prey items retained in the middle of the stomach may appear freshly consumed relative to those pressed against the stomach lining, suggesting that satiation could increase the number of identifiable items if stomach contents are assessed shortly after feeding. The digestion rate of prey has also been shown to depend on the number of items, size of the prey, size of the predator, and temperature, among other factors (Windell et al. 1976; Folkvord 1993; Bromley 1994; Knutsen and Salvanes 1999; Vinagre et al. 2007; Yamamoto et al. 2007; Legler et al. 2010). Size of the predator can affect rates of digestion where larger predators tend to require more time to digest a meal (Jobling et al. 1977) and are capable of consuming more prey items when preferred prey sizes are similar regardless of predator size (Gill and Hart 1994).

This study addressed effects of predator size and prey type on digestion in lionfish.

Descriptive scales of prey digestion stages can assist researchers in determining levels of prey digestion and the correct taxonomic rank that should be assigned, while also standardizing the procedure. Currently, one five-point digestion scale exists for lionfish (Green et al. 2012b), but it rates only the digestion level of fish prey. Prey items ingested at relatively the same time will digest at varying rates, dependent upon their composition (Randall 1967; Jackson et al. 1987). For example, shrimp, with an exoskeleton, have been found to require twice as much time to digest as fish (Andersen 1999; Berens and Murie 2008). Given that at least 28% of prey by number in stomach contents represent invertebrates (Morris and Akins 2009; Valdez-Moreno et al. 2012), a digestion rating scale for these organisms, specifically shrimp (a common prey item), is necessary. Two main problems arise when performing morphological identification on partially digested invertebrate specimens. The first obstacle is a question of how to accurately estimate the extent of digestion when no applicable scoring system is currently available for invertebrates. Taxonomic characters used to identify shrimp often include structures located on their appendages (e.g., presence or absence of snapping claw), which are frequently missing from a partially digested specimen, particularly if the claw was lost during prey capture (Randall 1967). Additionally, colors and patterns often used to discriminate between species of the same genus or family are often never retained through digestion. The second issue concerns the variation introduced through observer bias when assigning a digested specimen to a particular category (Baker et al. 2014), an issue shared among all prey types. However, to be reliable, researchers need to be confident and consistent about the taxonomic rank they assign to a partially digested specimen during morphological identification.

The purpose of this study was to evaluate lionfish digestion rates as a function of different prey types (fish and shrimp) and predator size. This study addressed three goals: 1) to

identify the time at which known prey items are digested beyond identification, 2) to identify if the digestion of these prey items differs based on lionfish size, and 3) to suggest improvements to future lionfish feeding ecology studies based on this study's empirical analysis of prey digestion rate.

## **Materials & Methods**

### **Experimental Design**

Live lionfish were collected with clear vinyl hand nets with mesh bottom from various reefs of La Parguera (17°58'29" N, 67°02'47" W) (Figure 3.1), then transported to an outdoor aquarium facility (6.1m x 1.5m x 0.9m) with continuous flow-through seawater system. This facility is exposed to natural light cycles, temperature fluctuations and sea water chemistry and contains hard bottom with artificial substrates to promote natural behaviors from fish during acclimation. Lionfish from two representative size classes, small (<80 to 160mm) and medium (161 to 215mm), commonly observed in the La Parguera insular reef systems were used for the study. A floating basket (0.4m x 0.3m) with small-mesh screen was used as an experimental housing while each fish was subjected to a feeding trial. The basket allowed the researcher to observe feeding from above, and provided the lionfish with room to exhibit all natural forms of prey capture without hindrance from the enclosure. Fish prey items (i.e., Haemulidae, Pomacentridae, Scaridae, Labridae, Holocentridae) were collected from the back reef of Laurel and Enrique reefs, while euryhaline shrimp (*Macrobrachium rosenbergii*) were purchased from a local hatchery. Lionfish were starved for a minimum of three days prior to the start of each feeding experiment. Water temperature was recorded at each trial.

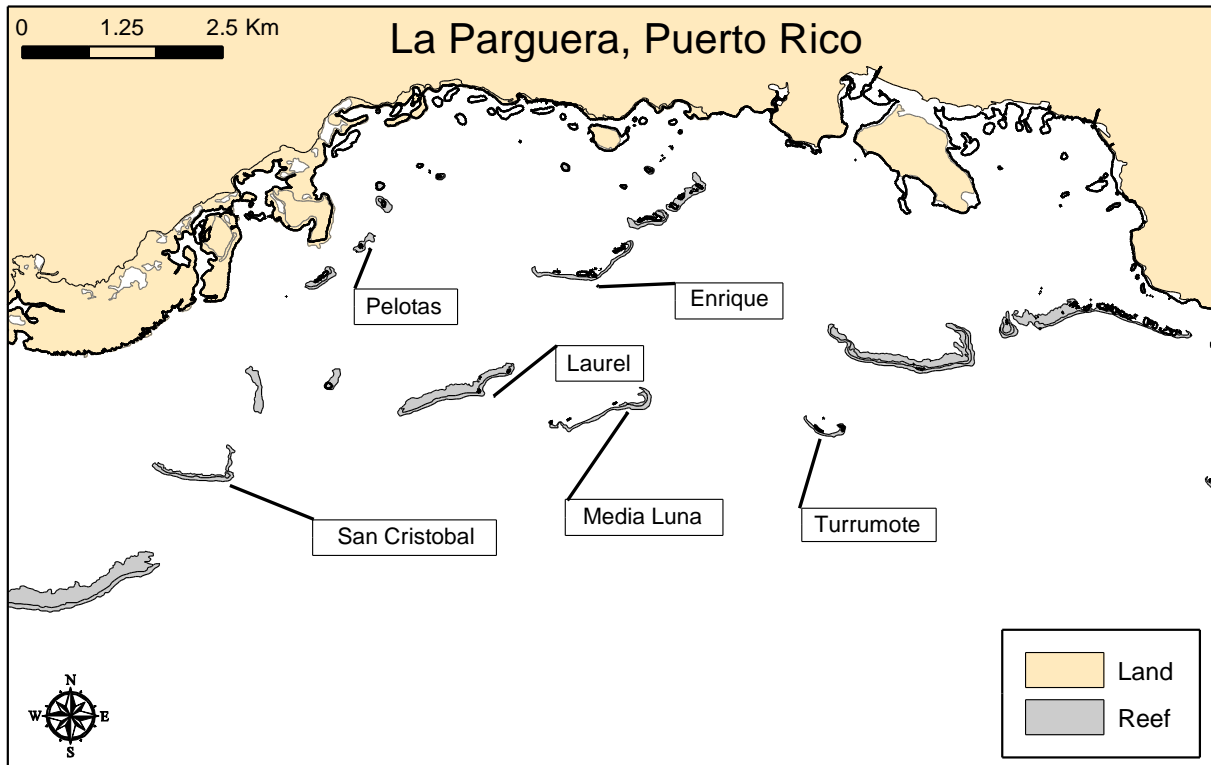


Figure 3.1 Map of La Parguera, Puerto Rico and various reefs of the insular shelf. Lionfish collection took place at these various sites. The map was generated from ArcMap v. 10.1

One lionfish was randomly selected from those in the holding tank, placed in the experimental basket and allowed to acclimate to the basket for one to two days before prey items were introduced. Lionfish were presented with fish or shrimp of similar sizes to avoid effects of differential digestion based on prey size. Additionally, prey sizes were controlled relative to the size of the lionfish, with body depth of the prey kept smaller than the maximum possible (i.e., the maximum body depth of prey that can be consumed relative to the maximum gape of the lionfish), as generally observed for piscivores (Lawrence 1958; Gillen et al. 1981; Nilsson and Brönmark 2000). The feeding trial commenced when the first prey item was consumed. Prey items were then continually introduced to the basket until satiation was reached (i.e., fish would feed *ad libitum* until hunting ceased and live prey items remained). Feeding trials consisted of a

designated period of time allowing for digestion to occur based on previous research (Doupé and Knott 2010; Legler et al. 2010; Green et al. 2011). Only one type of prey (fish or shrimp) was offered for the duration of each feeding trial. Time to reach satiation and the number of prey required varied by lionfish, but ranged from 30 seconds to 5 minutes (with a maximum of 15 minutes) and required at least three to seven prey items. One feeding trial per designated time (durations of 1 hour and 45 minutes with 30 minute or 1 hour intervals thereafter, up to 9 hours) per size class was conducted, with some exceptions. At the end of the designated time, the fish was sacrificed for gut content analysis. A total of 36 feeding trials were conducted, but these were not evenly distributed across the prey types and lionfish size classes. Larger fish require more time to digest, respectively, larger prey (Legler et al. 2010; Baker et al. 2014), and shorter times initially used were found to be inadequate for digestion. As a consequence, these were dropped when assessing shrimp digestion rates.

The stomach was removed, and each prey item was weighed and subsequently measured for total length (TL) and scored for extent of digestion. Green et al.'s (2012b) five-point digestion scale was used to identify the level of digestion of the known fish prey items. Two independent scorers ranked the digested prey by documenting morphological changes from an undigested state. As the fish scale did not provide adequate identifiers to correlate with invertebrate metrics, a similar five-point digestion rate scale for shrimp was developed (Table 3.1). However, this scale differed from that for fish in that (1) more descriptive characters for each possible score were included, making the scale more interpretative and standardized, and (2) descriptions for half rankings (i.e. 1.5, 2.5 etc.) were added to reduce observer bias. Half scores alleviate the condition of uncertainty in total digestion rank when some characteristics varied across prey items in their degree of digestion over time.

The digestion rate of fish and shrimp prey items in lionfish stomachs was determined by calculating the amount of time elapsed for the prey items to become unrecognizable for morphological identification (i.e., time to scale rating of 5). Similar to other studies (Legler et al. 2010), digestion was considered complete when no prey items could be identified.

Table 3.1 A general guide to rate the level of digestion of shrimp (*Macrobrachium rosenbergii*) within *P. volitans* stomachs. Scale refers to the rank given to a prey item where 1 is the least digested and 5 is the most digested.

Scale	Description
1	Exoskeleton and appendages (e.g. legs, antennae and rostrum) are present and intact. Natural color and markings are present; appears freshly eaten. Species level identification.
1.5	Exoskeleton is intact. Natural colors and markings are still visible but may be slightly fainter. Some appendages could be missing. Exoskeleton does not appear fresh. Species level identification.
2	Exoskeleton is intact. Natural colors and markings may be faint. Some appendages are missing or fragmented. Eyeballs are intact but some pigment may be missing. Species level identification.
2.5	Exoskeleton disjointed or connection is obviously weakened and indented, particularly at the carapace/abdomen juncture. Most appendages are missing or fragmented. Natural colors and markings may be faint. Genus or Family level identification.
3	Exoskeleton disjointed. All appendages may be missing or fragmented. Eyes may be deflated and some pigmentation may be missing. No colors or markings. Flesh beginning to degrade. Family level identification.
3.5	Exoskeleton disjointed. All appendages missing. Possibly no eyes, colors or markings. Pieces of exoskeleton are missing but the entire animal is still connected in some area. Body likely flaccid. Family level identification.
4	Pieces of animal may be loosely connected as to identify as an invertebrate; but all other defining taxonomic characters are missing. Body is flaccid. Tail may be absent. Invertebrate-only identification.
4.5	Can be identified as an invertebrate; pieces of exoskeleton may be present but no full animal is intact. No exoskeleton on the body. Invertebrate-only identification.
5	Animal is fully digested. Tissue present cannot be distinguished as vertebrate or invertebrate.

### Statistical Analysis

A proportional odds ordinal regression model (McCullagh 1980; Agresti and Kateri 2011) was used to analyze the relationship between predator size, prey type, and time since ingestion on digestion in lionfish (SPSS v. 19). To assess potential effects of temperature variation on digestion during feeding trials, a partial correlation was used to compare

temperature during each feeding trial per size class with the corresponding digestion rate for fish prey items, controlling for time (SPSS v. 19). The effect of temperature on shrimp digestion was not assessed as all trials were conducted in the same month and temperature did not fluctuate. The time required to digest each prey type to an unidentifiable state was plotted to illustrate potential trends. For each lionfish, the median rank of digestion for all prey items in its stomach was used to create one value of digestion for that specific prey type; this allowed each item to be considered in the overall weighting of digestion rank.

## **Results**

In all cases, as more time elapsed, the digestion level of the prey items increased towards an unidentifiable state (Figure 3.2), although shrimp were found to require more time than the study allotted to fully digest past recognition as an invertebrate. Similarly, larger lionfish required longer periods of time to achieve the corresponding digestion rank observed with smaller lionfish. Linear trends were observed in all treatments and slopes were almost identical for fish prey types within each size class of lionfish (Small/Fish:  $R^2 = 0.86$ ,  $y=0.0085x + 0.92$ ; Medium/Fish:  $R^2 = 0.90$ ,  $y=0.00841x + 0.35$ ), and similar to small lionfish consuming shrimp ( $R^2= 0.72$ ,  $y=0.0058x + 0.19$ ), but marginally different from medium lionfish consuming shrimp ( $R^2=0.93$ ,  $y=0.0114x + -2.12$ ). The range of digested prey items illustrated the varying degrees of digestion contributing to the median rank obtained (Table 3.2).



