DIET AND FORAGING ECOLOGY OF THE RED-FOOTED BOOBY (SULA SULA) PROVISIONING CHICKS AT ULUPA`U CRATER, O`AHU

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Chapter 1

Review of tropical seabird foraging ecology and diet: towards the study of the Red-footed Booby (*Sula sula*)

INTRODUCTION

The goal of this paper is to summarize understanding of tropical seabird diet, by evaluating the advantages and disadvantages among various methods used to quantify diet in the past. This literature review provides historical context and regional background for a new diet study of Red-footed Boobies (*Sula sula*) rearing chicks on O'ahu, Hawai'i.

Defining Tropical Seabirds

Tropical seabirds are species that reside within tropical waters, where sea surface temperature is ≥ 23ºC, for one, or more, seasons of the year (Ballance & Pitman 1999). Taxonomically, three of the four recognized orders of seabirds occur in the tropics: Procellariiformes (albatrosses, storm-petrels, shearwaters, petrels), Pelecaniformes (pelicans, boobies, frigatebirds, tropicbirds, cormorants), and Charadriiformes (terns, gulls, skimmers). Only the penguins (order Sphenisciformes) are not present in tropical waters, although with one species restricted to island-associated upwelling areas around the Galapagos at latitude 1° South (Schreiber & Burger, 2002).

Despite great taxonomic diversity, tropical seabirds demonstrate striking convergent evolution of life-history and foraging adaptations (Harrison 1990). In particular, these species are characterized by distinct eco-morphological (body design) and behavioral (foraging guild) adaptations, attributed to the widespread low productivity and over-dispersed prey resources in tropical ocean ecosystems (Ainley & Boekelheide 1983; Spear & Ainley 1998; Ballance &

Pitman 1999). Tropical seabirds are characterized by having low wing loading, resulting in high flight proficiency and low locomotion costs (Ballance et al. 1997; Ballance & Pitman 1999).

Some far-ranging species, like albatrosses, travel to higher productivity areas within sub-polar and temperate latitudes to forage, and then return to feed their young in the breeding colonies on tropical islands. Thus, reliance of foraging areas outside of the tropics allows these species to support the high energetic demands of their developing chicks (Fernandez et al. 2001).

For those species that forage within tropical waters, six pervasive adaptations have been widely documented across the world's oceans: (1) use of some feeding methods (plunging and aerial capture) to the detriment of others (diving), (2) relatively less prey overlap among species, (3) opportunistic feeding, (4) nocturnal feeding, (5) reliance on physical features that aggregate and make prey available, and (6) reliance on subsurface predators (SSPs) to locate and aggregate prey near the surface (Harrison et al. 1983, Ballance & Pitman 1999).

Foraging of Tropical Seabirds

Since flight and diving abilities are inversely related, due to the eco-morphological constraints of wing loading, the ratio between body size and wing span, tropical seabirds are largely restricted to foraging slightly above, on, or slightly below the ocean's surface (Ashmole & Ashmole 1971; Ballance & Pitman 1999). Marine ornithologists use the reliance of the various foraging methods and the degree of association with different SSPs (tunas, marine mammals), to characterize the foraging ecology of tropical seabird species (Ashmole 1971, Hebshi et al. 2008).

Tropical seabirds use a variety of foraging methods (Figure 1): plunge diving (tropicbirds, boobies), surface plunging (tropicbirds, terns), surface seizing (shearwaters, petrels), piracy (frigatebirds), scavenging (albatrosses, petrels), aerial feeding (tropicbirds, frigatebirds), and pursuit diving (shearwaters) (Ashmole & Ashmole 1971, Harrison 1990, Spear & Ainley 1998, Ballance & Pitman 1999).

Another widely-used foraging technique is to associate with subsurface predators (SSPs), such as tunas and marine mammals, which provide foraging opportunities for non-diving seabirds (Ashmole & Ashmole 1967a, Ballance & Pitman 1999). This is a prevalent tactic within the tropics, where volant prey species, such as flyingfish (Exocoetidae) and flying squids (Ommastrephidae), comprise most of the tropical seabird diet (Ashmole & Ashmole 1967a, b, c; Harrison et al. 1983; Ballance & Pitman 1999). The SSPs chase these prey from below and force them close to the surface. In response, the prey leap out of the water, becoming accessible for the birds (Ashmole & Ashmole 1967b; Ballance & Pitman 1999). Additionally, the birds also benefit from eating prey injured by the SSPs during their subsurface pursuit and by scavenging any left-over scraps (Ballance & Pitman 1999).

Foraging Ecology of the Red-footed Booby

The Red-footed Booby (RFBO, *Sula sula*) is a non-migratory tropical seabird, with a year-round residence within tropical regions. RFBO have been observed to use five of the six foraging adaptations described above. Individuals are not known to rely on night-time foraging, but provisioning adults do occur at sea occasionally throughout the night (J. Adams USGS unpublished data). These birds forage primarily by day and most often, return to the colony by dusk. This diurnal pattern restricts foraging trips to a maximum distance of 150 km – 276 km from the colony (Weimerskirch et al. 2005; Friedlander et al. 2009; Young et al. 2010). Based on 15 observations made onboard research cruises taken throughout the Pacific, RFBOs forage by using two distinct methods: plunge diving (60% of observations) and aerial pursuit (40% of observations) (Ainley & Boekelheide 1983). Although RFBOs also forage in association with a variety of SSPs, including large predatory fishes (Skipjack Tuna, *Katsuwonus pelamis*) and marine mammals (Spotted Dolphin, *Stenella attenuate* and False Killer Whale, *Pseudorca crassidens*), Hebshi et al. (2008) found there were no significant species-specific associations, indicating RFBOs to be generalists, without strong associations with any specific SSP.

Whereas knowledge about the foraging techniques of RFBOs provide insights into the mechanisms they may use to find and capture prey, additional information can be obtained from studies of their diet and foraging movements; however, few such studies have been completed to date. A pioneering study was completed over 50 years ago on O'ahu, HI, whereby 12 opportunistically-collected regurgitated diet samples from breeding adults were analyzed, revealing that flying squid (Ommastrephidae) had the greatest presence within RFBO diet (39% by volume and 36% by number), with flyingfishes (Exocoetidae, 38% by volume and 14% by number) and snake mackerels (Gempylidae, 9% by volume and 30% by number) providing the remainder of the prey (Ashmole & Ashmole 1967a; 1968). Overall, 17 studies completed during the last five decades throughout the Indo-Pacific region have documented similarities in RFBO diet, despite the use of different analytical methods (Table 1) (Ashmole & Ashmole 1967a; Schreiber & Hensley 1976; Diamond 1983; Duffy & Jackson 1986; Seki & Harrison 1989; Ballance 1995; Le Corre 1997; Burger & Gochfeld 2000; Lewis et al. 2004; 2005; Weimerskirch et al. 2005; 2006; Cherel et al. 2008; Yin et al. 2008; Guo et al. 2010; Young et al. 2010; Mendez et al. 2015).

Due to the small number of RFBO diet and foraging studies and their disparate methodologies, during the past 50 years in different ocean basins (Table 1), intraspecific comparisons across study sites are largely precluded. Thus, the degree to which the diet and the foraging ecology of RFBO are influenced by regional and temporal changes in the underlying oceanography remains poorly understood. Although there is no single “correct” way to quantify seabird diets, the lack of methodological consistency across studies only allows limited comparisons (Duffy & Jackson, 1986). Standard analytical methods, therefore, are needed to compare and contrast diet samples within and amongst seabird species, over space and time.

APPROACH

This review summarizes the methods used to quantify the diet of tropical seabirds, and to facilitate development of standardized methods for my thesis research on RFBO. First, I describe the various methods previously used for collecting and storing diet samples, with an emphasis on RFBO studies. Next, I summarize the advantages and disadvantages of the various methods used for analyzing and quantifying prey items. This critical evaluation will inform the selection of specific, standardized approaches and metrics for my thesis research.

Historical Overview - Collection and Storage of Diet Samples

Researchers have directly sampled seabird diet in three ways: (1) by euthanizing birds, (2) non-lethally capturing live birds (using emetics or lavage, regurgitation, dropped prey), and (3) opportunistically obtaining stomachs from naturally deceased birds (Duffy & Jackson, 1986) (Table 2).

Currently, the non-lethal sampling of diet from live birds is preferred, for both ethical and scientific reasons. Upon capture of the bird, a diet sample may be collected via four methods: (1) using emetics to induce regurgitation, (2) lavage to flush out the stomach contents, (3) collection of samples regurgitated by birds handled during banding or tagging, and (4) the opportunistic sampling of dropped prey items found in the colony (Barrett et al. 2007; Duffy & Jackson 1986; Karnovsky et al. 2012). While the two latter methods cause the least disturbance and risk to the individual bird, their opportunistic nature relies on chance and may introduce potential biases (Ashmole & Ashmole 1967a, b; Duffy & Jackson 1986; Young 2010).

Following sample collection, preservation is needed to stop the digestion process and store prey items in stable conditions that ensure their integrity until they can be analyzed in the laboratory. Storage of samples varies depending on specific conditions and logistics of the field site, in which, preservation can be achieved by either rapid freezing or via chemicals. Storage in a portable cooler and freezing upon return to the lab does require electricity, which in some field sites is not available. In extreme field conditions, the prey may be sorted in the field with only reference specimens preserved for identification (Diamond, 1983). Chemicals are useful because they do not require electricity, but involve other logistical issues, including transportation out to remote field sites and the use of personal protective equipment (PPE).

Formalin (usually a 10% solution) and ethanol (70% concentration) are commonly used for short-term preservation of prey samples and for long-term storage of sorted items, but each has advantages and limitations (Table 3) (Ashmole & Ashmole 1967a, b; Schreiber & Hensley 1976; Diamond 1983; Duffy & Jackson 1986; Barrett et al. 2007).

Preferably, sample sorting and quantification should follow shortly after collection, to avoid any distortion of individual prey items that may occur over long periods of time (mass, size and color) (Duffy & Jackson 1986). Of the three short-term preservation agents routinely used, freezing seems to be the most benign, preserving the physical (colors) and chemical features (DNA, proteins) of the prey items. After sorting, complete and identifiable prey items may be preserved for the long-term, using deep-freezing (-29 to -62 ºC) or chemical agents (alcohol), for future use in genetics and pollutant studies or as ID voucher specimens.

Methods for Sorting Samples

Before sorting, it is recommended to obtain a size measurement of the entire sample, in terms of weight or volume. Depending on the condition of the sample, sorting involves two separate steps: one initial pass removes foreign (non-food) items that may have been collected accidentally with the sample (e.g., sand, gravel, leaves, sticks), and a second pass separates the diet items into broad categories for further quantification. For example, each prey item is classified into distinct prey classes, or groups: “fish”, “squid”, “crustaceans” and “other” (including parasites and other invertebrates). Unidentifiable items are also categorized, and all groups are quantified, in terms of their mass or volume (Ashmole & Ashmole 1967a, b; Schreiber & Hensley 1976; Diamond 1983; Baduini et al. 2001). In addition to complete prey items, incomplete or partial items may be given estimated size values based on the measurable morphologies present and their size relation to complete, intact individuals (Ashmole & Ashmole 1967b, c; Diamond 1983).

Hard structures from fully digested prey, including squid beaks, euphausiid eye-balls, and fish otoliths, provide a biased long-term dietary record, which complements the perspective derived by undigested prey (Duffy & Jackson 1986; Baduini et al. 2001). While these items routinely occur within stomach samples, their quantification depends on the research goals (i.e., broad diet description or quantification of species-specific size classes eaten) and the quality of the prey samples (i.e., degree of digestion). Ultimately, the species-specific natural history influences the quality and quantity of prey that can be obtained from any given sample. For instance, while terns and alcids deliver a bill load of fairly intact fish and squid, petrels deliver a mix of highly-digested stomach oil and partially digested prey items. Moreover, because indigestible prey items are collected in the gizzard, they are much more prevalent in those species with a two-chambered stomach, (e.g., order Procellariiformes), and very rare in those species with a one-chambered stomach (e.g., Suliformes).

Different studies have quantified the presence and abundance of indigestible prey items in a variety of ways (Duffy & Jackson 1986; Karnovsky et al. 2012). Some researchers merely record occurrence (presence and absence) within the stomach samples, regardless of numbers. Other researchers count undigested items and apply a correction factor to estimate an expected number of individuals consumed (e.g., divide the total number of euphausiid eye-balls by two, to estimate the minimum number of individuals) (Baduini et al. 2001). Additionally, otoliths and squid beaks removed from complete prey items may also be used to assist in species identification (Furness et al. 1984). Finally, some researchers discard all incomplete items and focus the dietary analysis on identifiable and complete items, which are weighed and measured individually (Schreiber & Hensley 1976; Diamond 1983; Seki & Harrison 1989).

All diet studies use a combination of descriptive classification and quantitative measurements to characterize the prey, while considering the different types of prey items, as discussed before, together or separately: complete or incomplete, and undigested prey or hard parts. This quantification involves determining the following metrics for each specific item type and taxonomic grouping: (1) the frequency of occurrence (FO), (2) the count of individuals / items (N), and (3) the size, volume or mass, of individuals within each prey group (Ashmole & Ashmole 1967a, b; Schreiber & Hensley 1976; Diamond 1983; Baduini et al. 2001).

- Frequency of occurrence (FO): To determine the relative frequency of a prey item in the diet, the proportion (i.e., frequency) of each prey type present within all samples is calculated. Frequently, researchers often use indigestible items to correct for the minimum observations of undigested prey items, by accounting for previously digested prey (Ashmole & Ashmole 1967a, b, c; Karnovsky et al. 2012). For instance, the Ashmoles (1967a, b), Diamond (1983) and Oehm (2016) marked the presence and absence of hard structures for frequency analysis, but Diamond did not use these structures to account for prey within a sample due to their possible long residence (weeks) within the stomach.