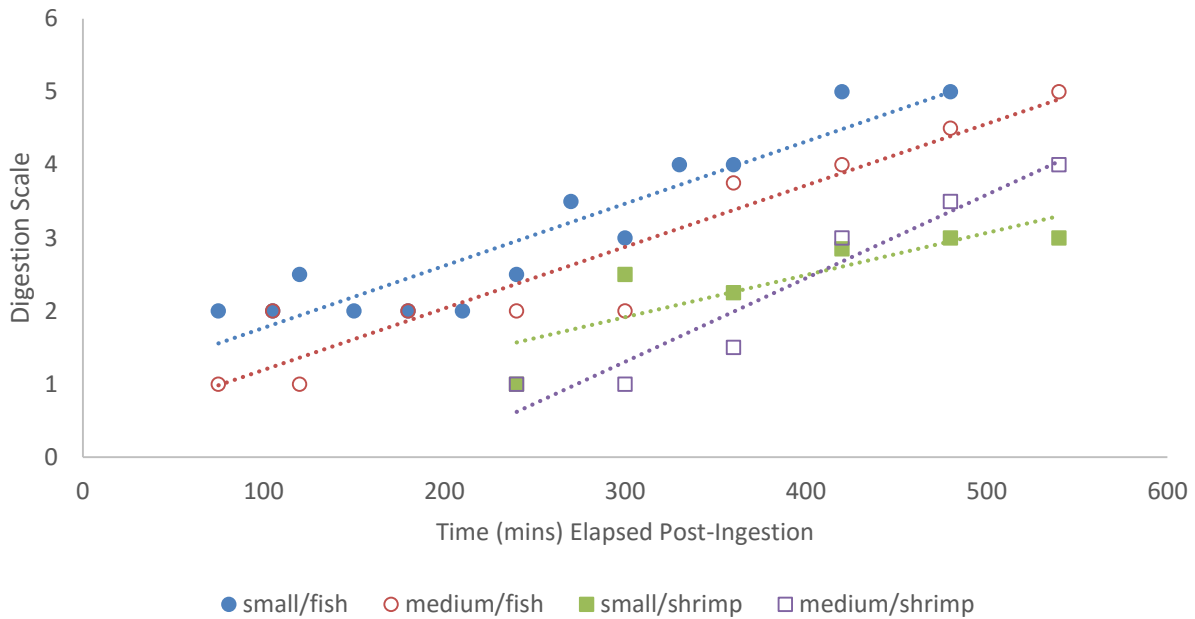


Figure 3.2 The median digestion level of fish and shrimp prey items for both small and medium size classes (<80 to 160mm, 161 to 215mm, SL respectively) of lionfish. Time elapsed represents the feeding trials conducted, where one value represents one fish. Dotted lines represent least squares regression lines for each size class/ prey type combination

Table 3.2 State of digestion over predetermined times (post-ingestion) of fish and shrimp prey consumed by *Pterios volitans*. Values are the median digestion state (1-5) among multiple prey items in an individual stomach. Range of digestion is in parentheses. Each combination of prey type, time and lionfish size is represented by a single trial (=fish). Small (<80 to 160mm) and Medium (161 to 215mm) refer to size of the lionfish. Temperature is recorded in Celsius

Time Feeding Trials	Fish Prey				Shrimp Prey			
	Small	Temp	Medium	Temp	Small	Temp	Medium	Temp
1:30	2 (1-4)	26.3	2 (1-2)	26.4	-	-	-	-
2:00	2.5 (1-4)	26.3	1 (1-2)	28.7	-	-	-	-
2:30	2 (2-4)	26.4	-	-	-	-	-	-
3:00	2 (2-3)	27	2 (1-2)	29	-	-	-	-
3:30	2 (2-3)	27	-	-	-	-	-	-
4:00	2.5 (2-3)	27	2 (1-3)	29	1 (1-1.5)	28	1 (1-1.5)	29
4:30	3.5 (3-4)	27.7	-	-	-	-	-	-
5:00	3 (1-4)	27.7	2 (2-3)	27	2.5 (2-2.5)	29	1 (1-4)	29
5:30	4	28.7	-	-	-	-	-	-
6:00	4 (1-5)	26	3.75 (3-5)	29	2.25 (2-2.5)	29	1.5 (1-2)	29
7:00	5	29	4 (3-5)	27	2.85 (1.5-4.5)	29	3 (1.5-4)	29
8:00	5	29	4.5 (4-4.5)	28	3 (3-3.5)	29	3.5	29

9:00	-	-	5	28	3 (1.5-5)	29	4 (3.5-4.5)	29
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The assumption of proportional odds for the ordinal regression model was met, as assessed by the full likelihood ratio comparing the fitted model to a model with varying parameters ( $\chi^2 = 16.087$ ,  $df= 30$ ,  $p >0.05$ ). The deviance goodness-of-fit test indicated that the model was a good fit to the observed data ( $\chi^2= 90.681$ ,  $df= 371$ ,  $p>0.05$ ) even though most cells contained zero frequencies, an effect of using a continuous variable as a covariate (Hosmer and Lemeshow 2000). The final model showed a significant relationship between the independent variables (prey type, lionfish size and time since ingestion) and the dependent variable (digestion rank of prey items), over the intercept-only model ( $\chi^2= 64.413$ ,  $df= 3$ ,  $p <0.05$ ). The regression explained 85% of the variability in prey digestion rank based on the variables (Pseudo  $R^2 = 0.852$ ) and all factors significantly influenced digestion rank (Table 3.3). An increase in time since ingestion was associated with an increase in digestion rank of prey items. Additionally, fish prey type was more likely to influence digestion rank than shrimp prey (i.e., fish prey were digested more rapidly). Finally, smaller lionfish were more likely to have higher digestion ranks of prey compared to medium lionfish. The partial correlation to test for potential effects of temperature on digestion of fish prey resulted in no association in small lionfish ( $r_s=0.183$ ,  $N=10$ ,  $p>0.05$ ) or medium lionfish ( $r_s= -0.149$ ,  $N=7$ ,  $p>0.05$ ), when controlling for time.

Table 3.3 Results of the parameter estimates from the ordinal regression (SPSS v. 19) assessing the relationship of prey type, predator size and time since ingestion on the median digestion rank of prey items in lionfish (*Pterois volitans*) stomachs. Significant directional statement of effect requires an OR >1 for this scenario. Confidence intervals correspond to OR.

Factor	Estimate ( $\beta$ )	Odds Ratio (OR)	95% Confidence Intervals	Wald $\chi^2$	d.f.	P value
Time	0.035	1.036	1.036 -1.049	29.938	1	<0.01
Prey Type	6.382	591.013	44.698-7814.5	23.470	1	<0.01
Predator Size	1.981	7.250	1.683-31.230	7.069	1	<0.01

## **Discussion**

The lionfish is predominantly a crepuscular feeder (Morris and Akins 2009; Green et al. 2011; Cure et al. 2012), yet logistical constraints often prevent researchers from harvesting and assessing specimens until later in the day, resulting in partially or completely digested stomach contents. Lionfish feeding ecology studies indicate that prey richness is likely greater than estimated due to a significant portion of unidentifiable stomach contents (70% in Morris and Akins 2009), which can underestimate their ecological impact on native fauna (Côté et al. 2013). The assessment of digestion of both fish and shrimp prey items in lionfish stomachs provides future feeding ecology studies with insight to adjust sampling times in an effort to standardize methods and interpretations utilizing morphological identification as the sole method of gut content analysis. In this study, 4 hours 30 minutes was the minimum time period in which the lowest resolution of identification (species) was lost for fish prey (3 on the scale, Green et al. 2012b). However, after 2 hours 30 minutes to 5 hours it was still possible to identify prey to species or family in some cases. This indicates that other factors apart from those assessed in this study contribute to the rate of digestion, such as number of prey items consumed. Six hours post-ingestion leads to identification solely as a vertebrate, which is quantitatively useless to identifying specific prey preference or addressing impacts to certain fish communities. If lionfish begin feeding before sunrise (approximately 7:00) but are not collected until after 8:00, then the first prey items consumed would have already been digested past species level recognition by the time the fish has been dissected a few hours later.

Overall, the factors investigated (prey type, predator size and time since ingestion) significantly influenced digestion rate. Differences were observed between size classes, where larger lionfish were found to require longer periods of time to achieve the corresponding rate in smaller lionfish. Likewise, shrimp prey items required a longer time period for initial digestion

past “species” identification. These linear trends were similar between prey type and predator size. In the field, lionfish are typically found to be either resting or hunting. Based on this study, it is likely that lionfish require substantial time to complete digestion, which may correspond to the amount of time spent resting, especially since our experimental lionfish were exposed to natural climatic variations.

Nevertheless, digestion rate will depend on additional factors than those tested here. For example, in nature, lionfish would likely consume shrimp and fish randomly and opportunistically, resulting in mixed prey types in the gut ingested over a longer period of time, which would also affect digestion rate. Fish may require less handling time (Juanes et al. 2001) and are easier to digest than shrimp (Andersen 1999; Berens and Murie 2008) which could result in a higher percentage of fish in the stomach. Additionally, invertebrate consumption has been correlated with size of lionfish (smaller lionfish consume proportionally more invertebrates) (Morris and Akins 2009), a trend observed for other carnivorous fishes (Rooker 1995; Cocheret de la Morinière et al. 2003). Thus the rate of digestion will be dependent on prey type, number of prey consumed, and order of ingestion (Baker et al 2014; Bromley 1994), of which lionfish size and prey type were only controlled in this study. Digestion is also dependent upon abiotic factors like temperature and salinity. Temperature is known to affect digestion rate in fish (Vinegre et al. 2007; Legler et al. 2010). In this study, warmer temperatures corresponded to higher digestion rankings by effect of experimental design where longer feeding trials were conducted in the warmer months. However, in this study temperature was not observed to be an influencing variable on the rate of digestion, thus supporting the significant effect of time. For shrimp prey, it is important to note that all feeding trials were conducted in the warmest months

of the year. Digestion may be longer during the cooler months. Temperature effects could be tested in future studies by randomizing the feeding trials or controlling the temperature.

As lionfish diet has become extensively studied, the focus of such studies has shifted away from gross characterizations based on family level identification of prey items and towards identifying what key species may be at risk or what species are actually preferred. Despite their generalist feeding strategy, specific traits of prey are likely targeted by lionfish (Green and Côté 2014) as well as specific species (Rocha et al. 2015), as has been observed for other fish predators (Nilsson and Brönmark 2000; Beukers-Stewart and Jones 2004). For example, piscivorous pike (*Esox lucius*) was found to prefer smaller than maximum ingestible prey (i.e., less than the maximum body depth relative to predator gape) and preferred shallow bodied prey over deep bodied prey (Nilsson and Brönmark 2000). Similarly, lionfish have been reported to prefer shallow bodied prey (Green and Côté 2014) However, deep bodied prey, like the family Pomacentridae, have been frequently observed in lionfish stomachs (Harms-Tuohy et al. in press) and when controlled for size were readily consumed in our experimental trials (n=9). Additionally, these individuals were often more digested than small-bodied prey in the same stomach, perhaps as a consequence of their greater surface-to-area ratio or potential reduced handling time in prey capture. Prey preference and the impacts of predation will depend not only on ease of capture and nutritional value, but also on the relative abundance of potential prey. A study investigating prey size preference in largemouth bass (*Micropterus salmoides*) found deep-bodied and shallow-bodied prey to be equally preferred when prey abundances were relatively equal (Hambright 1991). Thus, perhaps the generality of prey preference towards shallow-bodied prey could be overshadowed by the potential reduction in handling time of certain deep-bodied prey when body depths are relatively similar and encounter rates of all prey types are equal. This

scenario could be a likely situation for opportunistic lionfish that appear to be unconstrained by prey availability (Anton et al. 2014; Bejarano et al. 2015). These observations encourage the need to obtain the highest resolution dietary analysis to address both perceived and real impacts. Thus, studies should design sampling strategies that aim to collect and dissect lionfish within the first two hours after feeding to maximize identification to the lowest possible taxon.

While the majority of the feeding ecology literature concerns lionfish effects on reef fish communities, few studies have addressed their consumption of benthic marine invertebrates (Morris and Akins 2009; Jud et al. 2011; Layman and Allgeier 2012; Valdez-Moreno et al. 2012), and only one study to date has focused solely on these taxa (Layman et al. 2014). The most common invertebrate families reported from gut content analysis include shrimp families Palaemonidae, Penaeidae (Barbour et al. 2010; Jud et al. 2011; Layman and Allgeier 2012), and Alpheidae (Valdez-Moreno et al. 2012; Layman et al. 2014). These shrimp serve important ecological roles to coral reef ecosystems as prey for many native fishes (Duarte and Garcia 1999; Yeager and Layman 2011) and as fish parasite cleaners (Becker and Grutter 2004). Thus, the reduction of these invertebrates would likely have detrimental effects on coral reef ecosystems (Alexander and Haynes 2011; Stella et al. 2011). Stark differences between exposed teleost tissue and shrimp exoskeleton, among other physical differences (Jackson et al. 1987), make the use of the existing fish digestion scale ineffective for invertebrate digestion ranking. Primarily, the exoskeleton of crustaceans seems to maintain structure, and thus protect the muscle underneath to a higher degree than exposed vertebrate tissue (Jackson et al. 1987). This study's descriptive guide provided a basis to rank shrimp digestion and assisted in reducing variation between observers. The descriptive characteristics for a given rating (1-5) would frequently apply comprehensively to a given shrimp prey item. However, in some instances, digestion

excluded all descriptors from being applied explicitly. For example, a shrimp with a rating of “three” on the digestion scale would meet only some of the descriptive requirements, and some characteristics associated with other adjacent ranks. Thus, the half rankings were introduced to alleviate this dilemma and provide a cushion to reduce the variance of observer bias. When the ranking system was subsequently applied to an analysis of lionfish gut contents, independent ranking of the state of shrimp digestion by different observers resulted in almost identical conclusions.