- Count of Individuals / items (N): Both partial prey and indigestible items are often used to correct the total observations of undigested prey items. For instance, the number of unique structures can help identify the total number of prey within a sample, using the greater number of structures within the sample (e.g., 6 squid heads with 5 intact squid mantles, suggests 6 squids were recently eaten). Yet, this total is limited to those structures that are relatively intact and can be matched to individuals. For example, while every flyingfish has one tail and one head, the number of pectoral fin rays varies between species. While the number of heads and tails can provide a conservative estimate of the minimum number of flyingfish eaten (e.g., using the greater count: heads or tails), the number of fin rays cannot provide such an estimate. The use of fish heads and tails to provide a minimum abundance may also be misleading. Deciphering whether a head belongs to a tail when all the pieces in between are missing can be hard; thus, the structure that has the highest abundance (heads or tails) accounts for the minimum number of fish present. As for indigestible hard parts, they are regularly reported as an indirect metric of the abundance of a specific prey type and should not be used within diet sample analyses but within overall prey consumption analyses (Ashmole & Ashmole 1967b; Diamond 1983; Karnovsky et al. 2012).

- Size measurements of individual prey: Measurements (or derived estimates) of prey length, volume (V), and mass (W) provide additional information for dietary studies. These measures have been used to determine age class and relative contribution of different sized prey to the overall diet. Moreover, size provides the basis for analyzing incomplete prey items using structural morphometric relationships, whereby the size or mass of incomplete prey items may be estimated using data from morphometric measurements taken from reference samples (i.e., the relationship of otolith length (OL) to teleost total length (TL) (Ashmole & Ashmole 1967a,b,c; Schreiber & Hensley 1976; Diamond 1983; Duffy & Jackson 1983; Barrett et al. 2007; Karnovsky et al. 2012).

These following measurements are used to quantify the size of complete prey items (Figure 2):

- Length: Standard length is used for fish due to diverse caudal morphologies of epipelagic species, and the complete mantle length in squid with the head not included.
- Mass: Wet weight, and occasionally dry weight after drying in a convection oven.
- Volume: Measured by displacement (reported less frequent than mass).

  Note: Volume can also be calculated using wet mass and prey density.

Degree of digestion of prey items indicates loss of tissue and potential for an underestimate of the true fresh mass). To address this bias, Ashmole and Ashmole (1967b) used a three-level ranking system to classify the freshness of the prey items: (1) “perfectly intact” or good condition, (2) “slightly digested” in fair condition, and (3) “incomplete” individual (size was estimated based on reference to intact prey measurements) (Table 4, Figure 3 and 4).

When prey items are in a compromised condition, specific morphometric measurements of incomplete prey items can be used to estimate their overall length. Diamond (1983) built upon the method of Ashmole and Ashmole from 1967, by measuring all incomplete fish to reconstruct their length (Figure 2 from Diamond 1983), based on complete samples collected during the field study or archived in the British Museum.

For squids, the beaks were removed from complete individuals and the rostral lengths were compared with mantle lengths, to establish a taxa-specific statistical correlation, whereby the rostral length increased as the squid length increased (Diamond, 1983). These size estimates were then used to identify different age classes, within a given species.

Prey Analyses

There are five main indices of prey importance used in non-parametric analyses of seabird diets: relative presence by number (%N), relative weight (%W) or relative volume (%V), relative occurrence (%O), the index of relative importance (%IRI) and the modified index of relative importance (%MIRI) (Duffy & Jackson 1986; Liao et al. 2001; Brown et al. 2012). Because each of these metrics is subject to specific biases, which highlight the importance of different prey items, researchers frequently use multiple approaches to quantify the importance of different prey items or taxa. For example, if the prey importance was calculated based on prey size, the %MIRI and %W will be biased towards the larger prey, and %N will be biased towards the smaller prey (Liao et al. 2001). The %IRI and O% would be best suited for this comparison because they eliminate these biases associated with prey size (Duffy & Jackson 1986; Liao et al. 2001). A new index, the prey specific index of relative importance (%PSIRI), only compares importance of prey species in diet samples that contain that same prey species to not under estimate importance of individual species that may not be present often, which occurs within the

%IRI (Brown et al. 2012). Taxonomic level of identification is overlooked within many indices, which is why the use of properly defined indices such as the %PSIRI are necessary to remove this bias. Ultimately, the selection of one or more indices will be based on their inherent biases and on the degree of freshness and digestion of the prey items. Although using multiple indices provide insights into these inherent biases in the prey consumption data, a multi-variate approach is preferable to the use of a single metric (Liao et al. 2001).

ANCILLARY ANALYSES

Increasingly, genetic analyses capable of identifying highly-digested items (Barrett et al., 2007) are augmenting results derived from quantification of identifiable prey items. The advent of novel molecular methods has provided a complementary perspective by identifying incomplete prey organisms to coarse (family) or fine (species) taxonomic levels. Currently, two approaches are applied to diet studies: prey identification and environmental genetics.

Prey Identification

Molecular analyses have facilitated the identification of prey items from stomach samples of African Penguin *Spheniscus demersus* and Great Cormorants *Phalacrocorax carbo*, and from the feces of Macaroni Penguins *Eudyptes chrysolophus* (Barrett et al. 2007; Deagle et al. 2007; Tollit et al. 2008; O'Rorke et al. 2012; Oehm et al. 2016).

DNA analyses involve Polymerase Chain Reaction (PCR) methods (Deagle et al. 2007; O'Rorke et al., 2012). PCR's ability to specify where to cut and replicate DNA has increased the ability to identify prey from diet samples, resulting in greater species richness. For example, the use of PCR to analyze pinniped diet samples yielded a 22% increase of prey species known to be consumed (Tollit et al. 2009). The genetic identification of prey can also help to prevent overlooked food groups that were misidentified using hard parts; thus, expanding the potential for understanding food web interactions (O'Rorke et al. 2012).

Next-Generation Identification

Recently, innovative PCR enriched techniques, (e.g., group-specific methods through primer exclusion of predator DNA segments to prey DNA segments), such as next generation sequencing, have facilitated the mapping of detailed food-web interactions (Gómez-Díaz & Gonzáles-Solís 2007; O'Rorke et al. 2012; Evans et al. 2015). In particular, metabarcoding is a next generation sequencing method that has been applied to fisheries studies aiming to assess biodiversity of an ecosystem through the barcoding of all prey and predators present within water samples and stomach contents (Evans et al. 2015). Thus, metabarcoding seems ideal for identifying prey species within diet samples that contain significant amounts of unidentifiable prey. Yet, an *a priori* list of the possible prey and predators species (and their DNA) in the food web is required to interpret the analysis of the isolated DNA sequences (O'Rorke et al., 2012). Therefore, exhaustive lists of the potential prey items ingested by tropical seabirds will be critical to compile the genetic sequences and primers available on the National Center for Biotechnology Information (NCBI or genbank) databases. In many cases, researchers will need to add the sequences from known and potential prey species to these databases, so these species can be considered in these genetic studies of seabird diets**.**

Unresolved Issues

Previous studies have described the dietary segregation of tropical seabirds, associated with foraging guild differences and the underlying positive relationship between the size of the ingested prey and the size of the birds' beaks (Ashmole & Ashmole 1967b; Schreiber & Hensley

1976; Harrison 1990; Young et al. 2010). While these studies have provided static snapshots of seabird diets, additional research is required to quantify the foraging ecology of these species in a dynamic context, involving their behavior to prey availability and selection, and investigating year-to-year and longer-term differences in relation to oceanographic variability. Therefore, as suggested in previous diet studies, future research should focus on a more comprehensive perspective of foraging ecology, relating prey class and size with the behavior of the birds, relevant SSPs, and oceanographic variables (Diamond 1983; Hebshi et al. 2008; Hyrenbach et al. 2013).

A critical component of this integrated perspective requires quantifying the digestion rates and nutrition (i.e., quality) of different prey items (Duffy & Jackson 1986). Determining the rate of digestion of each prey type would help to understand the birds foraging patterns and the timing of prey capture during foraging trips (Duffy & Jackson 1986). The nutritional value of the prey, found through chemical analysis of fat and protein content (Baduini et al. 2001), would help to determine how certain prey items benefit the birds (Lewison et al. 2012). For digestion and nutrition information to be accurate, genetic identification down to species would be needed, rather than to family. This would help determine the biases associated with differential digestion of different prey samples.

Another aspect not resolved from the analysis of diet samples alone involves determining the actual locations of prey concentrations and feeding events, within the larger areas used by the foraging birds (Hunt et al., 1999). Frequently, oceanographic or fisheries research cruises are used to survey the distribution and abundance of sub-surface predators that commonly feed on the same prey as seabirds (e.g., tunas, dolphinfish, pinnipeds) (Ballance & Pitman 1999; Hunt et al. 1999; Hebshi et al. 2009; Lewison et al. 2012). Increasingly, researchers are able to link the diet samples and movements of the birds during foraging trips, and this integrated perspective provides insights into important foraging areas and potential cues, such as physiographic features (shallow banks and seamounts) and oceanographic features (e.g., fronts and eddies) that influence changes in prey availability (Weimerskirch et al. 2005; Young et al. 2010; Lewison et al. 2012). Altogether, this information can reveal changes in foraging behavior (distances covered, areas used, degree of association with subsurface predators), that are often not evident from the evaluation of the ingested prey alone.

CONCLUSIONS

The degree to which the diet and the foraging ecology of seabirds, like the RFBO, are influenced by regional and temporal changes in the underlying oceanography remains poorly understood. Although there is no single "correct" way to quantify seabird diets, the lack of methodological consistency across studies only allows for limited comparisons. Standard analytical methods are therefore needed to compare and contrast diet samples within and amongst seabird species, over space and time.

While this synopsis of studies has provided static representations of seabird diets, additional research is required to quantify the foraging ecology of these species in a dynamic context, involving their behavior to prey availability and selection, and investigating year-to-year and longer-term differences in relation to oceanographic variability. Therefore, as suggested in previous diet studies, future research should focus on a more comprehensive perspective of foraging ecology, relating prey class and size with the behavior of the birds, relevant SSPs, and oceanographic variables.

In conclusion, studies aiming to provide a complete description of the foraging ecology of a seabird species and ability to compare diet studies, require standardized methods for quantification and identification of diet samples. If possible, diet studies should be augmented with genetic analysis and ancillary information on foraging behavior at sea (e.g., tracking or complimentary oceanographic cruises) to completely understand foraging ecology.

As a result of this literature review, two complementary studies were done to develop a comprehensive understanding of the foraging ecology of the Red-footed Booby (*Sula sula*, RFBO) breeding on O`ahu, Hawai`i, by integrating three different perspectives: (i) the quantification of their diet using morphological and genetic analysis; (ii) the analyses of their foraging behavior using GPS tracking and depth recorders and (iii) the driving environmental parameters using concurrent data about atmospheric and oceanographic conditions.

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TABLES AND FIGURES

Table 1. Studies of Red-footed Booby (*Sula sula*) diet and foraging ecology (1967 – 2015).

Location	**Analyses**	**References**
Oahu, HI	Frequency/Number/Size	Ashmole & Ashmole 1967
Oahu, HI	Observational Foraging Surveys	Hebshi et al. 2008
French Frigate Shoals (NWHI)	Frequency/Number/Size; IRI (Index of Relative Importance)	Seki & Harrison 1989
Midway Island (NWHI)	Heavy Metal Pollutants	Burger & Gochfeld 2000
Johnston Atoll (Central Pacific)	Flight Energetics/Metabolic Rate	Ballance 1995
Johnston Atoll (Central Pacific)	Temperature & Depth Loggers	Lewis et al. 2004
Johnston Atoll (Central Pacific)	Bird Morphometrics; Temperature & Depth Loggers	Lewis et al. 2005
Christmas Island (South Pacific)	Frequency/Number/Size; Bird Morphometrics	Schreiber & Hensley 1976
Palmyra (South Pacific)	Frequency/Number/Size; Tracking (GPS); Morphometrics; Stable Isotopes	Young et al. 2010
Aldabra Island (Indian)	Frequency/Number/Size; (OI) Overlap Index	Diamond 1983
Dong Island (South China Sea)	Heavy Metal Pollutants	Yin et al. 2008
Dong Island (South China Sea)	Post-fledging Care; Causes of Juvenile Independence	Guo et al. 2010
Europa Island (Mozambique)	Depth Recorders	Le Corre 1997
Europa Island (Mozambique)	Tracking (PTTs; GPS; IMV2)	Weimerskirch et al. 2005
Europa Island (Mozambique)	Frequency/Number/Size, Tracking (PTTs; GPs; IMV2)	Weimerskirch et al. 2006
Europa Island (Mozambique)	Frequency/Number/Size; Stable Isotopes	Cherel et al. 2008
Europa Island (Mozambique)	Tracking (GPS)	Mendez et al. 2015

Table 2. Advantages and disadvantages of the collection methods used for sampling seabird diets (Duffy & Jackson 1986; Barrett et al. 2007).

Method	**Examples**	**Advantages**	**Disadvantages**
Lethal	Collection of specimens for necropsy and diet analysis (Shooting / Mist-nests)	Provides full stomach sample; Allows measurement of bird morphometrics; Quantifies bird condition and health	Kills individual; reduce population size
Non-lethal	Induced Regurgitation: (Lavage / Emetics)	Partial to full stomach sample; Allows measurements of bird morphometrics	Lavage: causes substantial stress; Emetics: causes substantial to lethal stress; May only collect partial sample
Non-lethal	Voluntary Regurgitation	Partial to full stomach sample; Allows measurements of bird morphometrics; Causes little stress on the birds	Potential biases: only birds with large loads regurgitate
Non-lethal	Opportunistic collection of discarded prey at colony	Partial stomach sample; Potential biases: only large prey items are regurgitated and sampled	Lack certainty of species / age class of bird providing sample
Non-lethal	Naturally Deceased Specimens used for necropsy and diet analysis	Full stomach sample; Allows measurements of bird morphometrics; Quantifies bird condition and health; Causes no stress on the birds	May not describe diet accurately based on cause of death (e.g., sick and starving birds);

Table 3. Effects of preservatives on prey from diet samples.

Preservative	**Effects**	**References**
Formalin	Discoloration, added mass, health hazards, otoliths dissolve quickly	Ashmole & Ashmole 1967; Diamond 1983; Schreiber & Hensley 1976; Barrett et al. 2007
Alcohol	Breakdown of proteins / DNA, discoloration	Diamond 1983; Barrett et al. 2007
Frozen	Possible continuation of digestion after thawing	Duffy & Jackson 1986

Table 4. Criteria used to determine freshness of prey items (Ashmole & Ashmole 1967)

Freshness scale	Criteria
1	"Perfectly intact": good condition, fully intact with colorful skin/scales. Fish with head attached, Squid with buccal mass and finlet (but not necessary) could just be mantle with pink purple skin
2	"Slightly digested": fair condition, partially intact with colorful (may begin to be discolored) skin/scales. Usually fish head or tail present, tissue missing, still able to collect information
3	"Incomplete": not fresh, fish tail with vertebrae with little to no muscle, just head present or whole skeleton. Part of mantle tissue, not firm, extremely discolored (gray/ white/ pale pink)

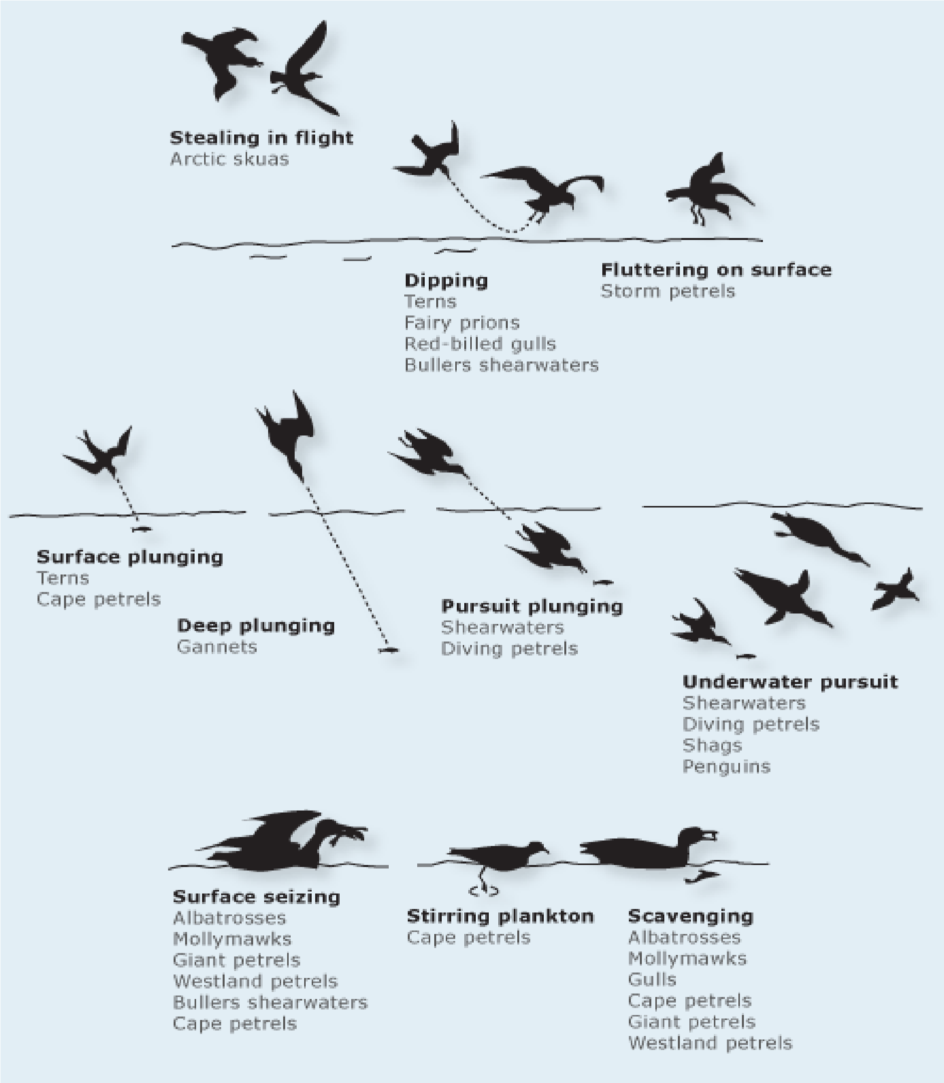

Figure 1. Seabird foraging strategies (Ashmole 1971).

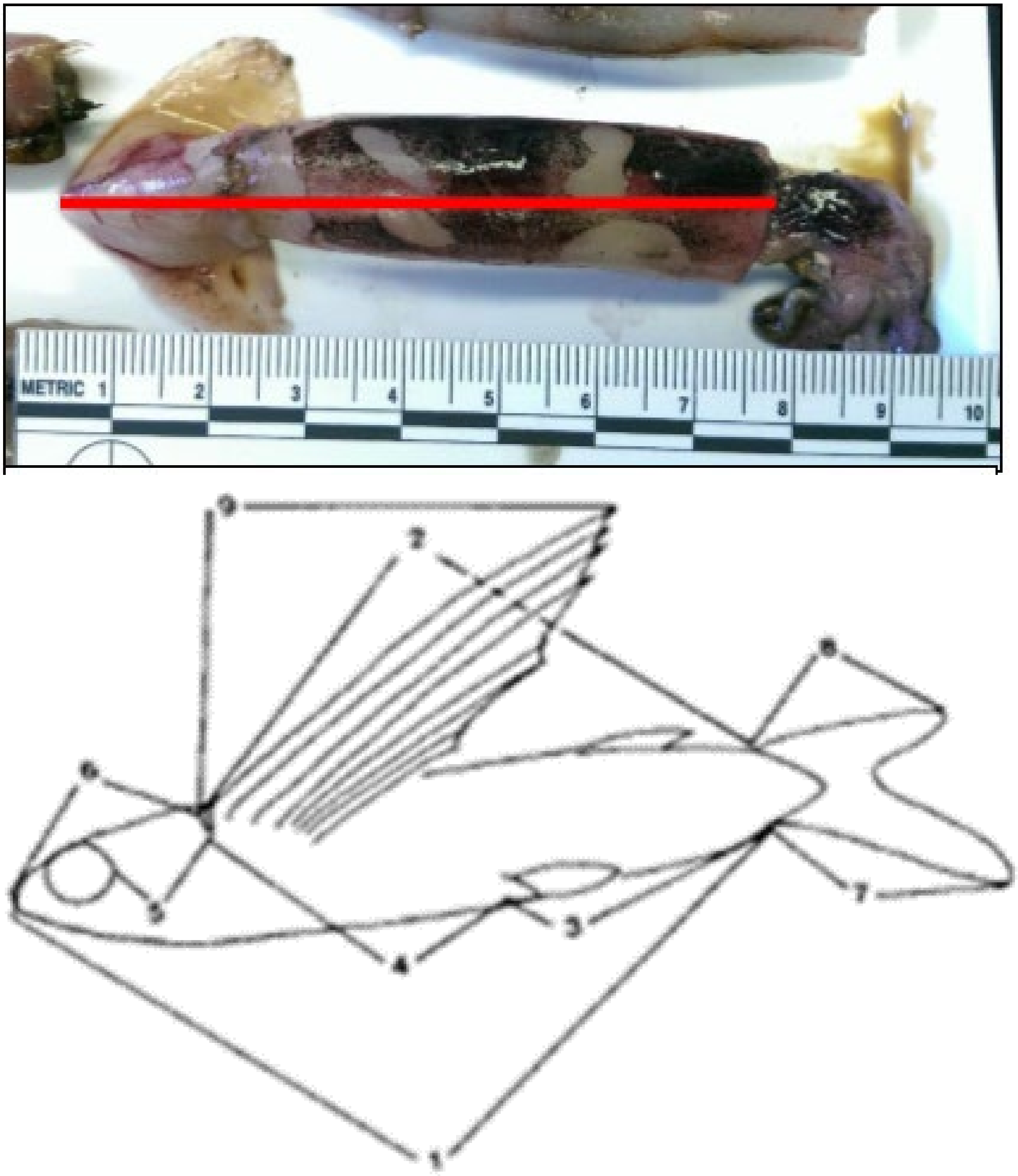

Figure 2. Size measurements of complete prey items. The mantle length for squids is shown by the red line. Size measurements for flyingfish involve eight morphometric measurements: (1) standard length, (2) base of pectoral fin to base of upper caudal fin, (3) base of pelvic fin to base of lower caudal fin, (4) base of pectoral fin to base of pelvic fin, (5) shortest distance between the eye and the base of the pectoral fin, (6) from tip of nose to base of pectoral fin, (7) length of lower lobe of the caudal fin, and (8) length of upper caudal fin (picture from Diamond 1983).

Figure 3. Fish examples of the freshness scale (Ashmole & Ashmole 1967) based on photos taken of prey samples collected from Red-footed Boobies (*Sula sula*) nesting at Ulupa`u Crater, MCBH, O`ahu, July 2015. Figure 2A: Top prey item is a species of flyingfish with a freshness rank of 2; Bottom prey item is another flyingfish with a freshness rank of 1. Figure 2B: A more damaged fish having a 2 on the freshness scale. Figure 2C: What is considered rank 3.

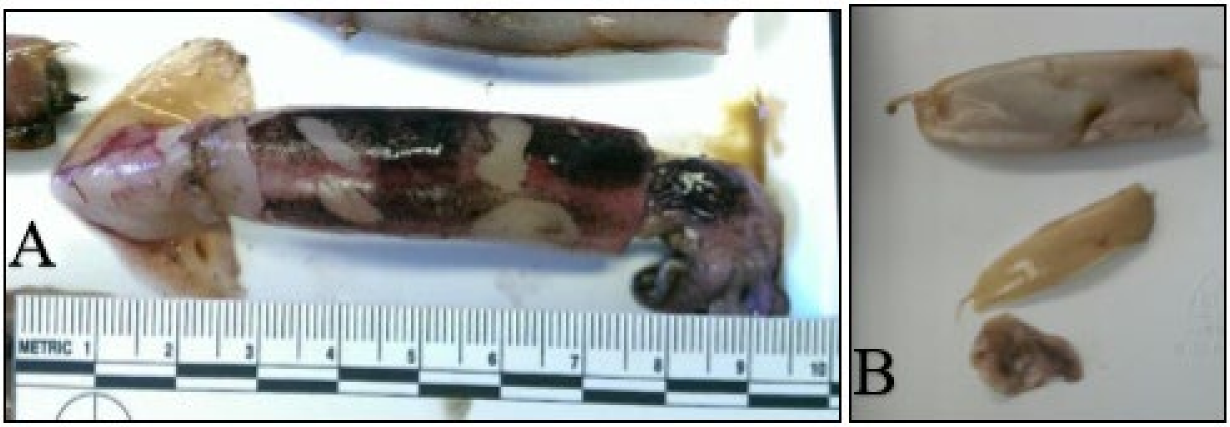

Figure 4. Squid examples of the freshness scale (Ashmole & Ashmole 1967) based on photos taken of prey samples collected from Red-footed Boobies (*Sula sula*) nesting at Ulupa`u Crater, MCBH, O`ahu, June 2014. Figure 3A: Purpleback flying squid (*Sthenoteuthis oualaniensis*) with a freshness rank of 1. Figure 3B: Top and middle squids are classified as a rank of 2 while the bottom squid mantel piece is considered a 3.

Chapter 2

Genetic analysis to evaluate diet of Red-footed boobies (*Sula sula*) provisioning chicks at Ulupa`u Crater, O`ahu

ABSTRACT

The diet of Red-footed Boobies, (*Sula sula*) provisioning chicks at Ulupa'u Crater, O`ahu, Hawai`i, was quantified using 106 regurgitations collected opportunistically from 81 adults for two years: 2014 (1–7 June) and 2015 (17 June–27 July). A total of 1007 prey items were sorted into 3 broad categories: Fish, Squid, and Other (extremely digested "mush" and parasitic isopods). The average number of prey items per sample was 8.1 ± 5.7 (2014) and 10.6 ± 8.4 (2015). Prey items were assigned a categorical freshness value: 1 (perfect condition), 2 (superficial digestion), or 3 (highly-digested and incomplete). Next, 492 items (freshness 1 and 2) were measured and sampled for genetic identification 492 items (freshness 1 and 2). 82% (401 of 492) prey items were genetically identified. Rarefaction analyses revealed robust estimation of species richness in the diet, which involved 29 fish and 3 squid species. Moreover, 98.7% (232 of 235) of identified squids were Purpleback flying squids (*Sthenoteuthis oualaniensis*). Despite the dominance of fish in 2014 (%PSIRI = fish 32.3%, squid 15.1%) and 2015 (%PSIRI = fish 29.9%, squid 22.3%), diet composition varied significantly between the two study years. Squid were more important in the diet and significantly larger in 2015 (7.2 ± 1.1 cm) than in 2014 (6.3 ± 1.7 cm), suggesting that differences in relative abundance and size may have been associated with El Niño conditions, similar to results from French Frigate Shoals (Seki & Harrison 1982). Altogether, we suggest that, due to their diverse diet, Red-footed Boobies can be used to sample variability in epipelagic nekton assemblages during periods of contrasting oceanographic conditions. Moreover, their heavy reliance on the Purpleback flying squid, makes them an ideal vehicle for studying the abundance and size distributions of immature squid.

INTRODUCTION

The Earth's oceans are experiencing large scale changes in temperature, that impact the structure of marine food webs and the distribution of upper-trophic-level predators (Lehodey et al. 1997; Mantua et al. 1997; Wolter & Timlin 2011; IPCC 2014). In particular, seabirds respond to year-to-year variability associated with the El Niño Southern Oscillation (ENSO) phenomenon and longer-term decadal variability (Schreiber & Schreiber 1983; 1984; Montevecchi & Myers 1997; Hyrenbach & Veit 2003). For instance, Red-Footed Booby (*Sula sula*, RFBO) diet at French Frigate Shoals, in the Northwestern Hawaiian Islands (NWHI), shifted from flyingfish to flying squid during the 1982-1983 El Niño event (Seki & Harrison 1989). This result was interpreted to reflect a shift in central Pacific prey distributions, when epipelagic squids became more available to central-place foraging seabirds, during warm-water conditions.

Tropical seabirds rely on a variety of behavioral and physical mechanisms to locate and capture prey. For example, many species engage in night-time feeding on vertically-migrating mesopelagic fish and squid, associate with subsurface predators (SSPs) that drive epipelagic prey to the surface or into the air, and forage along oceanographic features, like eddies and fronts, that support high localized productivity and create surface convergences (Harrison et al. 1983; Ballance & Pitman 1999; Spear et al. 2007). In particular, species in the "tuna bird" foraging guild, including frigatebirds, terns, noddies, shearwaters, and boobies, often forage in association with cetaceans and large predatory fishes (Harrison et al. 1983; Au & Pitman 1986; Hebshi et al. 2008). Therefore, the distribution and foraging behavior of seabirds provide a broader ecological context for studying the effects of oceanographic processes on the food webs that support other economically-important upper-trophic-level marine predators, like tunas (Au & Pitman 1986; Seki & Harrison 1989; Lyday et al. 2015).