Considering all controlled and uncontrolled elements of prey digestion in lionfish, this study suggests that utilizing visual inspection as the sole method of gut content analysis should sample lionfish as close to dawn or dusk as possible, and immediately place lionfish on ice to slow digestion. Preliminary observations of prey abundance and lionfish density at sampling sites should be considered, as aggregations of lionfish would encourage cooperative hunting where prey abundances are high, resulting in fuller stomachs at a faster rate. Feeding to satiation could enhance observation of less digested prey because items will be exposed to varying levels of digestive enzymes (Essington et al. 2000). Ideally, more than one method of gut content analysis should be applied to amplify the diversity of prey observed, as well as to determine the contribution of certain prey items to the overall diet. For example, a dietary study of the arrow squid (*Nototodarus gouldi*) used both a morphological and DNA-based approach, showing that morphological identification alone was not suitable but the combination of the two methods was found to be highly useful in characterizing the diet of this generalist predator (Braley et al. 2010). The molecular method of DNA barcoding has been used recently within lionfish feeding ecology (Barbour et al. 2010, Munoz et al. 2011, Valdez-Moreno et al. 2012, Côté et al. 2013), as well as the new method of metabarcoding (Harms-Tuohy et al in press). These studies, when combined

with visual inspection, will provide the most accurate assessment of the dietary profile of lionfish.

There are limitations to the application of using gut content analysis and digestion rates to understand prey selection, specifically as it applies to the life history of the fish. For instance, lionfish are gape-restricted predators so their ability to consume certain prey items is a factor of jaw morphology. Juvenile lionfish typically consume more invertebrates, which is consistent with the well-documented ontogenetic dietary shifts of piscivores (Wainwright and Richard 1995). Additionally, adult lionfish are predominantly piscivorous and may be unable to capture invertebrates that are hidden deep within the reef. However, no small juveniles or post settlers were used in this study, so there is a lack of understanding of the rate at which invertebrates digest at this life history stage. Thus, the digestion rates determined here apply to the size classes investigated and cannot be extrapolated to post-settlers, which are likely feeding on a variety of smaller invertebrates besides shrimp.

Overall, our study identified the time to digestion for two of the most commonly observed prey items in lionfish stomachs, while suggesting that digestion can be predicted based on prey type, predator size and time since ingestion. When collecting lionfish in the field, only one of these factors can be selected for (predator size), however visual census of the prey communities can assist with predictions of digestion rate and drive the experimental design to sample at appropriate times to maximize identifiable prey items.

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**Chapter Four:**  
**The effectiveness of small-scale lionfish removals as a management strategy:  
effort, impacts and the response of native prey and piscivores**

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## **Abstract**

Lionfishes (*Pterois volitans* and *P. miles*) are now established in all regions of the Caribbean, Gulf of Mexico and the Western Atlantic. As they continue to spread and colonize, reef ecosystems could be undergoing significant changes in species composition. The only means of control seem to be targeted removals, but most organized removal efforts occur on large spatial scales, or involve many volunteers or personnel. This study sought to address the effectiveness of lionfish removal over small spatial scales on a linear coral reef in La Parguera, Puerto Rico. The study area mimicked the size and complexity of small marine protected areas, and quantified the effort necessary to diminish and maintain low lionfish densities. Three removal events took place over one month by 11 skilled lionfish hunter-volunteers per day to cull a 0.6 km area. Timing of recovery varied by area in the removal region, but biomass was reduced. The recovery of lionfish in post-removal months was due to re-colonization via recruitment or ontogenetic migration rather than by lateral immigration. No detectable effects of the removal were observed on native prey or piscivore densities. This project demonstrated that while removals are an effective means of controlling lionfish, native fish abundance may not be impacted when lionfish densities are relatively low. Removal efforts with the goal of increasing native biodiversity should consider lionfish density relative to the densities of the native faunal communities before committing resources to this strategy.

**Keywords:** *Pterois volitans*, management, control, invasive species, Caribbean

## **Introduction**

Since the mid-1980s, two Indo-Pacific lionfishes, *Pterois volitans* and *P. miles* have established a significant presence in the Western Atlantic, including the Caribbean Sea and the



Gulf of Mexico. Second only to habitat loss, invasive species are often cited as the largest global threat to biodiversity (Mooney and Cleland 2001), and the presence of lionfish has raised significant concerns regarding their impacts on native fish species. In general, lionfish are found to significantly affect ecosystem function and associated ecosystem services (Albins and Hixon 2008; Morris and Akins 2009; Green and Côté 2009; Arias-González et al. 2011; Green et al. 2012a; Albins 2013; Albins and Hixon 2013). These negative impacts of lionfish on marine ecosystems in the Western Atlantic and Caribbean emphasize the need for sustained research on their biological and ecological dynamics. Recent research has indicated that biotic resistance (i.e., relying on native predators to control lionfish) is not currently a sufficient means of lionfish control (Hackerott et al. 2013; Valdivia et al. 2014) despite anecdotal accounts of predation. Management focus has thus shifted to consider targeted population control as the only means of reducing lionfish impact (Barbour et al. 2011; Green et al. 2012a).

Many studies have identified specific habitats and areas where targeted removals could mitigate potential effects of lionfish and provide the greatest management. These include habitats such as rugose reefs and the edges of drop-offs (Bejarano et al. 2015) as well as sites of high priority management including marine protected areas (Akins 2012). Other studies have assessed the effectiveness of lionfish culling in different regions throughout the Caribbean (Frazer et al. 2012; de León et al. 2013; Côté et al. 2014; Green et al. 2014). These studies address large scale removal efforts both in terms of effort (e.g., derbies) and spatial scale (e.g., those that span large areas or multiple reef patches). The conclusions are unanimous in showing that culling is effective.

However, many of these studies defined effectiveness solely as the ability to maintain low lionfish densities. In contrast, only one empirical study has directly evaluated the impact of

lionfish removal on the potential to restore effected fish communities (Green et al. 2014, but see Valderrama and Fields 2015). Green et al. (2014)'s study produced a model to predict target lionfish densities necessary to allow for native prey biomass to recover in a depressed-density scenario. However, the observed increases in prey biomass and the corresponding target lionfish densities were found to vary greatly across the study's patch reef system due to inter-reef variation in prey biomass. While this robust model provides a basis for estimating threshold densities and incorporates biotic and abiotic factors influencing prey production, its use of lionfish biomass rather than density limit its application in a true management scenario. Furthermore, as evidenced in Benkwitt (2015), the effects of lionfish scale non-linearly with increased densities; thus a universal rule to management cannot be applied to all areas of the invasion simply based on scaling up or down the impacts observed in high density areas. A recent assessment of lionfish impacts in Venezuela found no detectable effects on the native fish assemblages, even at high lionfish densities (max observed 0.05 fish/m<sup>2</sup>), indicating that healthy reef systems may be able to mitigate impacts naturally without the intervention of removals (Elise et al. 2014). Effects of lionfish at varying densities, including low densities, would be beneficial in determining if, where and under what conditions lionfish removal would be effective and efficient. No current studies have dealt with practical issues of small-scale targeted removal, such as the necessary frequency of re-culling and its relation to the mechanisms of lionfish recolonization, or addressed the impacts of lionfish at relatively low densities.

This study assessed the effectiveness of small-scale removals on a linear reef in southwest Puerto Rico to address three goals: (1) To assess the temporal and spatial effectiveness of localized lionfish removal through an experimental approach, including the quantification of catch/effort relative to population size and density, (2) To assess the effectiveness of a lionfish

removal event on the degree and rate of recovery of native reef fish species commonly found in lionfish stomachs and the response of native piscivorous predators, and (3) To assess the time frame and mechanisms of lionfish re-colonization.

## **Methods**

### **Study Site**

The study was conducted along the fore reef of Pelotas Reef in La Parguera ( $17^{\circ}57'26''$  N,  $67^{\circ}04'13''$  W) from May 2013 to June 2014. The study site was approximately 1.2 km in length with a maximum depth of 18 m. This area was chosen because it mimics the size and complexity of other established no-take marine protected areas in Puerto Rico (particularly the Tres Palmas Marine Reserve in Rincón, which receives frequent culling), and because Pelotas Reef was not a specific target for recreational divers who frequently cull lionfish at popular dive sites in La Parguera and was therefore assumed to have a relatively undisturbed lionfish population. The study site was partitioned into three removal areas with two controls, one on Pelotas Reef and one on the adjacent Conserva Reef, which is separated from Pelotas by a minimum 30 m wide, 10 m deep channel and lacks mangrove habitat (Figure 4.1). The removal region (0.6 km) was separated into three zones (A-C) to equally space both sampling allocation and diving/removal effort. The zones were delineated underwater with a series of semi-permanent rope lines that were laid perpendicular to the reef crest that extended from a depth of 3 m to the base of the fore reef. Additionally, subsurface marker buoys were attached to each rope.

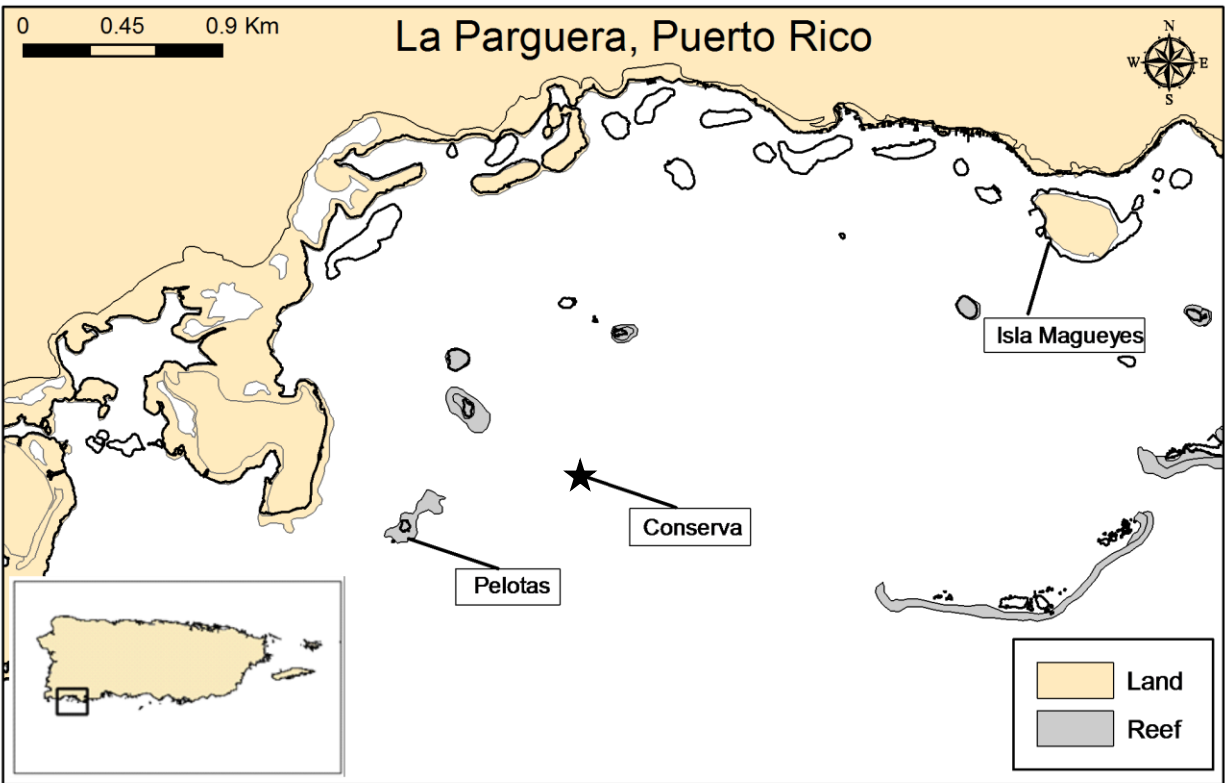


Figure 4.1 Location of the study site in La Parguera, Puerto Rico. The removal was conducted on Pelotas reef, the controls were on Pelotas and Conserva reef. Conserva is a submerged reef with no mangroves, indicated with the star symbol

### Sampling & Experimental Design

Underwater visual census (UVC) was used to assess the size class and density of lionfish and density of native piscivores (e.g., snappers, groupers, jacks, etc.). Quadrat sampling was used to determine species richness and abundance of native juvenile and cryptic reef fish species which are typical prey commonly observed in lionfish stomachs in Puerto Rico (Harms-Tuohy et al. in press). Sampling was conducted in each of the removal and control regions, and carried out one month before and one, three, six, nine and 12 months after the removal, representing a Before-After-Control-Impact (BACI) design (Table 4.1). Reef rugosity in each area was calculated using the method in Weil et al. (2005), and the slope of the reef was measured from still images using Image J (v. 1.49), while habitat characteristics were noted qualitatively.

Table 4.1 Timeline denoting tasks for the duration of the project.