This study focuses on the use of the RFBO diet as an indicator of year-to-year variability in epipelagic prey availability and oceanographic conditions, by virtue of four key traits: 1) their ability to sample localized prey distributions during the breeding season, when the foraging ranges are restricted around their colonies (mean = 67.52 km ± 44.17 S.D., Palmyra Atoll; Young et al. 2010; 201.6 km ± 87.1 SD for multi-day trips, Oahu; Adams et al. Unpublished) ; 2) their generalist foraging habits and opportunistic diet; 3) their demonstrated ability to respond to changes in the marine environment; and 4) the logistical ease involved in the study of their diet and movements, due to their non-migratory habits and colonial breeding.

More specifically, I contend that RFBO diet can be used to detect changes in the prey available within the surface oceanic environment surrounding their breeding colony on the island of O`ahu, Hawai`i. In particular, the goal of this study is to quantify the RFBO diet during two years with contrasting oceanographic conditions (2014, 2015) to address three hypotheses, with the following predictions:

1) Sampling Biases:

   To account for methodological biases, the difference between the total mass and sample freshness based on collection time will be assessed. I expect that samples after tag retrievals (post-foraging) will be larger (greater mass) and fresher (lower Weighted Average of Freshness, WAF) than samples before tag deployments (pre-foraging).

2) Inter-annual Colony Variability:

   RFBO change their diet during years of contrasting oceanographic conditions. Yet, due to high prey diversity in tropical marine ecosystems and the observed variability in RFBO diet (Ashmole & Ashmole 1967b; Harrison & Seki 1987; Harrison 1990), I expect that these dietary differences will be driven by key functional groups rather than specific species and that they will have relatively more squid in 2015 (a warm-water year) than in 2014 (a year of "average" water temperature).

3) Sexual Dimorphism:

   Because females are, on average, larger than males (954 g ± 41 SD for ♀; 839 g ± 44 SD for ♂, n=24), I expect sex-specific differences in the prey loads (overall mass, and composition by fish and squid) (Young et al. 2010).

METHODS

Study Area

The RFBO study colony (21.458394 N; 157.723361 W), is located on the eastern crest of Ulupa`u Crater, within the Marine Corps Base (MCBH), on the island of O`ahu, Hawai`i. The breeding pairs build their nests in the available Kiawe trees (*Prosopis pallida*) and Haole Koa shrubs (*Leucaena leucocephala*). The colony size estimates range from a minimum of 337 individuals in 2008, to a maximum of 2,380 birds in 1969 (Russell & Vanderwerf 2010). Currently, the RFBO breeding population is estimated at a minimum of 700 individuals (Unpublished data, T. Russell, MCBH).

Collection and Preservation of Diet Samples

Regurgitations were collected opportunistically from tagged and non-tagged adults, during field work to deploy and retrieve GPS tags. Overall, 80 birds were tagged (20 during each of four deployments) and 66 tagged and 15 untagged birds provided 106 regurgitations, totaling 9698.4 grams of sorted prey (Table 1). The regurgitations were immediately placed into a labeled zip-lock bag, stored on ice in a cooler, and placed in a freezer for long-term storage. No preservatives were used during sample collection and all samples remained frozen until they were sorted in the lab.

Diet Analysis

The diet was characterized using 106 regurgitations (diet samples), totaling 9698.4 grams of sorted prey, collected over two years: 51 in 2014 and 55 in 2015. The same person (SED) completed all sample processing and sorting, following a standardized five-step protocol (Figure 1): (1) Two passes for sorting, the first to remove foreign items and the second to determine prey classes; (2) Determination of prey freshness; (3) Quantification of the frequency, number and size of "fresh" prey (ranked 1 or 2 on freshness scale); (4) Genetic Analyses to determine identification and species abundance and diversity; and (5) Statistical analyses of the relative occurrence and relative abundance of the different prey classes and items.

Step 1: Determine Prey Classes

Sorting involved removing foreign (non-prey) items that may have been collected accidentally with the sample (e.g., sand, gravel, leaves, sticks), then separating each sample into distinct prey classes: "Fish", "Squid", "Crustacean" (isopods), and "Mush" (undistinguishable matter). The presence or absence (P/A) and mass of each prey class were used to quantify its incidence and relative abundance across all samples.

Step 2: Determine Prey Completeness & Freshness

Because digestion may lead to the underestimation of the mass of the stomach contents, diet studies often quantify the freshness of individual prey items (Ashmole & Ashmole 1967; Diamond 1983). Following previous studies, prey items were categorized as either complete (C) or incomplete (I), and according to their degree of digestion, using a three-level ranking system, or freshness scale (Table 2, Appendix 1). Four combinations of completeness and freshness codes are possible: C1, C2, I2, I3 (Table 3).

The prey category "Mush" was assigned a freshness rank of 3 because it consists of unidentifiable tissue, which could represent a mixture of the three other categories (fish, squid, crustacean). Thus, mush only contributed to the total mass and total freshness of a sample and did not contribute to the occurrence and the relative mass of fish and squid.

To investigate the potential influence of digestion on the apparent diet, a Weighted Average of Freshness (WAF) metric was used to determine an average freshness for each sample, based on the freshness of the prey items it contained. The WAF of each sample was calculated by averaging the freshness values of each individual prey item, the fish parts, the squid parts and the mush, weighted by their masses. Accordingly, WAF was calculated as follows:

$$\mathbf{WAF} = (\boldsymbol{\Sigma}(\boldsymbol{M} * \boldsymbol{F_{Pi}}) + (\boldsymbol{M} * \boldsymbol{F_{Fp}}) + (\boldsymbol{M} * \boldsymbol{F_{Sp}}) + (\boldsymbol{M} * \boldsymbol{F_{m}}))/\boldsymbol{TM} \qquad \textbf{Eqn. 1}$$

where M represents Mass, F: Freshness rank, Pi: Prey item, Fp: Fish parts, Sp: Squid parts, m: Mush and TM: Total Mass. The unitless WAF index ranges between 1 and 3, with 1 representing a fresh sample and 3 representing a highly digested sample.

Step 3: Ranked Prey Analyses

All prey samples were sorted and categorized using four metrics:

(1) Percent Frequency of Occurrence (*%FO*): The P/A of each prey class within and across samples based on the total prey items across classes;

(2) Percent Number (*%N*): The count of C and I individuals within each freshness rank category, relative to the total number of prey individuals;

(3) Percent Mass (*%M*): The wet mass (g) of C and I individuals within each freshness rank category, relative to the total wet mass of all prey items; and

(4) Size (Length or Mass): C prey items were measured using the standard length (for fish), due to diverse caudal fin morphologies of epipelagic species, and the mantle length (for squid) (Ashmole & Ashmole 1967a, b; Schreiber & Hensley 1976; Diamond 1983; Baduini et al. 2001). In addition to standard length, flyingfish morphometrics were quantified using nine measurements to predict the length of incomplete flyingfishes in future studies (Appendix 1c).

The Percent Prey Specific Index of Relative Importance (%PSIRI) was used to determine which prey class was most important during each year, as described in this equation:

$$\%PSIRI = (\%FO \times (\%PN+\%PM)) / 2 \qquad \text{Eqn. 2}$$

where %FO is percent frequency of occurrence, %PN is percent prey number (%PN is %N out of total number of samples that species is found in; %N is number of that species out of total number of prey), and %PM is percent prey weight, calculated by the total number of samples in which the species was present. The %PSIRI is considered an improvement over the Index of Relative Importance (IRI) and %IRI because it does not over- or under- compensate values based on prey class by including mass as a factor of importance. Moreover, by dividing the numerator by 2, it becomes a bounded index, ranging from 0% (the prey class never occurred in a sample) to 100% (the prey class occurred exclusively in every sample) (Brown et al. 2012).

Step 4: Genetic Identification

All genetic analyses relied on previously developed protocols and methods (Evans et al 2015; Renshaw et al 2015). Briefly, a comprehensive list of potential prey species was assembled using published lists for the MHI and the NWHI (Appendix 3). Cut genomic sequences (mitochondrion, cytochrome b, 12S, 16S, 18S, and Cytochrome Oxidase I, COI) for these species were compiled from NCBI (Genbank, https://www.ncbi.nlm.nih.gov/genbank/) and used to identify ingested prey items using Polymerase Chain Reactions (PCR), following standardized protocols (Appendix 4). The PCR products were cleaned and processed with a Sanger sequencer. Two sets of primers (COI and 18S) were used to ensure successful sequencing. The COI primers, designed for environmental DNA application (Leray et al. 2013), worked very well for the purposes of this study. For DNA samples where the COI primers did not work, 18S primers were used. The 18S did increase the Sanger sequence sample size but provided poor results because of either the similarities in 18S sequences across species or the lack of 18S information available on Genbank for the species involved.

Species diversity and richness were analyzed using three different metrics: (1) the Simpson diversity index **Simpson's D=1** $\boldsymbol{-\left(\frac{\Sigma\, n(n-1)}{N(N-1)}\right)}$ $\mathbf{Eqn.\,3}$, where n is the total number of organisms of a particular species and N is the total number of organisms of all species; bounded from, 0 (all items belong to the same single species; no diversity) to 1 (single item of every species; infinite diversity) (Simpson 1949), the bias corrected Chao1 and Chao2 indicators (estimation of the lower bound of species richness, accounting for species contributing one and two prey items (Chao 1987)), the Jack 1 and 2 richness estimators (resamples to reduce bias after removing unique species (found within only one sample; Jack 1) and duplicates (species found in exactly two samples; Jack 2), (Burnham & Overton 1978; 1979)). These indices and rarefaction curves were calculated using the EstimateS biodiversity statistical software (EstimateS 9.1.0, Colwell 2013)

Step 5: Statistical Analyses

All statistical tests were performed using the SYSTAT13 (Systat Software, Inc., San Jose, CA) and significance was assessed using an alpha of 0.05. Normality was determined using the

Kolmogorov – Smirnov and Shapiro – Wilk tests and the homogeneity of variances was determined with the Levene's test (Zar 1984).

Hypotheses Testing: Sampling Biases

To account for the potential biases associated with regurgitations collected at different times (before and after tagging), a subset of 23 individuals that yielded two regurgitations (obtained during tag deployment and recapture) were used to compare the total mass and freshness of these samples from the same individual birds using paired t-tests.

Hypotheses Testing: Colony-comparison

The statistical analysis of the diet data followed a hierarchical approach using three different datasets with decreasing sample sizes. First, the "colony-wide" analysis of the entire sample set aimed to describe broad patterns from year to year: incidence and importance of different prey functional groups and the presence and size of "indicator" prey species. This analysis involved 106 regurgitations from 81 adults (2014, N = 51 samples; 2015, N = 55 samples). First, the sample WAF was compared between years (2014, 2015) using one-way Analysis of Variance (ANOVA). Next, to explore the potential influence of the sample freshness on the overall mass and make-up of the samples, ANCOVA was used to compare Total Mass and Mush Mass during the two study years, while controlling for WAF.

Hypothesis Testing: Sex-differences

Next, the "sex-based" analysis quantified dietary differences using 82 diet samples from 59 birds of known sex: 34 diet samples from 23 birds (14 Male, 9 Female) in 2014, and 48 diet samples from 36 birds (19 Male, 17 Female) in 2015. Birds were sexed from a blood sample taken during tag retrieval periods. A Sexual Dimorphic Index (SDI) was used to quantify the relative difference in morphometric and dietary data between males and females (Angel et al. 2015). SDI is expressed as the following ratio below:

$$\mathbf{SexDimorphismIndex} = \left|-\left(\frac{\mathbf{\mathit{mean\ male}}}{\mathbf{\mathit{mean\ female}}}\right) + \mathbf{1}\right| \mathbf{x100} \qquad \mathbf{Eqn.\ 4}$$

where a value of 0 means that the sexes are equal, and “positive and negative values” mean that the females or the males are larger, respectively. Three morphometric measurements were used to help determine the sex of RFBO without blood samples; body mass (g), culmen length (mm), and wing chord (mm). Additionally, the SDI was applied to the relative abundance (% Total prey Mass) of the different prey classes to determine if the diet composition of males and females differed.

Exploratory Analyses

In addition to testing these three hypotheses, we used multivariate analyses to explore the prey data, using the statistical software PC-ORD 6 (McCune and Mefford 2006). All analyses compared prey mass, using a data matrix, with samples (regurgitations) as rows and prey (species / functional groups) as columns. To facilitate a successful ordination, those “rare prey species” that were present in only one sample, and all “empty” samples that did not contain identified prey items, were discarded (McCune & Grace 2002). The pair-wise distances between the resulting samples were relativized using the Relative Sorensen distance measure, to ensure each sample contributed the same weight to the analysis (Kruskal and Wish 1978).

Two tests were performed using the same “sample-species” matrix: a free ordination and a group comparison. First, a Nonmetric Multidimensional Scaling (NMDS) ordination was performed using random starting coordinates with 500 iterations with the real data and 999 runs with the randomized data to yield p values. A maximum of 6 axes was selected for the ordination, and the stability criterion was set to 0.000001. Next, a Multi-Response Permutation Procedure (MRPP) test was performed to compared different groupings using all the prey species at once. The categorical grouping variable "Sex by Year" was used as the second matrix, to assign individual samples to one of four groups: males 2014, females 2014, males 2015, and females 2015. Finally, following the significant MRPP results, Indicator Species Analysis (ISA) was used to identify significant indicator prey species and functional groups associated with the four "Sex by Year" groups.

To explore the potential influence of individual prey species, these analyses were performed twice (1) using individual prey species (68 diet samples and 20 identified prey species), and (2) using functional groups (62 diet samples and 4 functional groups) defined based on the species-specific habitats and habits: reef-associated fishes, pelagic-oceanic fishes, flyingfishes and squids. Flyingfishes included the families Exocoetidae and Hemiramphidae because they both leap out of the surface ocean to escape predation. The other mesopelagic fishes that don't fly were grouped into pelagic-oceanic. Reef-associated fishes are species found to live in association with reef habitat and squids were all the family Ommastrephidae, flying squids.