<b>Task</b>	<b>May 2013</b>	<b>June 2013</b>	<b>July 2013</b>	<b>Sept 2013</b>	<b>Dec 2013</b>	<b>Mar 2014</b>	<b>June 2014</b>
Lionfish Survey	X	X	X	X	X	X	X
Piscivore Survey	X		X	X	X	X	X
Prey Survey	X		X	X	X	X	X
Lionfish Tagging	X		X				
Removal Events		X					

Transects of 25x3m were placed parallel to the reef crest, with approximately 3 to 5 m in between each transect. Number and size class of lionfish and number of native piscivores were recorded on each transect. Lionfish size was categorized as Small Juvenile (< 80mm), Juvenile (81 to 160mm), Maturing (161 to 215mm) and Adult (>216mm; TL), where size at maturity is estimated at 189mm (Gardner et al. 2015). There were 12 transects in each region except one control (Pelotas) where only four transects could fit in the area (n=52, per month sampled). Fifteen minutes were spent surveying each transect. The total area surveyed was 14,526 m<sup>2</sup>, including controls on Pelotas and Conserva Reefs. The initial population size was calculated for the total removal region (9,840 m<sup>2</sup>) by extrapolating the observed lionfish density to the total reef area.

Native fishes representing potential prey species were sampled from five randomly placed 1x1 m canvas quadrat tents in each region (n=25, per month sampled). A fish anesthetic (15% quinaldine/seawater solution) was introduced underneath the canvas tent and allowed to soak for 1-2 minutes to anesthetize and retain target fishes (cryptics and juveniles) which were identified to lowest possible taxon and counted (Gibson 1967). This method was preferred over belt transects to more accurately account for nocturnal or cryptic fish that are commonly missed in underwater visual census but are known lionfish prey (Harms-Tuohy et al. in press).

To assess the possible movement of lionfish into the removal region from the two control regions, conventional anchor tags (FD-68B FF, 3/4 inch; Floy Tag & Mfg., Inc., Seattle, WA, U.S.A.) were inserted into 18 lionfish of various sizes using a Floy<sup>®</sup> pistol grip corrosion resistant tagging gun. Fifteen lionfish were tagged on Conserva Reef, and three fish were tagged on the non-removal area of Pelotas Reef. The tagging procedure involved a simplified version of the method used by Akins et al. (2014) and similar to Tamburello and Côté (2015). The anchor tag was chosen for its high visibility underwater, its sturdy design and reduced handling time with insertion. One diver would capture the lionfish between a clear vinyl hand net and a black mesh hand net. The black mesh net was used as a distracting device to back the lionfish into the clear net, after which it was secured between the nets until the diver could safely remove it with a gloved hand. The diver would carefully restrain the lionfish by the head within the mesh hand net in one hand and then reveal the dorsal musculature for tag insertion with the other hand. The tag was inserted at an anterior angle behind and 3 mm below the 5<sup>th</sup> dorsal spine. The procedure required less than two minutes, and the fish was immediately released in the exact location in which it was tagged. No trauma was ever detected, and fish were inspected before release by lightly tugging the tag to ensure it was secure. After the release, tagged fish were observed for up to 2 minutes for any unusual behavior. The tagging occurred prior to the baseline visual censuses and the experimental removal, but also continued for one month post-removal. All re-sightings of tagged individuals were documented during post-removal sampling and included location or habitat changes at re-sighting.

### **Removal Design**

The experimental lionfish removal spanned a one-month period (June 2013), with three removal events occurring at two-week intervals on Saturday mornings (08:00 – 10:00). Removal employed only experienced lionfish spearfishermen to both maximize and standardize effort and

to limit any possible damage to the reef or injury to other native species. A total of 20 scuba divers participated, with a daily average of 11 divers per removal event. As an incentive, and again to standardize effort, each diver received a spearfishing kit that included a 1.2-m pole spear, collection bag, spine-resistant gloves, underwater flashlight and spine clippers. All divers used the same pole spear throughout the removals. One or two paired-diver teams were dropped into each of the three removal areas and were followed at the surface via surface marker buoys. Divers hunted for approximately 60-90 minutes, roving within their boundaries and from depths of 3-18 m, covering approximately 15,840 m<sup>2</sup>. Upon surfacing, lionfish were placed on ice immediately to preserve them for gut content analysis. Samples were returned to the lab where they were measured for standard and total length (mm), weight (g), sex and maturation state (juvenile/mature) based on macro-examination of the gonad (Green et al. 2012b). After each removal day, a simplified transect survey (n=12 per area) assessing lionfish in only the removal region was employed to estimate the reduction in lionfish density from the day's fishing effort and the remaining population abundance.

### **Statistical Analyses & Interpretation**

The decline in the rate of population removal (changes in catch per standardized unit of effort = CPUE) was used to estimate initial population size (DeLury 1947) as a cross check of the baseline estimate from visual census. The DeLury method assumes that the proportion removed from a given unit of effort is constant across the study. The method provides a calculation for expanding upon this small scale design for application to any removal scenario. A unit of effort was defined as a diver in the water for 1.5 hours. Because sea conditions and bottom turbidity can affect the ability to detect lionfish, a correction factor was used to account for poor conditions on the first removal day. This was done by surveying (n =12 transects) a reef with similar lionfish densities under conditions characteristic of both the first and second/third

removal events. The proportional reduction in observed lionfish density under the poor conditions (24%) was multiplied to the total effort during the first removal to obtain relative effective effort.

For all statistical comparisons concerning fish densities, the control area on Pelotas Reef was not included in order to remove the effects of an uneven assessment as this control was not surveyed prior to the removals. However, the Pelotas control area was included when addressing lionfish movement into the removal region. To test the effectiveness of lionfish removal, the densities of lionfish were compared before and after the event for all sampled months, and inside and outside the removal region using a nested permutational multivariate analysis of variance (PERMANOVA, Primer 6). The response variable was counts of lionfish in each transect, and there were four factors (time [2] = before/after removal events, month [6], treatment [2] = removal or control, and area [4] = 3 removal, 1 control). In the design, month was nested in time and area was nested in treatment. Area represented the sampling divisions in the study site and was included as a factor to account for variability in the observed differences of lionfish densities and the expected differential response to removal, associated with inter-reef habitat differences and variations in initial lionfish densities. To test if lionfish re-colonization occurred via recruitment or immigration, the length-frequency distribution of lionfish within each size category was compared before and after removal and separately comparing all months after the removal using Chi Square analysis (SPSS v.19). The biomass of lionfish removed and recovered in each treatment (removal and control) was calculated using a length-weight equation with constants supplied from previous literature (Green et al. 2012a, 2014) to further evaluate reductions based on size. The same PERMANOVA design was used to address the response of native prey and piscivores. Prey evenness ( $H'/H_{\max}$ ) using Shannon Wiener Diversity ( $H'$ ) was



calculated for each month of sampling within the removal region (Pielou 1966). The densities of specific piscivores in this study were compared with those obtained in pre-lionfish years on Pelotas Reef (Nemeth 2013).

## **Results**

### **Lionfish Densities & Initial Population Estimates**

Thirteen lionfish were observed over all transects in the removal region in May 2013 with an average density of  $0.004/\text{m}^2 \pm 0.005$  (mean  $\pm$  SD) and an estimated population size of  $39 \pm 17$  (95% CI). Twenty-two lionfish were removed from Pelotas Reef during the removals of June 2013, while in the control on Conserva Reef lionfish densities did not significantly decline. Twenty-three days after the last removal event, only five lionfish were observed in the removal region, for an average density of  $0.002/\text{m}^2 \pm 0.001$  and a population size of  $19 \pm 3.4$ . One year post-removal, only 10 lionfish were observed, for an average density of  $0.003/\text{m}^2 \pm 0.001$  with an estimated population of  $29 \pm 3.34$ . Across the three removal events, CPUE showed a consistent decline ( $y = -0.1437x + 1.7278$ ,  $R^2 = 0.9952$ ,  $p = 0.04$ , Table 2) and the DeLury method estimated an initial population size of 39 lionfish within the removal region. Thus, both the survey method and the DeLury method estimated the same initial population size of lionfish for Pelotas Reef.

Approximately nine months were required before lionfish densities peaked near their initial density within all areas of the removal region. However, initial lionfish densities in all areas of the removal region were dissimilar, and the response to removal was not the same (Figures 4.2 – 4.4). Lionfish density in the control area was initially higher than in all of the removal areas, and while density also decreased over time, the pattern was fundamentally different from that observed within the removal region. The decline did not start until after July

(i.e., after the adjacent removal event), with minimum density not observed until six months after removal (Figure 4.5).

Table 4.2 Catch per unit effort (CPUE) for each of the removal events. One unit of effort is a diver in the water for 1.5 hours. CummeE is the cumulative effort.

	Catch	Effort(diver)	Catch/Effort	ln(C/E)	CummeE
<b>1-Jun</b>	10	2.82	3.54	1.264597	2.823529
<b>15-Jun</b>	10	10	1	0	12.82353
<b>29-Jun</b>	2	10	0.2	-1.60944	22.82353

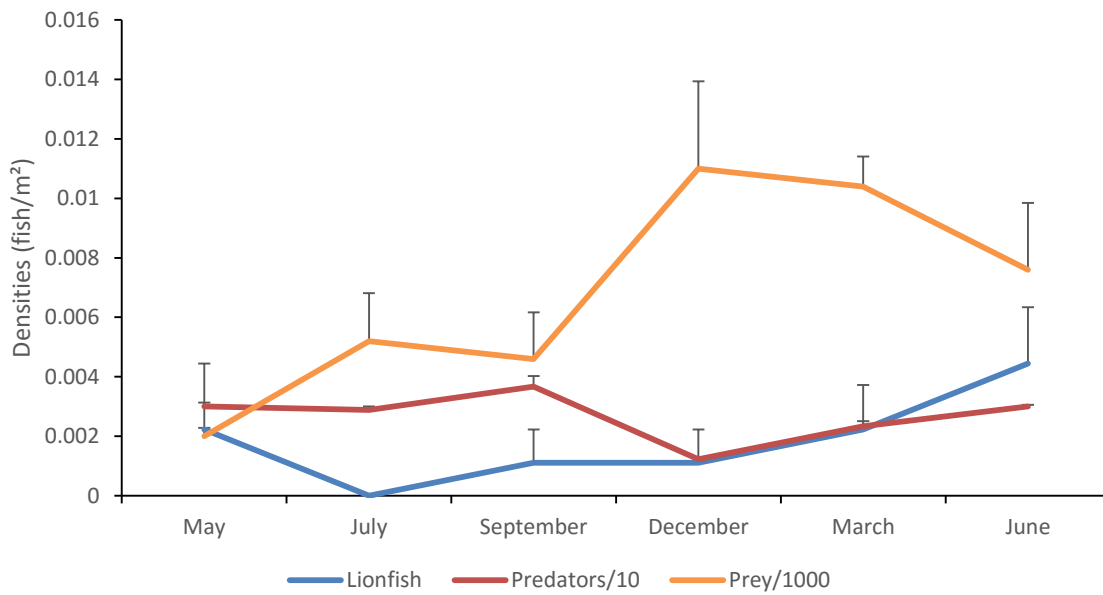


Figure 4.2 Estimated lionfish, other predators and prey densities within removal Area A before (May) and after the removal events (July - June). Error bars represent standard error of the mean. Predator densities were divided by 10 and prey densities by 1000 in order to plot trends on the same scale

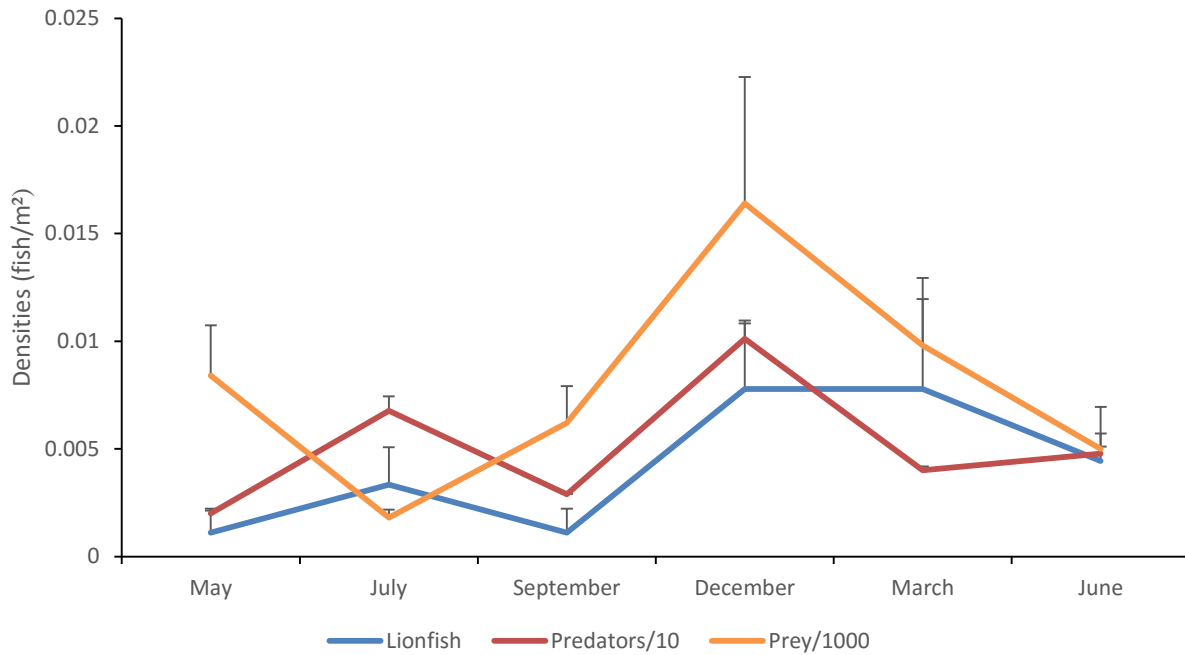


Figure 4.3 Estimated lionfish, other predators and prey densities within removal Area B before (May) and after the removal events (July - June). Error bars represent standard error of the mean. Predator densities were divided by 10 and prey densities by 1000 in order to plot trends on the same scale

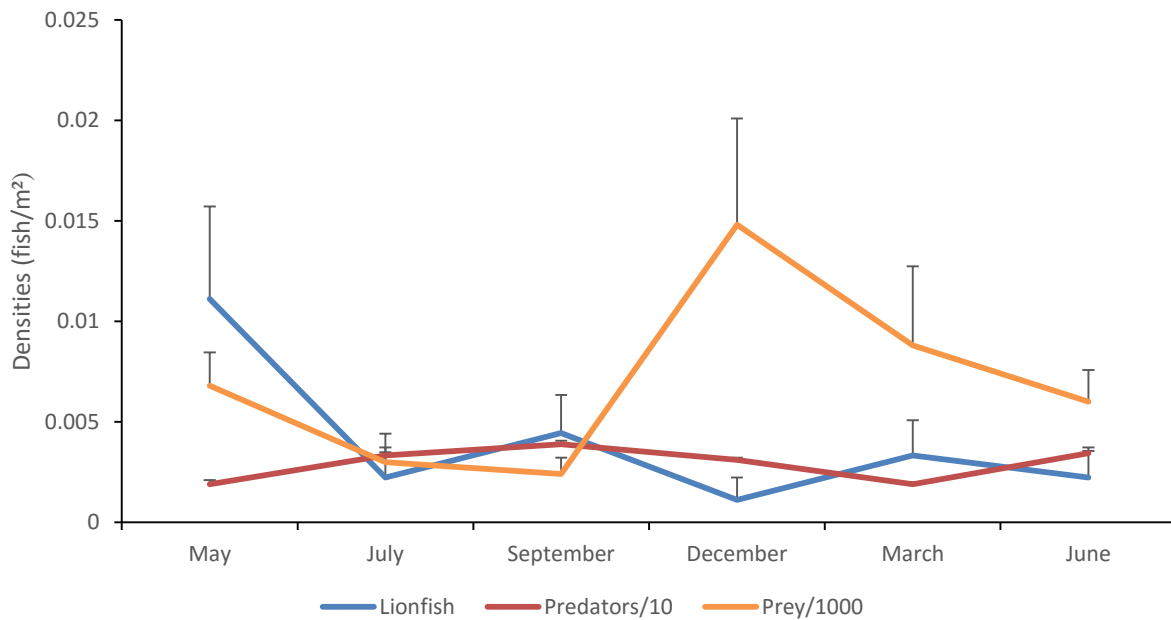


Figure 4.4 Estimated lionfish, other predators and prey densities within removal Area C before (May) and after the removal events (July - June). Error bars represent standard error of the mean. Predator densities were divided by 10 and prey densities by 1000 in order to plot trends on the same scale

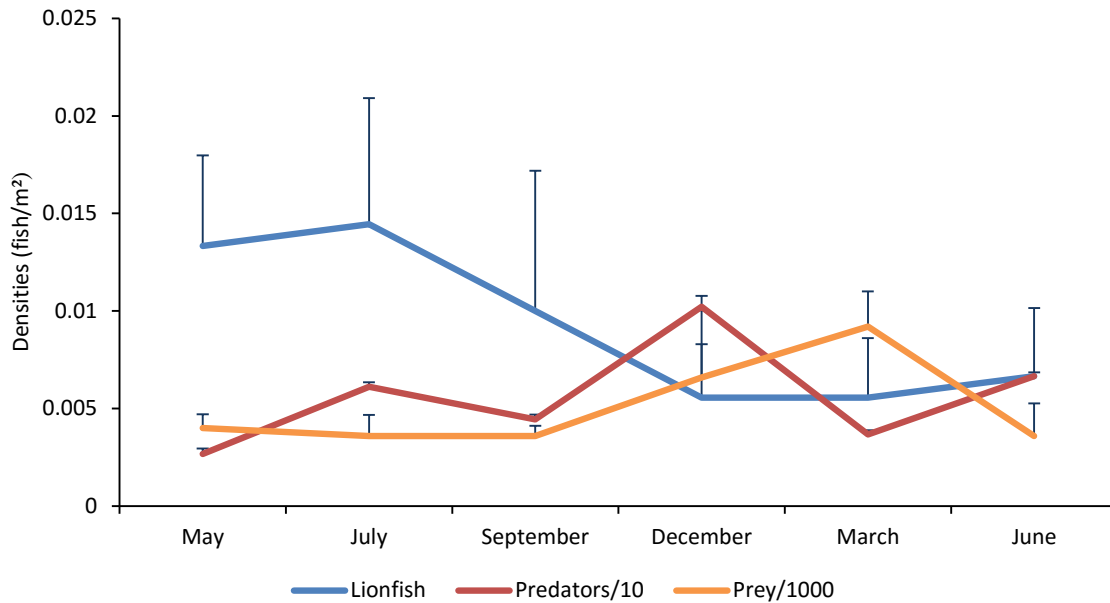


Figure 4.5 Estimated lionfish, other predators and prey densities within the control region (Conserva Reef) before (May) and after the removal events (July - June). Error bars represent standard error of the mean. Predator densities were divided by 10 and prey densities by 1000 in order to plot trends on the same scale

Despite the variability observed in Figures 4.2-4.5, the PERMANOVA showed no significant differences in lionfish abundance when comparing before and after the removal events, across time and between treatments (Table 4.3). However, there were significant differences in the densities of lionfish in each area, which differed before and after the removal ( $p < 0.05$ , Figures 4.2-4.4). A post-hoc pairwise comparison indicated that Area C differed in densities before the removal and immediately after (May vs. July; Monte Carlo  $p = 0.003$ , Anderson et al. 2008) further illustrated by the decrease in lionfish density at this time (Figure 4.4) which was not detected in other areas. Overall, lionfish densities were reduced in both treatments, where 62% of the population was extracted from the removal region and 63% observed to decline in the control. However, the temporal decline is drastically different, where the reduction in Pelotas Reef occurred in one month (0.004 to 0.001 fish/m<sup>2</sup>) versus the six-month decline on Conserva Reef (0.013 to 0.005 fish/m<sup>2</sup>). The similar, albeit gradual, decline in

lionfish within the control region provides a natural replication to the removal in that similar trends were observed despite the lack of any experimental manipulation. The lowest density observed in the control was effectively equivalent to the starting density in the removal region. Rugosity was similar among areas, except Area C, as well as habitat characteristics on both Pelotas and Conserva Reefs, however slope did marginally vary by area (Table 4.4).

Table 4.3 PERMANOVA results for the comparison of lionfish abundances before and after the removal and inside and outside the removal region, for all months. Month and Area are nested within Time and Treatment, respectively

<b>Factor</b>	<b>df</b>	<b>SS</b>	<b>MS</b>	<b>PseudoF</b>	<b>P</b>
Time	1	5.5007	5.5007	0.8288	0.5452
Treatment	1	24.284	24.284	3.1594	0.1311
Month (Time)	4	5.1417	1.2854	1.3472	0.3386
Area (Treatment)	2	9.0056	4.5028	4.7191	0.0608
Time*Treatment	1	1.284	1.284	0.22408	0.9504
Time*Area (Treatment)	2	13.006	6.5028	6.8151	0.0326
Month (Time)*Treatment	4	13.942	3.4854	3.6528	0.0602
Month (Time)*Area (Treatment)	8	7.6333	0.95417	0.40954	0.9132

Table 4.4 The average rugosity (Weil et al. 2005) for each area where 0 is least rugose and 1 is most rugose. A-C indicate removal areas on Pelotas. The slope is measured as the angle created from the interaction of the reef slope and a horizontal line parallel to the bottom.

<b>Factor</b>	<b>Conserva</b>	<b>A</b>	<b>B</b>	<b>C</b>
Rugosity	0.3516	0.3733	0.3633	0.4416
Slope (angle)	59.7	62.2	50.5	45.7

### Lionfish Size Patterns

During the removal events, lionfish of maturing and adult sizes initially dominated the catch but mean size gradually declined with subsequent removals. The final removal day resulted in two small juvenile lionfish. Surveys showed the same trend. Before removal, most individuals surveyed were in the maturing size class followed by the adult size class, while after removal (July) the distribution was significantly different ( $\chi^2=9.58$ ,  $df=3$ ,  $p=0.02$ ), with abundance dominated by the small juvenile size class with some maturing fish also present (Figure 4.6). In

the controls, no patterns were observed in size structure. Over time (July – September), the size class distributions inside the removal region was observed to gradually shift after removal ( $\chi^2=14.18$ ,  $df=3$ ,  $p=0.003$ ) from primarily small juvenile to juvenile individuals to primarily maturing-sized individuals in the last census, when the distribution was similar to that observed before removal ( $\chi^2=0.910$ ,  $df=3$ ,  $p=0.823$ ) except that it lacked the small proportion of small juveniles. Both treatments showed the highest proportion of small juveniles from July to September with none in December. In the control, size structure before and after the removal event were also different ( $\chi^2=7.924$ ,  $df=3$ ,  $p=0.045$ ), but the pattern was dissimilar from what was observed in the removal region. Similarly, July differed compared to the rest of the study ( $\chi^2=8.026$ ,  $df=3$ ,  $p=0.045$ ), but again the pattern differed from that observed in the removal region. Additionally, no significant differences in sizes were detected when comparing before the removal and the end of the study ( $\chi^2=4.251$ ,  $df=3$ ,  $p=0.236$ ). Converting abundances to biomass (Figure 4.7) shows even greater proportional declines from the removal event, while the control region illustrated the same gradual decline.

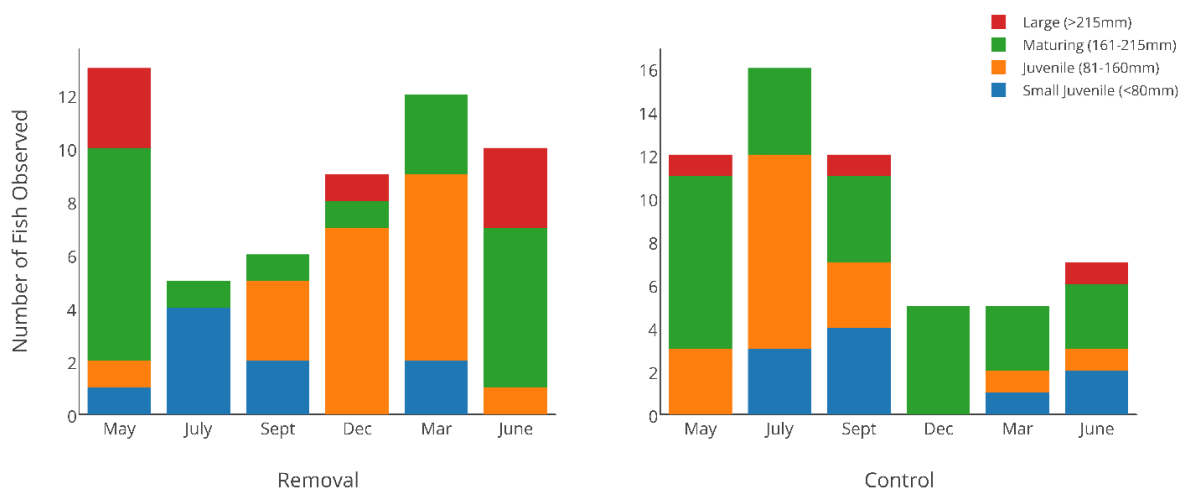


Figure 4.6 Size class distributions of lionfish within the removal and control area before (May) and after the removal events (July - June)