Environmental Parameters

Two basin-wide indices (the Pacific Decadal Oscillation (PDO) and the Multivariate El Niño Southern Oscillation Index (MEI)) and two local ocean – atmosphere indices (SST and wind speed) were compared to characterize environmental conditions during the two study years. These data were obtained from the following publicly available web-sites: MEI Index, http://www.esrl.noaa.gov/psd/enso/mei/table.html; PDO Index, https://www.ncdc.noaa.gov/teleconnections/pdo/; Ocean Watch LAS SST (GOES-POES) and wind speed (CCMP), http://oceanwatch.pifsc.noaa.gov/las/ .

The MEI quantifies ocean – atmosphere conditions along the Tropical Pacific (~10ºN to 10ºS) by integrating six cross-correlated variables: sea-level pressure, zonal and meridional surface winds, sea surface temperature, air temperature, and total cloudiness fraction within the sky (Wolter & Timlin 1993). "Average" conditions range from slightly positive (+ 0.5) to slightly negative (- 0.5) deviations from the long-term mean. An El Niño event is characterized by positive anomaly (≥ + 0.5), indicative of high sea surface and air temperatures in the Western Pacific with high sea level pressure in the Eastern Equatorial Pacific due to increased Westerlies along the Subtropical Pacific (~5ºN). La Niña is characterized by negative anomalies (≤ - 0.5) and reflects the opposite conditions of an El Niño event (Wolter & Timlin 1993). The PDO quantifies SST conditions across the North Pacific Ocean (North of 20ºN) (Mantua et al. 1997). SST anomalies are ± 0.5ºC. Monthly MEI and PDO index values during the two years, before and during the breeding season (January - August) of both study years (2014, 2015) were compared using paired t-tests.

In addition to these basin-wide indices, local oceanographic conditions were quantified using two variables, wind speed (WSP, m/s) and Sea Surface Temperature (SST, ºC)**.** Values were taken from a 5º×5º grid centered on the location of the NDBC Buoy 51000 **(**23°32'17" N 153°48'29" W)**:** Average daily observations during the breeding season (May-July) of both study years (2014, 2015) were compared using ANOVA.

RESULTS

Environmental Parameters

Both the basin-wide (MEI, PDO) and the local (SST, wind speed) conditions varied significantly during 2014 and 2015, suggesting that the two study years sampled periods of contrasting oceanographic conditions regionally and locally.

Basin-Wide Conditions

The monthly (Dec / Jan through June / July) MEI index values for the two study years (2014, 2015) were significantly different, with 2015 having higher values than 2014 (Paired T-test, $t = -10.733$, $df = 6$, $p < 0.0001$). In fact, a month-by-month comparison revealed that the MEI values were always higher in 2015 than in 2014 (Figure 2A). While the differences in the PDO were less obvious, , the monthly PDO index was significantly higher in 2015 than in 2014 (Paired t-test, $t = -2.698$, $df = 6$, $p < 0.036$) (Figure 2B).

Local Conditions

Higher daily wind speeds were observed during the RFBO breeding season (May – August) in 2014 than in 2015 (ANOVA, $F_{1,235} = 12.723$, $p < 0.0001$). There was also a significant month×year interaction ($F_{3,235} = 8.492$, $p < 0.0001$), with the month of June showing the largest year-to-year difference in wind speed, increasing in 2014 and decreasing in 2015 (Figure 3A).

Daily SST was not significantly different between 2014 and 2015 (ANOVA, $F_{1,235} = 0.268$, $p < 0.605$). However, there were different SST trends during the two study years, as evidenced by the significant month×year, interaction ($F_{3,235} = 86.021$, $p < 0.0001$). Namely, while May SST was colder in 2015 than in 2014, SST increased in subsequent months in 2015

(Figure 3B). Nevertheless, the two years were not significantly different, on average (2014 mean: 25.4 ± 0.5 SD and 2015 mean: 25.4 ± 1.0 SD).

Testing Sampling Biases in Diet Composition

The first step in the dietary analysis, involved investigating potential biases between prey samples obtained from tagged birds before or after tracked foraging trips, by comparing a subset of 23 pairs of regurgitations obtained from the same birds during deployment and recapture in the same years. This analysis involved 2 females and 9 males in 2014 and 1 female and 11 males in 2015, with 4 of these birds (2 males and 2 females) being sampled repeatedly during both study years.

The Total Mass of the deployment and recapture samples from the same individuals was not different (Paired T-test, $t = -1.004$, $df = 22$, $p < 0.327$), and the differences were normally distributed (one-sample Kolmogorov-Smirnov test, $n = 23$, Max Diff = 0.092, $p < 0.979$). On the other hand, the WAF was significantly lower for the recaptures than for the deployments (Paired T-test, $t = 2.124$, $df = 22$, $p < 0.045$), and the differences were normally distributed (one-sample Kolmogorov-Smirnov, $n = 23$, Max Diff = 0.090, $p < 0.984$). This result indicates that the food samples obtained from birds returning from a tracked foraging trip were fresher, as evidenced by the lower WAF. Nevertheless, since the Total Mass of the deployment and recapture samples did not differ, every sample was used for further analyses, regardless of the specific relative timing of the collection.

Diet Composition: Colony-Wide

Total Sample Mass

The colony-wide analysis used all 106 regurgitations collected from 81 birds. The total mass of the regurgitations was not significantly different across years (ANOVA, $F_{1,103} = 0.412$,

$p < 0.522$), and the residuals were normally distributed (one-sample Kolmogorov-Smirnov, n = 55, Max Diff = 0.065, $p < 0.976$). On average, the total mass of the regurgitations was 88.1 g ± 59.8 SD, (median = 66.2, range = 6.8 - 229.6) in 2014 and 95.6 g ± 61.0 SD (median = 89.4, range = 8.8 - 225.2) in 2015 (Figure 4). Nevertheless, the Total Mass of a sample was significantly related to its WAF score (ANCOVA, $F_{1,103} = 31.993$, $p < 0.0001$).

The WAF scores, which range between 1 and 3, were not significantly different during the two study years (ANOVA, $F_{1,103} = 0.051$, $p < 0.823$.) and averaged 2.2 ± 0.5 SD (median = 2.1, range = 3 - 1) in 2014 and 2.3 ± 0.5 SD (median = 2.1, range = 3 - 1) in 2015.

Prey Classes

Four different metrics underscored the dominance of fish across both study years, with squid playing a secondary role: relative prey masses, relative prey numbers, average number of prey items per sample, and %PSIRI.

Fish dominated the diet in both years. In 2014, fish accounted for 67.9%M and 54.2%N and squid accounted for 29.8%M and 45.8%N. A minor proportion of the sample mass (2.3%) was mush, which did not involve any discernible prey items. In 2015, fish accounted for 57.4%M and 53.8%N, and squid accounted for 42.1%M and 46.2%N. Mush only accounted for 0.5%M of the samples and did not include any discernible prey items.

The dominance of fish was also evident when the average masses were compared across samples (Figure 5). In 2014, the average fish mass per sample was 60.2 g ± 55.4 SD (median = 40.5; range = 0.8 - 219.7), and the average squid mass contributed less than half as much as fish (26.4 g ± 30.1 SD; median = 28.7; range = 0.0 - 225.2). In contrast, during 2015, the average mass per sample of fish and squid were more equivalent 54.0 g ± 57.8 SD and 39.6 g ±

50.9 SD, respectively (2014: median = 15.7, range = 0.0 - 128.6; 2015: median = 14.9, range = 0.0 - 202.8) (Figure 5). Overall, the average mass of fish (ANOVA, $F_{1,103}$ = 0.313, $p < 0.577$) and the average mass of squid (ANOVA, $F_{1,104}$ = 2.59, $p < 0.111$) in a regurgitation sample did not differ between years. However, the average mass of mush was higher in 2014 than in 2015 (ANOVA, $F_{1,103}$ = 7.676, $p < 0.007$).

In 2014, a total of 232 fish and 187 squid were sampled, with an average number of 8.1 ± 5.7 SD prey per sample (median = 7.0, range = 1 - 24). In 2015, a total of 315 fish and 270 squid were sampled, with an average of 10.6 ± 8.4 SD prey items per sample (median = 9.0, range = 1 - 34). While four combinations of completeness and freshness codes are possible (C1, C2, I2, I3), the majority (53.1%) of the prey items were categorized as I3, with 22.6% as I2, 14.4% as C1 and 9.9% as C2. Overall, 24.3% (255 of 1049) of the sorted prey items were complete and could be measured (Table 3). Based on the complete-freshness rank scale assigned to each prey item (C1, C2, I2, I3), squid freshness did not differ by year (χ^2 = 6.9489, df = 3, $p < 0.666$) but fish freshness did (χ^2 = 29.323, df = 3, $p < 0.0001$) with fresher prey items in 2014. The regurgitations were slightly fresher in 2014 though the difference in WAF across years was insignificant (ANOVA, $F_{1,103}$ = 0.051, $p < 0.823$).

Altogether, the %PSIRI indicated that fish was the most important prey class in 2014 (32.3%) and 2015 (29.9%). Squid had lesser %PSIRI in both years (2014: 15.1%; 2015: 22.3%), and mush had the least %PSIRI (2014: 0.5%; 2015: 0.4%) (Table 4).

Prey Size

Of the total number of prey items found in 2014, 108 (17 fish and 91 squid) complete items were measured, with fish standard lengths ranging from 5.0 - 19.2 cm and squid mantle lengths ranging from 3.0 - 10.3 cm. In 2015, 129 (19 fish and 110 squid) complete items were measured,with fish standard lengths ranging from 4.1 - 20.5 cm and squid mantle lengths ranging from 4.5 – 10.0 cm. The largest fish were flyingfish; a 19.2 cm and 120.7 g Narrowhead flyingfish, *Cypselurus angusticeps*, in 2014 and a 20.5 cm and 125.6 g Glider flyingfish, *Cheilopogon atrisignis,* in 2015. The largest squids were two Purpleback flying squids, *Sthenoteuthis oualaniensis* (2014: 10.3 cm, 35.1 g; 2015: 10.0 cm, 24.0 g).

While fish size frequencies were not tested due to small sample sizes (< 20 prey items per sample year), the mantle lengths of the squid sampled during the two study years were compared using a single-factor ANOVA. Squids were significantly larger in 2015 than 2014 (ANOVA, $F_{1,199}$ = 17.658, p < 0.0001) (Figure 6). In 2015, the size distribution was confined to the mid-size squids with 96.9% (31/32) of the values ranging from 4 to 8 cm and 60% ranging between 6 to 8 cm. In 2014, the size distribution ranged from 2 to 10 cm, with 75.8% of the individuals falling between 4 and 8 cm (Table 7, Figure 3). This result suggests a shift in the sizes of the consumed squid, with larger individuals taken in 2015.

Genetic Identification of Prey

Out of 1007 sorted prey items, 492 were complete and used for genetic identification. Prey with a freshness of 3 were not used due to the probability of the DNA being damaged or degraded from severe digestion. 82% (401) of these prey items produced successful COI sequences and were generically identified (Table 5). A total of 32 species (29 fish and 3 squid) were identified, belonging to 9 fish families and 2 squid families. Three families (Acanthuridae, Molidae, Onychoteuthidae) occurred solely in 2014. The other fish and squid families were sampled in both study years.

%PSIRI (Table 5) indicated flyingfishes (Exocoetidae) and flying squid (Ommastrephidae) were the most important in 2014 (15.6% and 10.8% respectively) and in 2015

(15.0% and 10.6%, respectively). The fish species with the highest importance were Tropical two-winged flyingfish (*Exocoetus volitans*, 9.4%, 2014), and Glider flyingfish (*Cheilopogon atrisignis*, 8.3%, 2015). Purpleback flying squid comprised 98.7% (232 of 235) of the identified squids and had the greatest importance (10.7% in 2014 and 10.5% in 2015) (Table 5). The other three identified squids were two Neon flying squid (*Ommastrephes batramii*, one from each year), and one *Onykia* species in 2014.

Rarefaction analyses of the pooled species data, which included 29 fish and 3 squid species, revealed robust estimation of species richness (Figure 7). Three species estimators (Chao 1, Chao 2 and Jackknife 2) curved down toward the asymptote of the observed species richness. The Jackknife 1 estimator reached an asymptote at 10 species greater than the observed species richness. Nevertheless, all these estimators remained within the 95% confidence intervals of the estimated richness.

The Simpson's Diversity Index indicated a diverse RFBO diet (D = 0.65, number of species: 32). When calculated by year, 2014 (D = 0.66, number of species: 27) was more diverse due to having 2 more species than 2015 (D = 0.63, n = 25) and by having more rare species (Table 6)

Sexual Dimorphism

Morphometrics

Overall, this analysis involved 56 birds of known sex with a complete set of morphometric data (body mass, culmen length, wing chord): 26 in 2014 (9 Females and 17 Males) and 30 (14 Females and 16 Males) in 2015 (Table 7, 8). Body mass was the most strongly sexually dimorphic trait in both study years, with SDI values of 13.1% (2014) and 13.5% (2015), followed by culmen length (2014: 3.9% and 2015: 3.8%), and wing chord (2014: 0.7% and 2015:

3.0%) (Table 7). All SDI values were positive, indicating that, on average, females were larger than males. Additionally, the morphometric data distributions were used to assign four birds of unknown sex using the z-scores of body mass (g), culmen length (mm), and wing chord (mm), where z = (value – sample mean) / (sample SD).

Total Sample Mass and Prey Class Comparisons

These 56 birds yielded a total of 79 regurgitations; 38 from 2014 (11 from females, 27 from males) and 41 from 2015 (16 from females, 25 from males). Female regurgitations had more mass than male regurgitations for both years, having an average Total Mass of 106.5 g ± 68.1 SD (2014; males: 90.8 g ± 59.9 SD) and 105.3 g ± 70.7 SD (2015; males: 88.7 g ± 64.2 SD) (Two-way ANOVA, $F_{3,75} = 14.421$, $p < 0.0001$) (Table 6). Year and the interaction between were not significantly different (Year: $F_{3,75} = 2.676$, $p < 0.102$; Year×Sex: $F_{3,75} = 1.243$, $p < 0.265$).