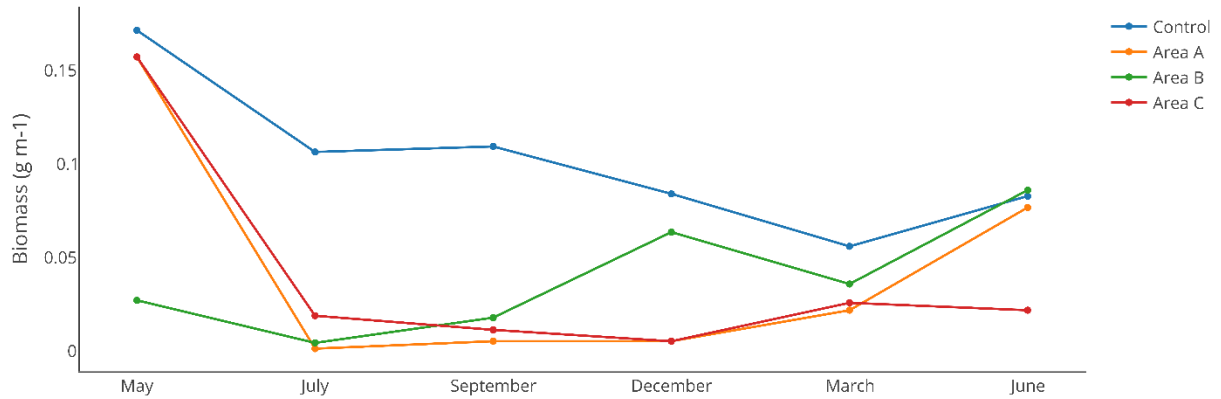


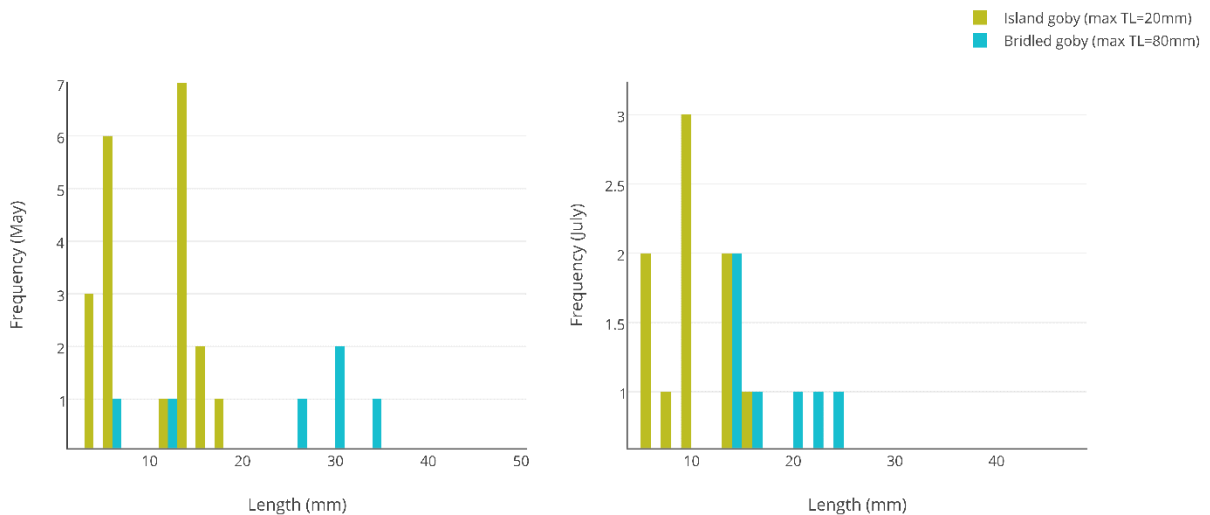
Figure 4.7 The biomass (g/m<sup>2</sup>) of lionfish in the three areas of the removal and the control over the study period

No tagged lionfish were ever observed in the removal region. In total, 83% were tagged on Conserva Reef (n=15) and 17% tagged on Pelotas Reef (n=3). Only five fish were re-sighted (27% recapture), each only once. Of those re-sighted, three individuals were detected up to 67 days at liberty in non-removal Pelotas and two individuals were observed up to 53 days in Conserva. Re-sighting of only two individuals on Conserva in July pre-dates the initial decline in lionfish densities in the control. Furthermore, all re-sighted lionfish were observed in the same location they were tagged within the control areas ( $\pm 0.5$  m). Tags were visually inspected at re-sighting, and none appeared to be dislodged or inadequately anchored to the fish however biofouling prevented the numbered tag from being read from a 0.5 m distance.

### Responses of Native Prey & Predators

Native prey abundances exhibited temporal variation by month, and differed in abundances before and after the removal events, however these differences were not the same for

all areas ( $p < 0.05$ , Table 4.5). An increase in prey abundance was observed for all areas, around the same time in December and March (maximum 16 fish/m<sup>2</sup>), and prey abundances were three orders of magnitude greater than lionfish densities (Figures 4.2-4.4). There was also a difference in prey assemblage composition before and after the removal (Table 4.6), but species evenness remained relatively constant throughout the study. By December and March, most areas were represented by a different prey species assemblage than what was present before the removal. Newly recruited individuals were observed in the collections, as illustrated by variations in the length-structure of two of the most abundant prey species *Lythrypnus nesiotes* and *Coryphopterus glaucofraenum* (Figure 4.8).





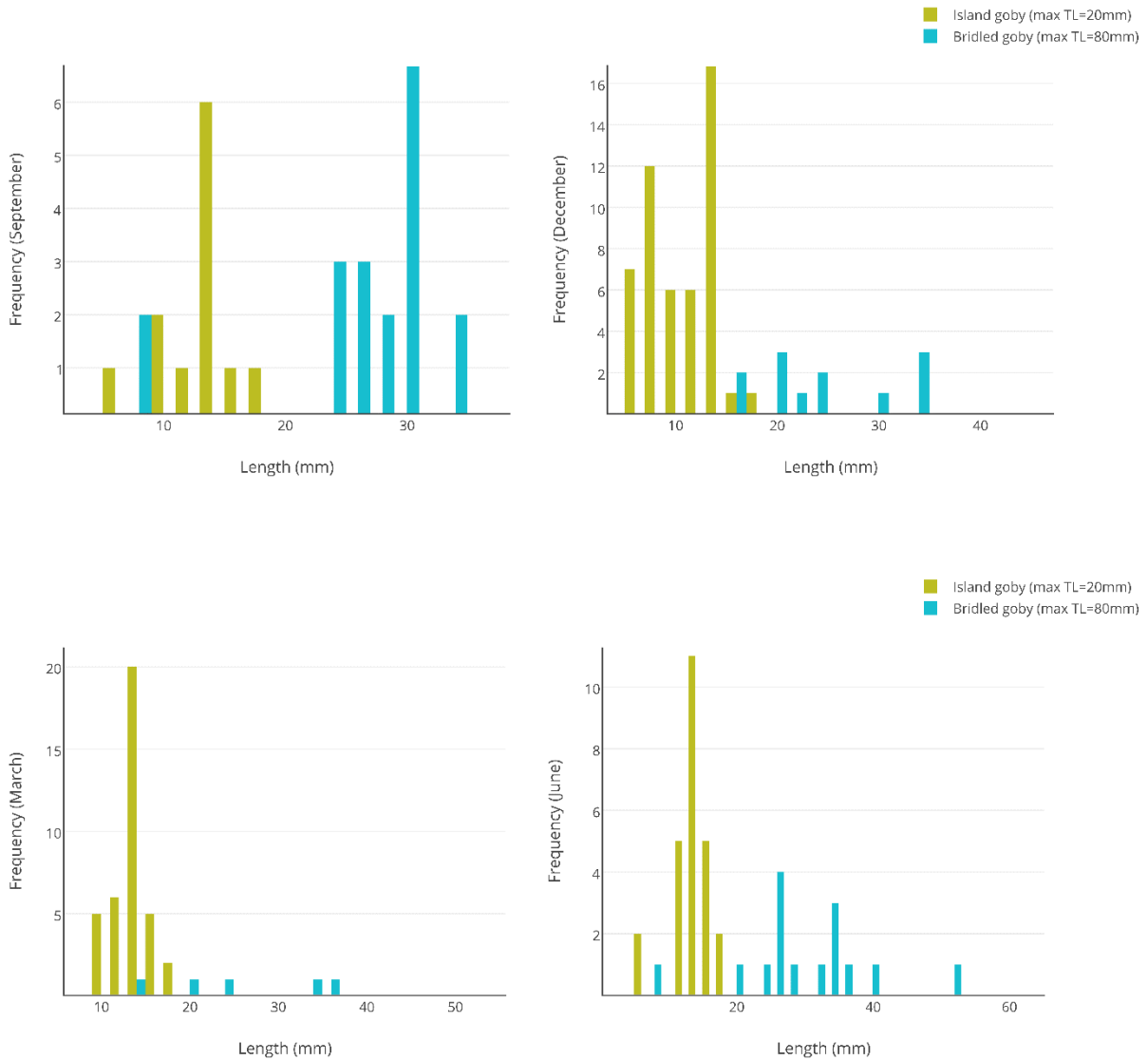


Figure 4.8 Length frequency distribution of the island goby, *Lythrypnus nesiotus*, and the bridled goby, *Coryphopterus glaucofraenum*, for each sampling month within the removal treatment. Max total length (TL) retrieved from Fishbase.org. Month of sampling is indicated in parenthesis on the vertical axis

Table 4.5 PERMANOVA results for the comparison of native prey abundances before and after the removal and inside and outside the removal region, for all months

Factor	df	SS	MS	PseudoF	P
Time	1	7730.4	7730.4	1.0246	0.4597
Treatment	1	1419.4	1419.4	0.6698	0.7423
Month (Time)	4	19379	4844.6	3.7873	0.0028
Area (Treatment)	2	5035.2	2517.6	1.9681	0.1174
Time*Treatment	1	1337.8	1337.8	0.47929	0.8989

Time*Area (Treatment)	2	7897.7	3948.8	3.087	0.039
Month (Time)*Treatment	4	6045.6	1511.4	1.1815	0.3435
Month (Time)*Area (Treatment)	8	10233	1279.2	0.8779	0.7057

Table 4.6 Density (fish/m<sup>2</sup>) of all native prey species observed in each sampling month within the removal region on Pelotas.

<i>Species</i>	May	July	September	December	March	June
<i>Acanthemblemaria aspera</i>	0.46	0	0.53	0.53	0.6	0.66
<i>Acanthemblemaria spinosa</i>	0.26	0	0	0.06	0	0.13
<i>Apogon townsendi</i>	0.13	0	0	0	0	0.06
<i>Astrapogon stellatus</i>	0.06	0	0	0	0	0
<i>Canthigaster rostrata</i>	0	0	0	0.13	0.06	0
<i>Coryphopterus dicrus</i>	0	0	0.06	0	0	0
<i>Coryphopterus eidolon</i>	0	0	0	0	0.06	0
<i>Coryphopterus glaucofraenum</i>	0.4	0.4	1.26	0.8	0.33	1
<i>Coryphopterus personatus</i>	0.06	0.6	0	6.73	3.86	0.46
<i>Elacatinus evelynae</i>	0	0	0	0.06	0	0
<i>Elacatinus genie</i>	0.46	0.46	0.33	0.13	0.60	0.46
<i>Emblemariopsis bahamensis</i>	0	0	0	0	0.06	0
<i>Emblemariopsis spp.</i>	0.13	0.06	0.13	0.06	0	0
<i>Enneanectes altivelis</i>	0.13	0	0	0.06	0.26	0.13
<i>Gramma loreto</i>	0	0	0	0.13	0	0
<i>Gymnothorax moringa</i>	0	0	0	0.06	0	0
<i>Haemulon aurolineatum</i>	0	0.2	0.6	0.06	0	0
<i>Haemulon flavolineatum</i>	0	0.06	0	0	0	0
<i>Hypoplectrus chlorurus</i>	0	0	0	0	0.06	0
<i>Hypoplectrus puella</i>	0.06	0	0	0	0	0
<i>Hypoplectrus spp.</i>	0.06	0	0.06	0.13	0	0.13
<i>Lythrypnus nesiotus</i>	1.2	0.53	1.06	3.46	2.46	1.73
<i>Malacoctenus boehlkei</i>	0	0	0	0	0	0.06
<i>Malacoctenus triangulatus</i>	0	0.06	0	0	0	0
<i>Ophioblennius macclurei</i>	0	0.06	0	0	0	0
<i>Paraclinus fasciatus</i>	0.06	0.06	0	0	0.06	0
<i>Paraclinus nigripinnis</i>	0.06	0	0	0	0	0
<i>Phaeoptyx pigmentaria</i>	0.86	0.26	0.13	0.93	0.26	0.66
<i>Sargocentron coruscum</i>	0	0	0	0	0	0.06
<i>Scarus iseri</i>	0.06	0.13	0	0	0.26	0
<i>Sparisoma aurofrenatum</i>	0	0	0	0.33	0.26	0.06
<i>Sparisoma viride</i>	0	0	0	0.13	0	0

<i>Starksia culebrae</i>	0	0	0	0.06	0	0.13
<i>Stegastes adustus</i>	0	0	0	0	0	0.06
<i>Stegastes diencaeus</i>	0.33	0.06	0.06	0.06	0	0.13
<i>Stegastes partitus</i>	0.26	0	0.13	0	0.06	0.2
<i>Stegastes planifrons</i>	0	0	0.06	0	0.06	0
<i>Synodus foetens</i>	0	0	0	0.06	0	0
<i>Tigrigobius saucrus</i>	0.2	0.2	0	0.06	0.06	0
<b>Total Prey Density</b>	<b>5.73</b>	<b>3.33</b>	<b>4.26</b>	<b>14.13</b>	<b>9.66</b>	<b>6.26</b>
<b>Species Richness</b>	<b>19</b>	<b>14</b>	<b>12</b>	<b>21</b>	<b>17</b>	<b>17</b>

Prey species abundance in the control region illustrated a similar signal of recruitment, albeit to a lesser degree (maximum 9 fish/m<sup>2</sup>), in December and March as within the removal region. Prey species were represented by six species in pre-removal surveys (May) compared to six to 12 species in post-removal months within the control region (Table 4.7). Although there was a change in prey assemblage composition within the control region, total prey densities did not appear to differ between treatments or times, but did, however, across months (Table 4.5).

Table 4.7 Density (fish/m<sup>2</sup>) of all prey species observed in each sampling month within the control region on Conserva reef

<i>Species</i>	<b>May</b>	<b>July</b>	<b>Septembe r</b>	<b>December</b>	<b>March</b>	<b>June</b>
<i>Acanthemblemaria aspera</i>	0.4	0.2	0.2	0.2	0.2	0.4
<i>Acanthemblemaria spinosa</i>	0	0	0	0.2	0	0.4
<i>Apogon townsendi</i>	0	0	0	0	0.2	0
<i>Astrapogon stellatus</i>	0	0	0	0	0.2	0.2
<i>Canthigaster rostrata</i>	0	0	0.2	0	0	0
<i>Coryphopterus eidolon</i>	0	0	0	0	0.4	0
<i>Coryphopterus glaucofraenum</i>	0	1.4	1	1	0.4	0.2
<i>Coryphopterus personatus</i>	0	0	0	1	2	0
<i>Elacatinus genie</i>	0.2	0.2	0	0.2	0	0.2
<i>Emblemariopsis bahamensis</i>	0	0	0	0	0.2	0
<i>Emblemariopsis spp</i>	0	0.2	0	0.2	0	0
<i>Enneanectes altivelis</i>	0	0	0	0	0.2	0
<i>Hypoplectrus chlorurus</i>	0	0	0	0	0	0.2
<i>Hypoplectrus spp.</i>	0	0	0	0	0	0.2
<i>Lythrypnus nesiotus</i>	1.4	1	1.8	2.6	4	1.2

<i>Ogilbia cayorum</i>	0	0	0	0	0	0.4
<i>Paraclinus fasciatus</i>	0.4	0.2	0	0	0	0
<i>Phaeoptyx pigmentaria</i>	1.2	0	0	1	0.6	0.2
<i>Scarus iseri</i>	0	0	0	0	0.6	0
<i>Starksia culebrae</i>	0	0	0	0.2	0	0
<i>Stegastes diencaeus</i>	0.4	0	0	0	0.2	0
<i>Stegastes partitus</i>	0	0	0.2	0	0	0
<i>Tigrigobius saucrus</i>	0	0.4	0.2	0.2	0	0
<b>Total Prey Density</b>	<b>4</b>	<b>3.6</b>	<b>3.6</b>	<b>6.6</b>	<b>9.2</b>	<b>3.6</b>
<b>Species Richness</b>	<b>6</b>	<b>7</b>	<b>6</b>	<b>10</b>	<b>12</b>	<b>10</b>

Native predator abundances differed among sampling months and sampling areas independent of each other ( $p < 0.05$ , Table 4.8). However, there were no clear trends in piscivore abundances between treatments. Piscivore densities were one order of magnitude greater than lionfish densities for all months sampled and in all areas in the removal region (Figures 4.2-4.4). Overall species assemblage differed in the pre-removal month (May) compared to the subsequent post-removal months (Table 4.9). Pre-removal species richness was six and was dominated by groupers (Epinephelidae), snappers (Lutjanidae) and the carangid *Caranx ruber*. In the post-removal surveys, 13 piscivorous species were observed immediately following the removal (July) and species richness ranged from seven to 12 species thereafter. The predator assemblage in the control was not the same as in the removal region indicated by the dominance of *Caranx latus* and *Lutjanus griseus* which did not significantly contribute to the predator assemblages in the removal region (Table 4.10).

Table 4.8 PERMANOVA results for the comparison of native piscivore abundances before and after the removal and inside and outside the removal region, for all months

<b>Factor</b>	<b>df</b>	<b>SS</b>	<b>MS</b>	<b>PseudoF</b>	<b>P</b>
Time	1	8715.4	8715.4	2.4068	0.0973
Treatment	1	949.35	949.35	0.48163	0.8804
Month (Time)	4	10566	2641.6	3.7461	0.0038
Area (Treatment)	2	4545.6	2272.8	3.2232	0.0362
Time*Treatment	1	1021.6	1021.6	0.70912	0.6982
Time*Area (Treatment)	2	2545.2	1272.6	1.8047	0.149
Month (Time)*Treatment	4	4649.7	1162.4	1.6485	0.109
Month (Time)*Area (Treatment)	8	5641.2	705.15	0.50574	0.9937

Table 4.9 Density (fish/m<sup>2</sup>) of all native piscivores observed in the sampling months in the removal region on Pelotas.

<i>Species</i>	May	July	September	December	March	June
<i>Calamus calamus</i>	0	0	0	0	0.003	0
<i>Caranx ruber</i>	0.01	0.004	0.003	0.004	0.001	0
<i>Cephalopholis cruentata</i>	0.003	0.008	0.008	0.009	0.005	0.009
<i>Cephalopholis fulva</i>	0.0004	0.0007	0.0004	0	0	0
<i>Epinephelus adscensionis</i>	0	0.003	0	0	0	0
<i>Epinephelus guttatus</i>	0	0.0004	0.0004	0	0	0
<i>Gymnothorax funebris</i>	0	0.0007	0	0	0	0.0004
<i>Lutjanus analis</i>	0	0	0	0.0004	0.0004	0.0004
<i>Lutjanus apodus</i>	0.006	0.01	0.007	0.007	0.01	0.01
<i>Lutjanus griseus</i>	0.002	0	0	0.004	0.0007	0
<i>Lutjanus jocu</i>	0	0	0	0.002	0.0004	0
<i>Lutjanus mahogoni</i>	0	0.0004	0.001	0.0007	0.0004	0
<i>Lutjanus synagris</i>	0	0.001	0.001	0.002	0.001	0.0007
<i>Mycteroperca bonaci</i>	0	0	0	0.0004	0	0
<i>Ocyurus chrysurus</i>	0.017	0.02	0.007	0.03	0.01	0.02
<i>Scomberomorus regalis</i>	0	0.001	0	0	0.0004	0
<i>Sphyaena barracuda</i>	0	0.0004	0	0	0	0
<i>Synodus intermedius</i>	0	0.001	0.001	0.002	0.001	0.0007
<b>Total Predator Density</b>	<b>0.03</b>	<b>0.05</b>	<b>0.04</b>	<b>0.05</b>	<b>0.03</b>	<b>0.04</b>
<b>Species Richness</b>	<b>6</b>	<b>13</b>	<b>9</b>	<b>11</b>	<b>12</b>	<b>7</b>

Table 4.10 Density (fish/m<sup>2</sup>) of all native piscivores observed in the control region on Conserva reef

<i>Species</i>	May	July	September	December	March	June
<i>Calamus calamus</i>	0	0	0	0	0.001	0
<i>Caranx crysos</i>	0	0	0	0.001	0	0
<i>Caranx latus</i>	0	0	0	0.05	0	0.007
<i>Caranx ruber</i>	0.001	0.01	0.01	0.002	0	0.03
<i>Cephalopholis cruentata</i>	0.002	0.008	0.01	0.01	0.006	0.01
<i>Cephalopholis fulva</i>	0.001	0	0	0.001	0	0
<i>Epinephelus adscensionis</i>	0	0.001	0	0	0	0
<i>Epinephelus guttatus</i>	0	0.001	0	0	0	0
<i>Gymnothorax funebris</i>	0	0	0.001	0	0	0
<i>Lutjanus analis</i>	0	0.001	0	0.002	0	0.002
<i>Lutjanus apodus</i>	0.004	0.01	0.01	0.001	0.002	0.008
<i>Lutjanus griseus</i>	0	0	0.002	0.03	0.02	0.02
<i>Lutjanus jocu</i>	0	0.002	0.001	0.002	0.002	0.002
<i>Lutjanus mahogoni</i>	0	0	0.002	0	0	0.004
<i>Lutjanus synagris</i>	0	0.01	0.007	0.006	0	0.008
<i>Ocyurus chrysurus</i>	0.02	0.02	0.02	0.007	0.005	0.02
<i>Scomberomorus maculatus</i>	0	0	0	0.11	0	0
<i>Scomberomorus regalis</i>	0	0	0	0.001	0.001	0
<i>Synodus intermedius</i>	0	0	0.004	0.002	0	0

<b>Total Predator Density</b>	<b>0.03</b>	<b>0.08</b>	<b>0.07</b>	<b>0.22</b>	<b>0.04</b>	<b>0.11</b>
<b>Species Richness</b>	<b>5</b>	<b>9</b>	<b>10</b>	<b>14</b>	<b>7</b>	<b>10</b>

## Discussion

Lionfish densities were observed to be negatively affected by the removal events in two of the three removal areas. The three days of removal effort over one month dramatically diminished the initial lionfish densities on Pelotas Reef, indicated by the significant decrease in catch/effort following each of these events and the 56% reduction in the population directly attributed to the removal (22 fish). Additionally, the reduction of adult and maturing lionfish, with a subsequent increase in small juvenile and juveniles, was indicative of a removal effect on the size structure of the population.

Spatial variability in lionfish density across removal areas was significant, with the greatest density change (and most lionfish removed) coming from Area C alone. These differences in initial density could be related to rugosity, slope and complexity of the reef (Luckhurst and Luckhurst 1978; Gratwicke and Speight 2005) and may have overshadowed the overall treatment effect. Similarly, lionfish have been observed to prefer rugose structure (Dahl and Patterson 2014). Specifically, lionfish prefer continuous coral reefs versus patch reefs, micro-habitat preferences of hard coral and overhangs (Biggs and Olden 2011) and macro-habitat preference to reef edges and drop offs (Bejarano et al. 2015). The overall rugosity, slope and habitat characteristics of the areas surveyed on Pelotas Reef were marginally different and the entire reef is patchily distributed with holes, crevices, overhangs and changes in slope, offering a suite of habitat characteristics preferred by lionfish. Area C was also the most rugose area. However, as lionfish re-colonized, they populated the two other areas of the reef where initial densities were not as high as Area C. Thus, we cannot conclude that changes in micro-

habitat complexity (i.e., rugosity and slope) in these areas was driving the differences in densities observed at the within habitat scale of linear fore reef with hard coral and complex structure.

After the removal, lionfish densities remained at low numbers within removal Area C but increased after the removal in the other two removal areas, although total biomass was reduced. Subsequently, reductions in lionfish densities were observed to follow a gradually decreasing trend in the control region. It is likely that fishing was occurring on Conserva Reef, which is a steeply sloped linear reef with similar habitat characteristics as Pelotas and occupied by many commercially important species (i.e., snapper and grouper). However, we received no reports of tagged lionfish caught by local fishermen. A second explanation for the low tag re-sighting rate could be the migration of lionfish to deeper/offshore reefs (Claydon et al. 2012). A recent tagging study reported lionfish to move, albeit infrequently, up to 800 m among discontinuous coral reef patches, and to vacate areas during natural perturbations (i.e., hurricanes) (Tamburello and Côté 2015). Similar movement patterns have been observed in grunts, where some individuals remain inshore, but most ontogenetically migrate offshore or to deeper inshore reefs (Appeldoorn et al. 1997, 2003, 2009).

Trends in the size distribution of lionfish at Pelotas Reef coupled with the results of tagging indicate that re-colonization following removal events occurred due to recruitment of juvenile individuals rather than from lateral migration of adults. Despite the low re-sighting rate, tagged lionfish from both control sites were never observed in the removal region and all re-sightings (up to 67 days post-tagging) were within 0.5 m of tagging sites. This pattern is consistent with previous tagging studies that show high site fidelity in lionfish (Jud and Layman 2012; Bacheler et al. 2015). In contrast, a greater number of small juvenile and juvenile lionfish were observed post-removal, even up to one year later. This suggests that post-settlement

individuals may have been missed during the removal, or that juveniles and smaller individuals moved into the removal region from the reef flat which is the preferred habitat of early settlement lionfish (Harms-Tuohy unpublished; Barbour et al. 2010; Biggs and Olden 2011).

Within both treatments, no small juveniles were recorded in December which may be indicative of the observed seasonal recruitment of lionfish. As lionfish have been found to grow in increments of up to 0.6 mm/day in lionfish smaller than 200mm TL (Akins et al. 2014), the observed size distributions indicate that juveniles seen during our study would have required a minimum of 7 months to reach the larger sizes observed, which was well within our study duration. In the control, the temporal pattern in changes of size distribution of lionfish was not the same as in the removal region. A greater proportion of juvenile and maturing lionfish were observed immediately following the removal, which transitioned into predominantly maturing fish for the remainder of the study. In the control, these observed differences in size structure were not an effect of removing a particular size class or observing re-colonization through ontogenetic migration.

The timing and density of peaks in prey abundance observed in the sampling areas demonstrated that recruitment was evident despite the presence of lionfish. In all areas and across treatments, prey abundance was greatest in December and March, irrespective of lionfish density or biomass. The similarity in the temporal pattern of density indicates a strong recruitment event and is evidence that lionfish were having no detectable effect on the prey communities. Single lionfish have been observed to reduce abundance of prey by up to 80% on small (1-4m<sup>2</sup>) artificial and translocated coral patch-reefs in the Bahamas (Albins and Hixon 2008) and predicted to reduce biomass of prey fish on natural reefs in high densities (0.039 fish/m<sup>2</sup>, Green and Côté 2009). While these studies may present valid scenarios for their



respective sites and design, predicative modeling to estimate lionfish densities that allow for the recovery of native prey has been controversial (Green et al. 2014; Valderrama and Fields 2015; Green et al. 2015). A recent study of the impacts of lionfish showed that effects of lionfish on prey abundances do not scale linearly with increased densities (Benkwitt 2015). Thus, the impacts of lionfish on native communities could be severely overestimated in scenarios that scale up impacts of small artificial patch reef/high lionfish density case studies. Artificial reefs provide valid comparisons to natural reefs in cases where biotic and abiotic factors influencing species richness and abundance are similar (Carr and Hixon 1997), but addressing impacts of novel predators on native communities based on results obtained from manipulative studies on artificial reefs must be carefully interpreted. For instance, prey abundance has been observed to decrease when artificial reefs do not provide the appropriate shelter from predators (Hixon and Beets 1989). Thus, the predation rate of lionfish on prey communities that may be more exposed on artificial reefs cannot be scaled to natural reef systems where this is less of an issue. Furthermore, manipulated high lionfish densities at artificial reef sites could encourage intra-specific competition resulting in overinflated estimates of consumption. In our study, removal of lionfish had no detectable impact on the native prey assemblage. These results concur with a study in Venezuela that found no impacts of lionfish on the fish communities of Archipelago Los Roques National Park, where average densities were similar to those in our study ( $0.003/\text{m}^2$  versus  $0.006/\text{m}^2$ , respectively; Elise et al. 2014). These densities are relatively low in comparison to other regions (on natural habitat), such as Eleuthera Island, Bahamas ( $0.052/\text{m}^2$ , Hackerott et al. 2013), New Providence, Bahamas ( $0.039/\text{m}^2$ , Green and Côté 2009) and Little Cayman Island ( $0.065/\text{m}^2$ , Frazer et al. 2012). This would indicate that small-scale removals aiming to increase prey fauna on reefs of similar structure and community assemblages as Pelotas Reef are not worthwhile unless lionfish densities are much greater. For this reason, Hackerott (2014)

suggested halting lionfish removal in Belize, as current densities elicit no impacts on native fish over a five year period.