The masses of fish and squid per sample were similar between sexes in 2014. In 2015, fish mass in males was less, coincident with a slight significant increase in squid consumption compared with 2014 whereas females showed the exact opposite, consuming significantly more fish than squid (Two-way ANOVA - Fish mass: $F_{3,75} = 5.299$, $p < 0.024$, $R^2 = 0.111$; Squid mass: $F_{3,75} = 3.725$, $p < 0.057$, $R^2 = 0.096$; Bonferroni Males-Females Fish mass 2015, df = 78, LCI = -97.423, UCI = -5.579, Dif = -51.501, $p < 0.02$; Bonferroni Males-Females Squid Mass 2015, df = 78, LCI = 0.071, UCI = 69.098, Dif = 34.584, $p < 0.049$).

Though Total Mass did not vary by year or sex, there was a strong interaction with prey class between year×sex where females of 2015 consumed fish significantly more than males whom consumed more squid (ANOVA, F$F_{1,78} = 14.421$, $p < 0.0001$, $R^2 = 0.029$).

Following the characterization of sexual dimorphism among morphometric traits, the SDI was used to characterize sexual dimorphism in the prey composition (average fish mass and average squid mass) of the male and female diet, using a sample size of 34 diet samples in 2014 (11 female and 23 male) and 48 diet samples in 2015 (18 female and 30 male). In 2014, the SDI results were similar to the previous ANOVAs of the regurgitation masses, with females consistently delivering food loads with more mass than males (Fish 15.0%, Squid 13.1%, Total Mass 14.7%). Whereas female diet samples were heavier in 2015 (15.8%), there were clear sex-differences; females delivered substantially more fish (59.2%), and males more squid (-191.4%).

The Simpson's Diversity Index indicated females had greater diversity within their diet than males when both years were combined (Females: D = 0.72, n = 18; Males: D = 0.58, n = 28). Males in 2014 had a more diverse diet than the females (Females: D = 0.56, n = 9; Males: D = 0.75, n = 21). This pattern switched in 2015 with females having a more diverse diet than males (Females: D = 0.85, n = 14; Males: D = 0.46, n = 15) (Table 6).

Prey Freshness and Size

The freshness, length and mass of prey items were compared between males and females during 2014 and 2015 using two-factor ANOVA tests. While freshness was not significantly different by Year or Sex, there was a significant year×sex interaction, with the most and the least digested prey items from 2015 females and 2014 males, respectively (Two-way ANOVA year×sex, $F_{1,290} = 5.934$, $p < 0.015$).

Nevertheless, the size of prey items was not significantly different by year or sex (Two-way ANOVAs Sex: $F_{1,132} = 3.284$, $p < 0.072$; Year: $F_{1,132} = 1.583$, $p<0.211$; Sex×Year: $F_{1,132} = 1.551$, $p < 0.215$)

Exploratory Analysis: Individual Species

NMDS revealed a 1-dimensional solution that explained 6.5% ($R^2 = 0.065$) of the variance between food loads and prey species composition. The final stress (12.09) for the NMDS indicated scaling results were “fair” and should be interpreted with caution. (Clark 1993; McCune & Grace 2002). While the NMDS ordination only explained a small amount of the overall variance, the correlations of the prey species with the single resulting axis indicated a fish – squid gradient, with samples being dominated (by mass) by either Glider flyingfish (tau 0.432) or Purpleback flying squid (tau -0.378).

The MRPP chance corrected within-group agreement, showed that the heterogeneity within groups was similar to that expected by chance ($A = 0.015$), and the groups were not significantly different ($p = 0.141$). Since the MRPP was insignificant, the pairwise tests were not considered, and no ISA was performed.

Exploratory Analysis: Prey Guilds (Functional Groups)

Because many rare species occurred infrequently in our samples, species data were pooled into functional groups, to facilitate characterization of the diet. Using the Fishbase species accounts (http://www.fishbase.org), functional groups were determined based on prey taxonomy and habitat characteristics (reef-associated, pelagic-oceanic, flyingfish or squid). Depth ranges for each species were not used because all prey reside within the euphotic zone and some prey were only identified to the genus level. (Table 9).

NMDS among functional groups generated a 2-dimensional solution, with a final stress of 4.46 which was considered an “excellent” ordination (Clark 1993; McCune & Grace 2002). Consistent with the individual species ordination, the 2-dimensional ordination revealed squids were highly correlated with axis 1 (tau 0.849) and axis 2 (tau 0.612) and flyingfish had a high negative correlation to both axes (axis 1: tau -0.790; axis 2: tau -0.556) resulting from individual birds delivering either flyingfish or flying squid guilds (Figure 8). The remaining two functional groups were not highly correlated with either axis; pelagic-oceanic fish were negatively correlated more with Axis 2 (Axis 1: tau -0.087, Axis 2: tau -0.338) and reef-associated fish were negatively correlated with Axis 1 and were positively correlated with Axis 2 (Axis 1: tau -0.114, Axis 2: tau 0.251).

The MRPP chance corrected within-group agreement, showed that heterogeneity within groups was larger than what was expected by chance (A = 0.107) and the groups were significantly different ($p < 0.002$). Pairwise comparisons, revealed three significant patterns: 2014 Males diet versus 2015 v Females (A = 0.076, $p < 0.02$), 2014 Males versus 2015 Males (A = 0.047, $p < 0.03$) and 2015 Females versus 2015 Males (A = 0.258, $p < 0.0003$). The Females from both years (A = 0.054, $p < 0.139$), Males and Females from 2014 (A = -0.017, $p < 0.705$) and 2014 Females and 2015 Males (A = 0.022, $p < 0.176$) were not different from each other.

Following the significant MRPP, an Indicator Species Analysis (ISA) based on 9999 iterations of the randomized data ($p < 0.0155$) confirmed that in 2015, females ate more flyingfish ($p < 0.0036$) and males ate more squid ($p < 0.0397$) (Figure 8).

DISCUSSION

RFBOs have been considered generalist foragers, because they associate with a wide range of sub-surface predators (tunas, dolphins, mahi-mahi), and are known to prey on a wide range of fish and squid prey (Harrison & Seki 1987, Hebshi et al. 2008). Moreover, because they are the most pelagic of the Sulidae, ranging up to 200 km from their colonies, they are thought to search for and consume widely distributed fish and squid prey they find in near surface waters (Adams et al. Unpublished).

Thus, the purpose of this study was to develop a comprehensive understanding of the diet of RFBOs breeding on O`ahu, Hawai`i, using standardized methods augmented with the use of genetic analyses. To this end, the project investigated the diet during two years of contrasting oceanographic conditions (2014, 2015), characterized by changes in basin-wide conditions (MEI and PDO) and regional environmental parameters (SST and WSPD).

Consistent with French Frigate Shoals RFBOs and birds from other locations (Harrison et al. 1983, Seki & Harrison 1989, Schreiber et al. 1996, Weimerskirch et al. 2006), the diet of the RFBOs of O`ahu consisted mostly of two main prey guilds (functional groups): flyingfishes (Exocoetidae) and ommastrephid squids, particularly the Purpleback flying squid. The Purpleback flying squid (*Sthenoteuthis oualaniensis*) was the most numerous squid species, accounting for 99% of all squids. In fact, this species was the most numerous identified prey, accounting, on average, for 21% (by mass) and 58% (by number), across both study years. Moreover, while RFBO ingested other reef-associated and epipelagic / mesopelagic fish species, flyingfish and flying squid were the main prey functional groups in the diet. These results are similar to those from other locations in the central Pacific (French Frigate Shoals, Palmyra, Christmas Island) and the Indian Ocean (Europa Island) (Ashmole & Ashmole 1967; Harrison et al. 1983, Seki & Harrison 1989, Schreiber et al. 1996, Weimerskirch et al. 2006; Young et al. 2010).

Nevertheless, because RFBO ingested a total of 29 fish species and 3 squid species, their diet was highly diverse, from the Simpson's Diversity Index, suggesting that RFBOs are generalist foragers, that eat a wide range of prey species, including reef-associated fish.

Overall, the RFBO diet changed during the El Niño onset of 2015, a period of warmer SST and lower winds, by eating more squid and less fish than during 2014. Moreover, the consumed squids were larger in 2015. While little is known about the environmental drivers of spawning and development of the Purpleback flying squid, the peak spawning period ranges are March to May in the Northwestern Indian Ocean and late spring to early summer around Eastern O`ahu (Young & Hirota 1998; Xinjun et al. 2006). Given the maximum size ranges of these squid in the Indian Ocean being (462mm for males and 612mm for females), and the smallest mature squids 160 mm (females) – 130 mm (males) around O`ahu, the squids delivered by RFBO at MCBH did not exceed 103 mm and were juveniles. Therefore, if squids of the central Pacific have a similar reproductive penology as squids in the northwestern Indian Ocean, RFBOs of O`ahu, during the June and July months, appeared to take squid spawned during the same year.

Given the observed changes in SST observed during the two study periods, the warmer water conditions in 2015 could have influenced the juvenile squid growth and survivorship, leading to more higher abundance and size for this prey species. Because little is known about central Pacific flyingfish responses to oceanographic variability, it is unclear whether their abundance or spawning was affected by changing SST in 2015. Nevertheless, a study from the Caribbean found that growth rates are faster where SST was higher and that juveniles hatched in warmer waters grew faster than juveniles in colder water (Oxenford et al. 1994).

Sexual Dimorphic Relations

Similar to other colonies in the central and south Pacific (Table 8) (Schreiber & Hensley 1976; Harrison et al 1983; Lewis et al. 2005, Weimerskirch 2005; Young et al. 2010), female RFBOs at MCBH were larger than males. When the diets of males and females were compared, larger-bodied females yielded significantly heavier food loads than their male counterparts. Nevertheless, the relative make-up of the diet was not different between males and females during the average environmental conditions of 2014. However, females and males ate disproportionally more fish and squid, respectively, in 2015. This disparity during the El Niño year suggests a segregation of foraging niches. Additional analyses of the tracking and diving data collected from these same individuals will provide farther insights into the potential spatial and behavioral segregation of males and females

This study documented temporal changes in RFBO diet during two years of contrasting oceanographic conditions, which underscore the potential use of seabirds to study broader shifts in the marine ecosystem structure, from year-to-year and over longer time frames. Unfortunately, the lack of standardized metrics and approaches have inhibited comparative studies of seabird diets over time. Therefore, the development and use of consistent methods and metrics is required for accurate comparisons of seabird diets over time (Duffy & Jackson 1986, Barrett et al. 2007).

This study used novel genetic methods to develop a comprehensive understanding of the RFBO diet composition, using newly developed metrics to describe prey-specific relative importance, species richness, and diversity. This comprehensive approach was applied to the RFBO on Ulupa`u Crater, on the island of O`ahu, the southern-most colony of this species in the Main Hawaiian Islands. The only historical data on RFBO diet at this site involve notes published in 1967 (Ashmole & Ashmole 1967a). Nevertheless, because this site is easily accessible, it is ideal for ongoing diet studies to characterize changing RFBO foraging habits during periods of changing oceanographic conditions. To date, the only other diet study of RFBOs of the Hawaiian Islands dates to the early 1980s at French Frigate Shoals. That study documented a RFBO dietary change during a period of unusual warm-water conditions. While squid remained abundant in the diet during 1982, a warm year leading into an El Niño event, fish became more abundance in 1981, during a relatively cooler year (Seki & Harrison 1989). Since 2015 was an onset to an El Niño year, like 1982, and so we expected a similar result for birds nesting on the Main Hawaiian Islands.

Studying the diet and foraging behavior of "SSP-dependent" seabirds can also provide evidence into how ocean productivity and prey availability are likely to affect other top predators over time (Harrison 1990; Harrison & Seki 1987). RFBOs are associated with a large variety of SSPs, including tunas, dolphinfish and cetaceans, throughout the Eastern Tropical Pacific (ETP) and CP (Harrison et al. 1983; Au & Pitman 1986, 1988; Hebshi et al. 2008). Dietary studies of breeding colonies in the NWHI have documented that RFBO consume prey up to 20 cm in length, which were attributed to feeding associations with large SSPs, including Skipjack tuna (*Katsuminus pelamis*), Yellowfin tuna (*Thunnus albacares*), Mahimahi (*Coryphaena hippurus*) and spotted dolphins (*Stenella attenuata*) (Harrison et al. 1983). While RFBOs are most commonly observed foraging with skipjack tuna around O`ahu, (n = 32: 80.0%), they are generalists and forage with a wide variety of SSPs: yellowfin tuna (n = 1: 2.5%), dolphinfish (n = 2: 5.0%) and cetaceans (n = 5: 12.5%) (Hebshi et al. 2008). Skipjack tuna are most numerous around O`ahu during the RFBO brooding season (spring – summer), further underscoring the ecological importance of their foraging association (Senina et al. 2008).

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TABLES & FIGURES

Table 1. Sample sizes over two study years (2014 and 2015): showing the number of regurgitation samples (mass of sorted prey) obtained per year, and the number of regurgitation samples obtained during tag deployments and recaptures of tagged birds and from non-tagged birds.

Year	Regurgitation Mass (g)	Regurgitation Samples	Deployment Samples (Dates)	Recapture Samples (Dates)	Tagged Birds	Untagged Birds
2014	4521.9	51	15 (June 1) 9 (June 4)	9 (June 4) 18 (June 5 – 7)	30	8
2015	5176.5	55	13 (June 17) 12 (June 29)	14 (June 22 – 23) 16 (July 6 – 7)	36	7
Total	9698.4	106	49	57	66	15

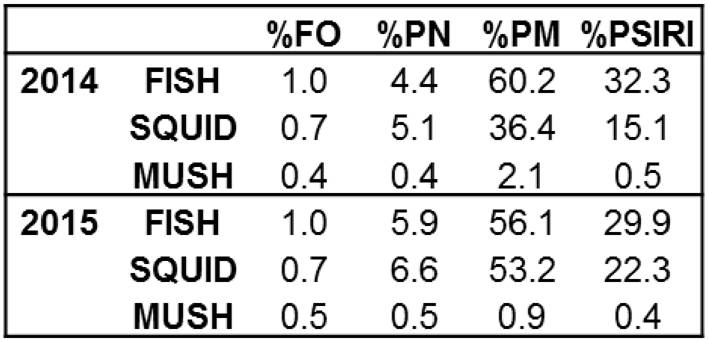
Table 2. Criteria used to determine the freshness of prey items (modified from Ashmole & Ashmole 1967b).