There is a well-established concern that competition among adult native predators and lionfish could have negative impacts on native predatory species (Albins and Hixon 2008; Morris and Akins 2009; Raymond et al. 2015). Recent studies, however, have demonstrated that some native predators with ecologically similar roles may not competitively interact with lionfish to a detectable degree (Hackerott et al. 2013; Elise et al. 2014; Valdivia et al. 2014; Bejarano et al. 2015). Our study reveals that native piscivore abundance is not affected by lionfish in this level of density. Piscivore abundances were observed to be an order of magnitude greater than lionfish and no increases in abundance were detected relative to lionfish removal. Therefore, the temporal changes in abundance observed in each area are likely a response of migration, prey availability, and seasonality factors (Cocheret De La Morinière et al. 2003; Appeldoorn et al. 2009). Piscivore densities common to this study and those sampled in the same months in 2006 (pre-lionfish colonization) on Pelotas Reef resulted in similar estimates (July: 0.13 and 0.14 fish/m<sup>2</sup>, September: 0.10 and 0.13 fish/m<sup>2</sup>, respectively; Nemeth & Appeldoorn unpublished).

Removal events or derbies have been shown to be effective at reducing lionfish densities (Frazer et al. 2012; de León 2013) and control of lionfish has been promoted for several years. These derbies or events attract media attention, engage stakeholders from multiple facets and improve awareness of both lionfish impacts and market potential but are primarily attended by participants in higher economic tiers with expendable income (Trotta 2014). However, not all countries affected by the invasion have the same resources or community awareness of lionfish, which leaves smaller island nations or countries with less tourist-driven economies to seek other means of control. Our standardized design proved effective at engaging local volunteer divers

and required limited funds and incentives, while the timeframe of removal (three days in one month) was adequate to reduce lionfish densities in our already low-density scenario. Volunteers were invited to participate based on their experience in culling lionfish, which allowed us to maximize our catch while engaging non-scientists (i.e., recreational divers, local fishermen) in the joint effort of control. The advantages of targeted, small-scale lionfish removals are three-fold: (1) they require less funding, promotion and incentives, (2) they require less manpower and (3) they are easier to manage for repeated culling events and assessment of native recovery. Our design can be modified to apply to specific lionfish populations. For example, our catchability coefficient ( $q = 0.1437$ ) represents the minimum proportion of the population removed from our 9,840-m<sup>2</sup> area by each additional unit of effort (1.5 man-hours), which can be used by managers to estimate effort required to reduce lionfish density in their region using the following equation: % Population Remaining =  $100 - e^{(0.1437 * E)}$  where E is the cumulative effort.

For high-priority conservation areas, such as marine protected areas (MPA), management can adopt this method to employ a limited number of personnel, with a few dedicated days of removal, and scheduled but intermittent surveying to assess effectiveness. Furthermore, implementing culling in buffer zones could significantly reduce the number of lionfish migrating into a culled MPA. These buffer zones could include nursery habitats for juvenile lionfish undergoing ontogenetic migration from seagrass, mangrove or shallow back reef habitats onto the fore reef of the MPA (Barbour et al. 2010; Biggs and Olden 2011), which the present study identified as the likely critical connection for re-colonization on Pelotas. However, if the goal of the removal or control program is to allow recovery of native fish, then initial densities of lionfish should be evaluated before investing in the effort. Predictive modeling has been suggested as a logical approach to determining the threshold needed to observe recovery (Green

et al. 2014), but management would benefit more from actual density estimates rather than biomass, allowing for the calculation of catch and effort necessary to achieve estimated densities. Additionally, site-specific variation in prey biomass may reduce the usefulness of a predictive model in application to all areas. Thus, the need for site-specific monitoring becomes all the more evident.

There is a limitation to the applicability of these results. While this study does indicate no detectable impacts on native fish communities at low lionfish densities, the outcome was dependent upon the spatial and temporal dynamics of the fish communities and habitat of the reef investigated. The removal and observation was conducted on one continuous reef and for one year. However, replication of this study on several reefs of similar structure and composition over two years would provide a more robust interpretation of lionfish impacts at low densities and would also provide temporal comparisons of the responses of native fish. Furthermore, an improvement to the study would investigate changes in abundance and biomass of specific prey species found in the lionfish diet, and biomass of native predators. Overall, expanding the study to address these limitations and improvements would provide stronger evidence to support no impacts at low lionfish densities.

This study was among the first investigations to address the responses of native fish communities to lionfish removals. A meta-analysis of published literature on aquatic invasive species showed that only 28% of studies (of 259 reviewed) actually addressed impacts on native faunal assemblages (Thomsen et al. 2014). Furthermore, these reports likely present a biased view given the reluctance to report non-significant treatments (Thomsen et al. 2014). This not only limits our ability to identify invasive species with negligible impacts on native communities (Thomsen et al. 2014), it fuels the perceived destructive impacts of invasive species which may

not be as pronounced as thought. This likely contributes to the strongly negative perspective of the lionfish invasion (Carballo-Cárdenas 2015).

## **Conclusion**

This small-scale removal design addressed the impacts of lionfish on native fish communities and found, in contrast to expectations, that at low to moderate densities, lionfish removal had no measurable effect on either native prey or other piscivorous species across a small fore reef habitat. Small scale removals were effective at reducing lionfish densities, even in a low lionfish density scenario. Re-colonization of lionfish to Pelotas Reef occurred via recruitment or ontogenetic migration and subsequent growth of juvenile and small individuals, rather than lateral migration from adult lionfish of nearby reefs. The temporal variation in native prey densities was indicative of natural recruitment cycles, rather than a response to changes in lionfish density. Similarly, the changes in abundance of native piscivores coincided with seasonality factors that typically drive piscivorous reef fish assemblage structure. Removals are an effective management tool at reducing lionfish densities, and this small-scale design is most appropriate for regions with less tourist-driven removal strategies or derbies, as well as for areas with high conservation priority like MPAs. However, agencies should first investigate if investing in a removal strategy is worth the effort if existing lionfish densities are too low to be impacting the native fish communities.

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## Chapter Five: Summary of Conclusions

This dissertation sought to address the management and feeding ecology of lionfish. Impacts of lionfish have been recently demonstrated to illustrate site-specific characteristics, driven largely by density differences (Elise et al. 2014; Green et al. 2014; Hackerott 2014, this study), which makes a single management strategy inappropriate for all invaded regions. Puerto Rico was first invaded in 2008, yet the studies conducted in this dissertation were among the first to investigate real impacts of lionfish. The results of these indicate that lionfish are not demonstrating the same alarming effects on native fish communities as what is indicated in heavier-density regions such as the Bahamas and Florida (Green et al. 2012a). Although lionfish diet assessment indicated the potential to significantly impact some ecologically important species, the assessment of impacts from lionfish removal overall on these native prey and predators elucidated no detectable response.

The dietary profile of lionfish was successfully determined through the use of next generation sequencing. The method of DNA metabarcoding was an effective and more accurate method of gut content analysis, affording the use of digested materials through which the presence of several previously unrecorded species were detected. Thirty-nine fish species were reported from the relatively small sample size of 63 lionfish. The metabarcoding approach is more cost effective in terms of sampling effort and post-processing analysis compared to traditional morphological identification or even DNA barcoding of recognizable tissues. Results indicated a prey preference to families Scaridae, Pomacentridae, Apogonidae and Gobiidae of which Pomacentridae and Scaridae have known ecological roles in transferring pelagic energy to the benthos and maintenance of coral reef health. In particular, the Scaridae consists of ecologically important herbivores (Burkepile and Hay 2008) that are a key functional group

related to coral reef resilience in algal-dominated reefs, such as found off of La Parguera (Pittman et al. 2010). While not the most frequently occurring in the stomach contents, their presence does indicate that future investigations of lionfish impacts should address impacts on parrotfish. When comparing lionfish stomachs between inshore and offshore reefs, certain species differences were noted, which correlated with known species distribution, such as the presence of the yellowhead wrasse (*Halichoeres garnoti*) restricted to offshore lionfish diet. The comparison of species information obtained from tissues versus digested material indicated the presence of species undetectable by one component alone – such as the identification of *Starksia williamsi*, a new range report for this blenny (Benjamin Victor, personal communication). Thus, the utilization of the entire stomach contents proved useful in obtaining the most inclusive high resolution species richness data. Apart from demonstrating the usefulness of this next generation sequencing technique to fish feeding ecology, the dietary profile of lionfish in the shallow-water coral reefs of Puerto Rico has now been documented. Further research should investigate any dietary differences among lionfish populations in other habitats, such as mesophotic systems, mangroves and seagrass beds. In conclusion, future work should also address the ability to quantify species information obtained via metabarcoding to provide a more robust interpretation of lionfish prey preference apart from frequency of occurrence.

Another aspect of feeding ecology involves the digestion of prey items in fish stomachs, which ultimately determines the ability to identify these items via morphological identification. As this method of gut content analysis is also prone to observer error and bias (Baker et al. 2014), the need to understand how quickly a prey item digests and what factors contribute to digestion is important for experimental design to reduce unidentifiable material. The second study of this dissertation evaluated the time to digestion of both fish and shrimp prey items,

while investigating the role of predator size and prey type on the outcome. Shrimp prey were observed to require longer time to digest beyond species level identification and to be unidentifiable, which is likely due to the nature of their composition (i.e., exoskeleton; Jackson et al. 1987). Fish prey required at least 4 hours to become unrecognizable at the species level, and this digestion was not correlated with temperature, albeit due to the design of the experiment. However, higher temperatures did correspond to greater digestion rank of prey items, but as an effect of experimental design. Predator size (i.e., small or medium lionfish), prey type and time were observed to significantly ( $p < 0.05$  for all factors) affect digestion rate such that they could be used to predict digestive state. Lastly, in an effort to standardize ranking applied to invertebrate (i.e., shrimp) prey items, a scale similar to the fish scale developed by Green et al. (2012b) was created to assist in digestion ranking for this study as well as future studies. The results of this study suggest recommendations for lionfish gut content analysis utilizing morphological identification, including: 1) observe the prey assemblage in the proposed study area to investigate potential prey items to expect within lionfish stomachs, 2) conduct collection at crepuscular hours of the day (i.e., dawn or dusk) to enhance opportunities of acquiring freshly ingested prey items and place lionfish on ice immediately to slow digestion, 3) combine morphological identification with another effective means of analysis (i.e., metabarcoding) to bolster results and confirm visually inspected specimens.

The management of lionfish has shifted focus to efforts of control via removals rather than aiming for complete eradication (Barbour et al. 2010). Removals, such as derbies and tournaments, have been demonstrated as successful techniques for reducing lionfish densities (Frazer et al. 2012; de Leon et al. 2013; Côté et al. 2014). However, only one study utilizing predictive modeling and a complementary manipulative approach has investigated the response

of native prey to lionfish removals, observing native prey abundance to increase in the absence of lionfish (Green et al. 2014). While this model may be useful to particular sites addressing biomass, it does not address what may be happening in areas with lower densities, especially since impacts of lionfish have been observed to scale non-linearly (Benkwitt 2015). Thus, this study addressed the effectiveness of small-scale removal on reducing lionfish, and monitored the response of native predators and prey for one year. The removal was found to be effective at reducing lionfish densities, which did not regain abundance until 9 months post-removal and never recovered biomass even after one year. Comparing size trends before and after the removal, coupled with tagging lionfish on a control site, also indicated that re-colonization occurred via ontogenetic migration rather than lateral movement from nearby reefs. This information will be useful to local management, such as the PR Department of Natural and Environmental Resources (DNER), which enforces lionfish removal around the island and is instrumental in developing management strategies for high-priority conservation areas such as marine protected areas (MPAs). However, upon monitoring the abundances of both piscivores and prey on Pelotas, no detectable trends were observed in response to the removal of lionfish. Furthermore, evidence of prey recruitment was observed in December and March for all areas surveyed, despite differential initial and post-removal lionfish densities. This study is not the first case to report no overall impacts of lionfish on native communities, as Elise et al. (2014) has noticed similar responses in low densities in Venezuela. For similar reasons, Belize has halted management-driven lionfish removals (Hackerott 2014). Lionfish can no longer be hypothesized to affect native fish communities in the same way across the invaded range, and a management strategy applicable to all areas is not practical. This study is evidence that site-specific monitoring will be key to detecting impacts, while suggesting that removals may not be necessary in areas where lionfish densities are low. In conclusion, this small-scale removal

design will be applicable to management agencies with limited funding and manpower as it required only 10-12 volunteer spearfishermen to remove lionfish for approximately 90 minutes over a three-day period in one month. However, these agencies should first assess lionfish densities and native fish abundances prior to investing in removal.

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