Freshness Scale	Criteria
1	"Perfectly intact": good condition, fully intact with colorful skin/scales. Fish with head attached, Squid with buccal mass and finlet (not necessary) usually just be mantle with pink purple skin
2	"Slightly digested": fair condition, partially intact with colorful (may begin to be discolored) skin/scales. Usually fish head or tail present, tissue missing, still able to collect information
3	"Incomplete": not fresh, fish tail with vertebrae with little to no muscle, just head present or whole skeleton. Part of mantle tissue, not firm, extremely discolored (gray/ white/ pale pink)

Table 3. Summary of the completeness (C-Complete, I-Incomplete) and freshness (1, 2, 3) of fish (“F”) and squid (“S”) prey items from RFBO sampled during two study years.

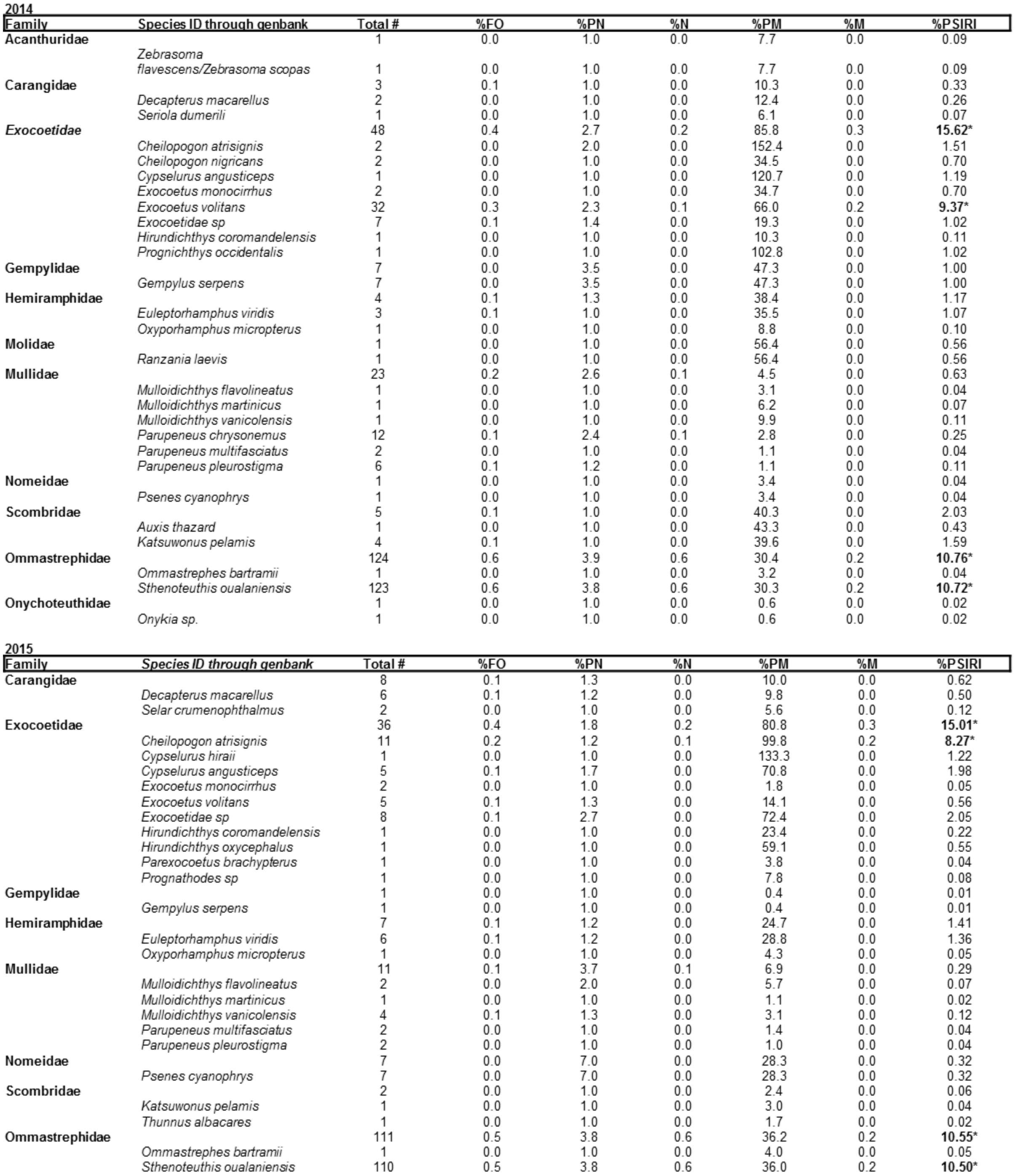
YEAR	CLASS	C1	C2	I2	I3	TOTAL
2014	S	54	38	38	57	187
	F	10	7	78	137	232
2015	S	84	43	57	88	272
	F	3	16	64	275	358
Total	S+F	151	104	237	557	1049

Table 4. Percent Prey Specific Index of Relative Importance (%PSIRI) of RFBO prey classes (fish, squid, mush) over 2 study years (2014 and 2015). The %PSIRI values integrate percent frequency of occurrence (%FO), percent average prey number (%PN) and percent average prey mass (%PM).



		%FO	%PN	%PM	%PSIRI
2014	**FISH**	1.0	4.4	60.2	32.3
	SQUID	0.7	5.1	36.4	15.1
	MUSH	0.4	0.4	2.1	0.5
2015	**FISH**	1.0	5.9	56.1	29.9
	SQUID	0.7	6.6	53.2	22.3
	MUSH	0.5	0.5	0.9	0.4

Table 5. Species list of 401 genetically identified prey items from 106 RFBO regurgitations, showing the number, frequency of occurrence (FO), (%PN) and prey-specific percent mass, percent number (%N), prey-specific percent number (%PM) percent mass (%M), and Percent Prey Specific Index of Relative Importance (%PSIRI). **Bolded** and starred (*****) %PSIRI indicate highly important prey families and species, with PSIRI values > 8% since most species were below 3%.



2014

Family	Species ID through genbank	Total #	%FO	%PN	%N	%PM	%M	%PSIRI
Acanthuridae		1	0.0	1.0	0.0	7.7	0.0	0.09
	Zebrasoma flavescens/Zebrasoma scopas	1	0.0	1.0	0.0	7.7	0.0	0.09
Carangidae		3	0.1	1.0	0.0	10.3	0.0	0.33
	Decapterus macarellus	2	0.0	1.0	0.0	12.4	0.0	0.26
	Seriola dumerili	1	0.0	1.0	0.0	6.1	0.0	0.07
Exocoetidae		48	0.4	2.7	0.2	85.8	0.3	**15.62***
	Cheilopogon atrisignis	2	0.0	2.0	0.0	152.4	0.0	1.51
	Cheilopogon nigricans	2	0.0	1.0	0.0	34.5	0.0	0.70
	Cypselurus angusticeps	1	0.0	1.0	0.0	120.7	0.0	1.19
	Exocoetus monocirrhus	2	0.0	1.0	0.0	34.7	0.0	0.70
	Exocoetus volitans	32	0.3	2.3	0.1	66.0	0.2	**9.37***
	Exocoetidae sp	7	0.1	1.4	0.0	19.3	0.0	1.02
	Hirundichthys coromandelensis	1	0.0	1.0	0.0	10.3	0.0	0.11
	Prognichthys occidentalis	1	0.0	1.0	0.0	102.8	0.0	1.02
Gempylidae		7	0.0	3.5	0.0	47.3	0.0	1.00
	Gempylus serpens	7	0.0	3.5	0.0	47.3	0.0	1.00
Hemiramphidae		4	0.1	1.3	0.0	38.4	0.0	1.17
	Euleptorhamphus viridis	3	0.1	1.0	0.0	35.5	0.0	1.07
	Oxyporhamphus micropterus	1	0.0	1.0	0.0	8.8	0.0	0.10
Molidae		1	0.0	1.0	0.0	56.4	0.0	0.56
	Ranzania laevis	1	0.0	1.0	0.0	56.4	0.0	0.56
Mullidae		23	0.2	2.6	0.1	4.5	0.0	0.63
	Mulloidichthys flavolineatus	1	0.0	1.0	0.0	3.1	0.0	0.04
	Mulloidichthys martinicus	1	0.0	1.0	0.0	6.2	0.0	0.07
	Mulloidichthys vanicolensis	1	0.0	1.0	0.0	9.9	0.0	0.11
	Parupeneus chrysonemus	12	0.1	2.4	0.1	2.8	0.0	0.25
	Parupeneus multifasciatus	2	0.0	1.0	0.0	1.1	0.0	0.04
	Parupeneus pleurostigma	6	0.1	1.2	0.0	1.1	0.0	0.11
Nomeidae		1	0.0	1.0	0.0	3.4	0.0	0.04
	Psenes cyanophrys	1	0.0	1.0	0.0	3.4	0.0	0.04
Scombridae		5	0.1	1.0	0.0	40.3	0.0	2.03
	Auxis thazard	1	0.0	1.0	0.0	43.3	0.0	0.43
	Katsuwonus pelamis	4	0.1	1.0	0.0	39.6	0.0	1.59
Ommastrephidae		124	0.6	3.9	0.6	30.4	0.2	**10.76***
	Ommastrephes bartramii	1	0.0	1.0	0.0	3.2	0.0	0.04
	Sthenoteuthis oualaniensis	123	0.6	3.8	0.6	30.3	0.2	**10.72***
Onychoteuthidae		1	0.0	1.0	0.0	0.6	0.0	0.02
	Onykia sp.	1	0.0	1.0	0.0	0.6	0.0	0.02

2015

Family	*Species ID through genbank*	Total #	%FO	%PN	%N	%PM	%M	%PSIRI
Carangidae		8	0.1	1.3	0.0	10.0	0.0	0.62
	Decapterus macarellus	6	0.1	1.2	0.0	9.8	0.0	0.50
	Selar crumenophthalmus	2	0.0	1.0	0.0	5.6	0.0	0.12
Exocoetidae		36	0.4	1.8	0.2	80.8	0.3	**15.01***
	Cheilopogon atrisignis	11	0.2	1.2	0.1	99.8	0.2	**8.27***
	Cypselurus hiraii	1	0.0	1.0	0.0	133.3	0.0	1.22
	Cypselurus angusticeps	5	0.1	1.7	0.0	70.8	0.0	1.98
	Exocoetus monocirrhus	2	0.0	1.0	0.0	1.8	0.0	0.05
	Exocoetus volitans	5	0.1	1.3	0.0	14.1	0.0	0.56
	Exocoetidae sp	8	0.1	2.7	0.0	72.4	0.0	2.05
	Hirundichthys coromandelensis	1	0.0	1.0	0.0	23.4	0.0	0.22
	Hirundichthys oxycephalus	1	0.0	1.0	0.0	59.1	0.0	0.55
	Parexocoetus brachypterus	1	0.0	1.0	0.0	3.8	0.0	0.04
	Prognathodes sp	1	0.0	1.0	0.0	7.8	0.0	0.08
Gempylidae		1	0.0	1.0	0.0	0.4	0.0	0.01
	Gempylus serpens	1	0.0	1.0	0.0	0.4	0.0	0.01
Hemiramphidae		7	0.1	1.2	0.0	24.7	0.0	1.41
	Euleptorhamphus viridis	6	0.1	1.2	0.0	28.8	0.0	1.36
	Oxyporhamphus micropterus	1	0.0	1.0	0.0	4.3	0.0	0.05
Mullidae		11	0.1	3.7	0.1	6.9	0.0	0.29
	Mulloidichthys flavolineatus	2	0.0	2.0	0.0	5.7	0.0	0.07
	Mulloidichthys martinicus	1	0.0	1.0	0.0	1.1	0.0	0.02
	Mulloidichthys vanicolensis	4	0.1	1.3	0.0	3.1	0.0	0.12
	Parupeneus multifasciatus	2	0.0	1.0	0.0	1.4	0.0	0.04
	Parupeneus pleurostigma	2	0.0	1.0	0.0	1.0	0.0	0.04
Nomeidae		7	0.0	7.0	0.0	28.3	0.0	0.32
	Psenes cyanophrys	7	0.0	7.0	0.0	28.3	0.0	0.32
Scombridae		2	0.0	1.0	0.0	2.4	0.0	0.06
	Katsuwonus pelamis	1	0.0	1.0	0.0	3.0	0.0	0.04
	Thunnus albacares	1	0.0	1.0	0.0	1.7	0.0	0.02
Ommastrephidae		111	0.5	3.8	0.6	36.2	0.2	**10.55***
	Ommastrephes bartramii	1	0.0	1.0	0.0	4.0	0.0	0.05
	Sthenoteuthis oualaniensis	110	0.5	3.8	0.6	36.0	0.2	**10.50***

Table 6. Simpson's Diversity Index (D) for prey in different groups of RFBO diet samples.

Group	# Species	Simpson's Diversity Index (D)
Colony	32	0.65
2014	27	0.66
2015	25	0.63
Females	18	0.72
Males	28	0.58
2014 Females	9	0.56
2014 Males	21	0.75
2015 Females	14	0.85
2015 Males	15	0.46

Table 7. Comparison of average female and male morphometric measurements and prey classes using the Sexual Dimorphic Index (SDI) (Angel et al. 2015), whereby positive (negative) values indicate that females have larger (smaller) values than males. Samples sizes are shown in parentheses.

Morphometrics	**2014 (n=26)**			**2015 (n=30)**		
	F (n=9)	**M (n=17)**	**SDI (%)**	**F (n=14)**	**M (n=16)**	**SDI (%)**
Mass (g)	1131.7 +/- 83.7	983.8 +/- 70.2	13.1	1084.6 +/- 63.2	938.4 +/- 63.1	13.5
Culmen (mm)	85.0 +/- 2.2	81.7 +/- 2.9	3.9	84.5 +/- 3.9	81.3 +/- 2.0	3.8
Wing chord (mm)	402.1 +/- 8.6	399.2 +/- 7.3	0.7	405.1 +/- 13.3	392.9 +/- 8.3	3.0
Diet	**n=11**	**n=23**	**SDI (%)**	**n=18**	**n=30**	**SDI (%)**
Fish (g)	69.1 +/- 53.7	58.7 +/- 56.0	15.0	87.1 +/- 71.3	35.6 +/- 48.5	15.8
Squid (g)	33.8 +/- 39.2	29.4 +/- 30.0	13.1	18.1 +/- 32.4	52.7 +/- 55.6	59.2
Total Mass (g)	106.5 +/- 68.1	90.8 +/- 59.9	14.7	105.3 +/- 70.7	88.7 +/- 64.2	-191.4

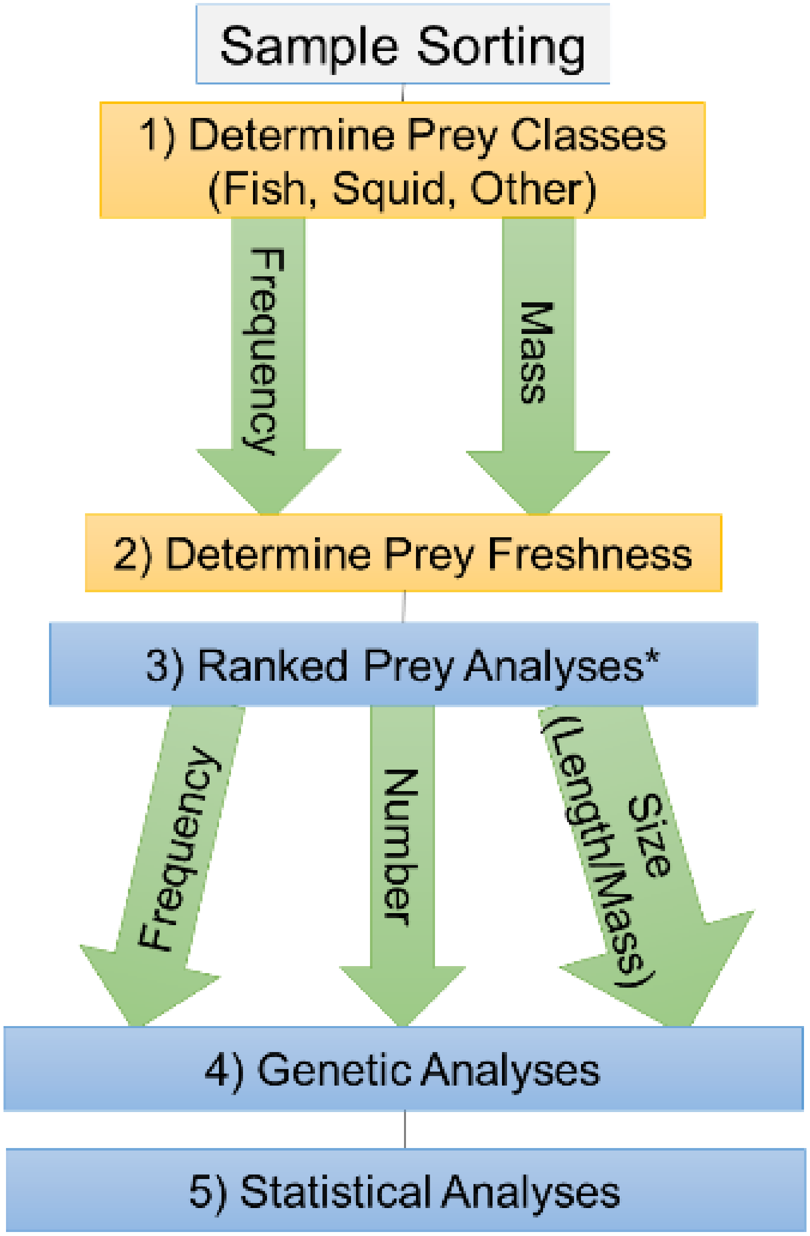
Table 8. RFBO size measurements from different geographic locations and sampling periods. Bird sample sizes were recorded differently and are presented as a single number in parentheses (n).

			Size Metric (Mean +/- SD)			
Location	Sample period	Sex	Mass	Culmen	Wing	Reference
O`ahu, HI	June-July 2014, 2015	Males (32)	960.5 +/- 70.4	81.3 +/- 2.3	396.3 +/- 8.4	Present study
		Females (23)	1103.0 +/- 73.4	84.7 +/- 3.3	404.0 +/- 11.5	
Palmyra Atoll	May-Oct 2007 Sept-Nov 2008	Males	839 +/- 44	8.2 +/- 0.4	3.7 +/- 0.2	Young et al. 2010
		Females	954 +/- 41	8.4 +/- 0.2	3.6 +/- 0.1	
Johnston Atoll	March 2003	Males (16)	1035 +/- 70	81 +/- 3	405 +/- 10	Lewis et al. 2005
		Females (15)	1182 +/- 86	84 +/- 4	417 +/- 11	
Christmas Island	Feb 1963- June 1964	Males	825 (700-1050)	82 (75-86)	-	Shrieber & Hensley 1976
		Females	1025 (850-1200)	76 (71-82)	-	
Laysan, NWHI	April - August 1978 -1981	Both	1110 +/- 12 SE (905-1400) (80)	83.8 +/- 0.10 SE (10)	-	Harrison et al. 1983
Europa Island	August- September 2003	Males	891 +/- 64.4 (34)	81.8 +/- 3.3 (33)	1467 +/- 24 (15)	Weimerskirch et al. 2006
		Females	1020.4 +/- 50.1 (46)	84.8 +/- 2.6 (43)	1499 +/- 33 (22)	

Table 9. Classification of the 32 identified prey species into four functional groups (reef-associated, pelagic-oceanic, flyingfishes, squids) defined based on taxonomic and habitat characteristics from Fishbase (www.fishbase.org).

Species	Reef-associated	Pelagic-oceanic	Flying fishes	Squids
Zebrasoma flavescens	*			
Decapterus macarellus		*		
Selar crumenophthalmus	*			
Seriola dumerili	*			
Cheilopogon atrisignis			*	
Cheilopogon nigricans			*	
Cypselurus angusticeps			*	
Cypselurus hiraii			*	
Exocoetus monocirrhus			*	
Exocoetus volitans			*	
Exocoetidae sp			*	
Hirundichthys coromandelensis			*	
Hirundichthys oxycephalus			*	
Parexocoetus brachypterus			*	
Prognichthys occidentalis			*	
Gempylus serpens		*		
Euleptorhamphus viridis		*		
Oxyporhamphus micropterus		*		
Ranzania laevis		*		
Mulloidichthys flavolineatus		*		
Mulloidichthys martinicus		*		
Mulloidichthys vanicolensis		*		
Parupeneus chrysonemus		*		
Parupeneus multifasciatus		*		
Parupeneus pleurostigma		*		
Psenes cyanophrys		*		
Auxis thazard		*		
Katsuwonus pelamis		*		
Thunnus albacares		*		
Ommastrephes bartramii				*
Sthenoteuthis oualaniensis				*
Onykia sp.				*

Figure 1. Flow chart illustrating the sequential steps of diet analysis.



*Rank of 3 may not have enough tissue or important hard parts present for identification; only mass recorded

Figure 2. Average (+/- S.D.) monthly Multivariate ENSO Index (MEI) (A) and monthly Pacific Decadal Oscillation (PDO) (B) values before and during the sampling period (January -July) of the two study years (2014 and 2015).

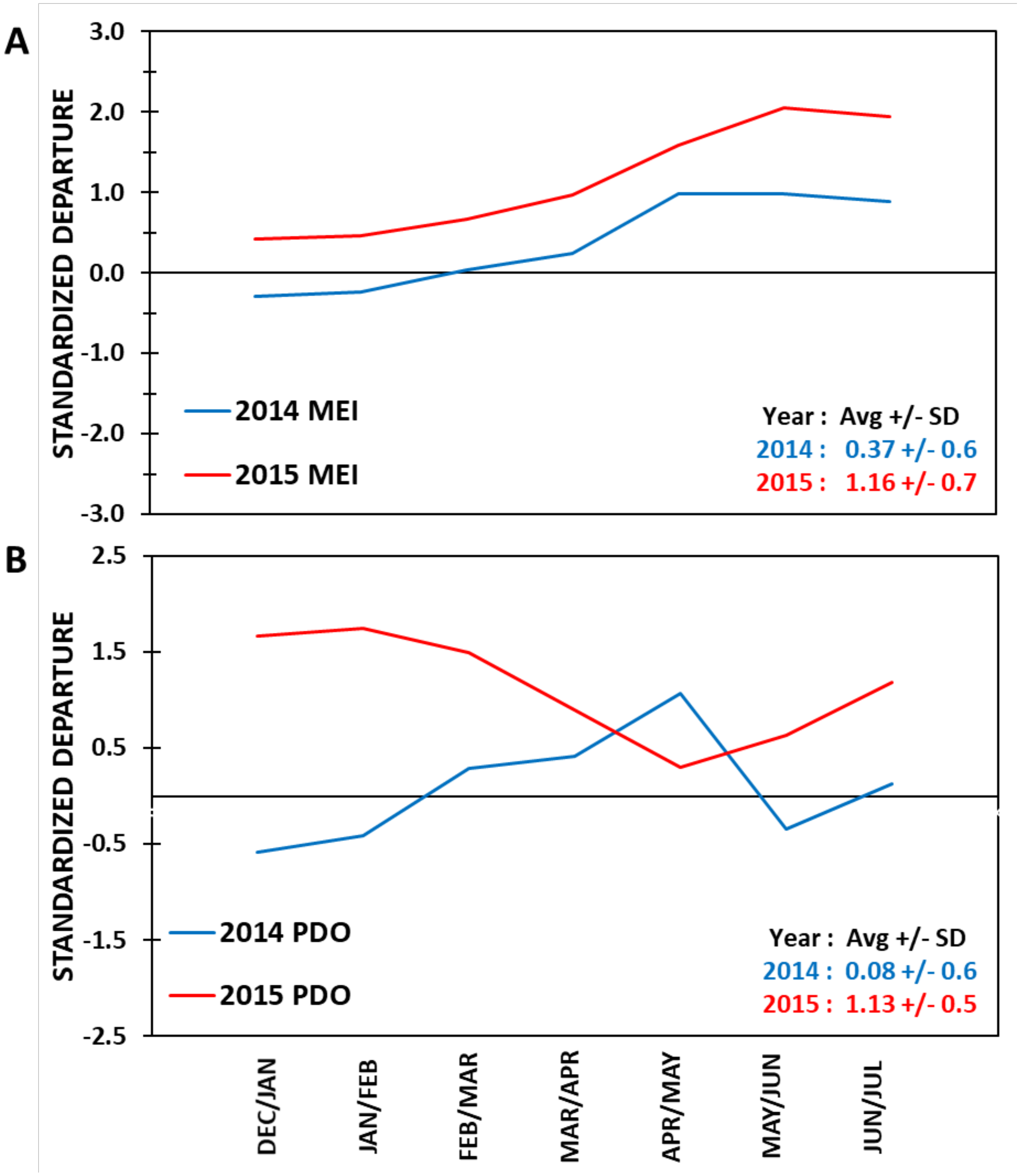
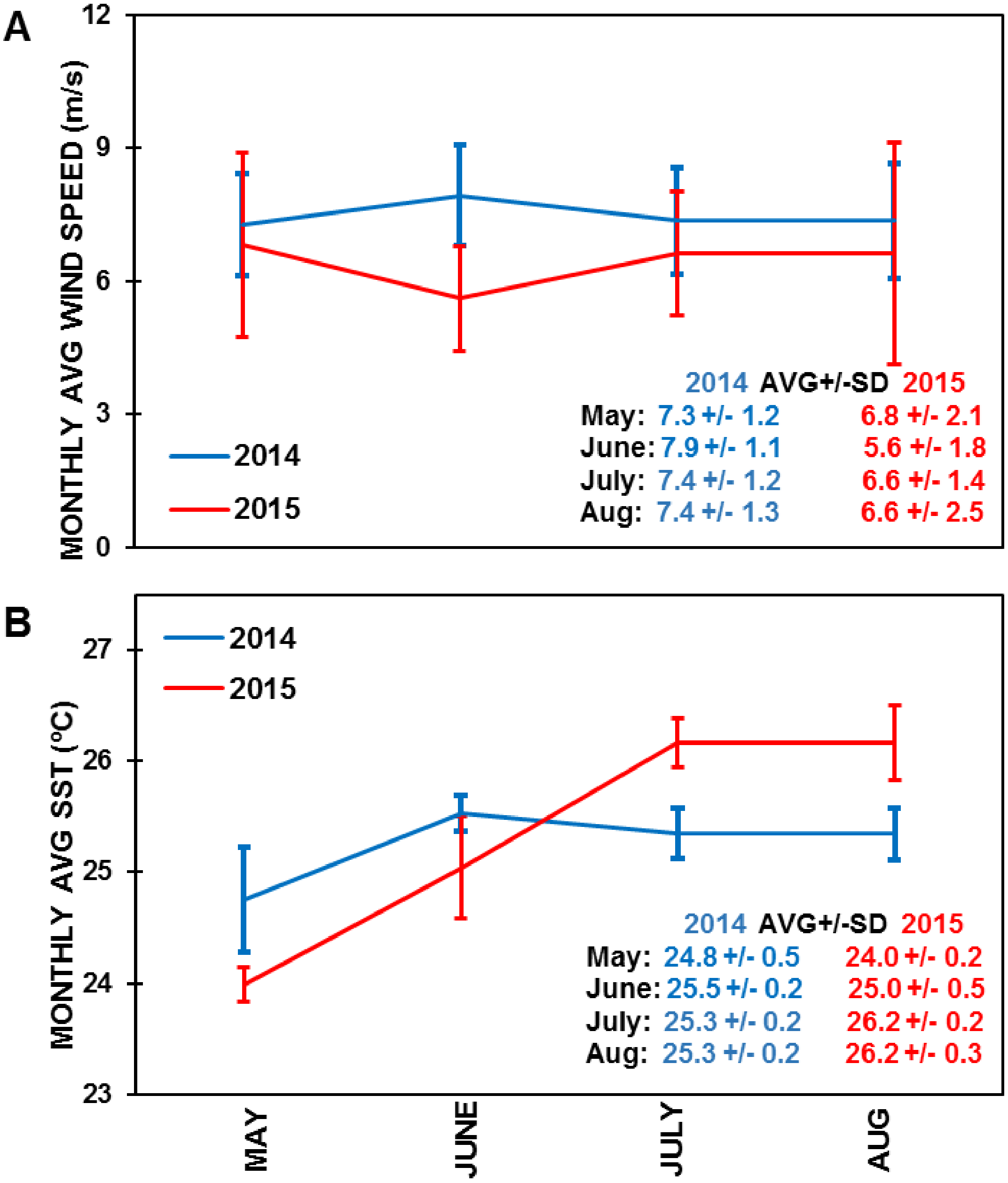


Figure 3. Average (+/- S.D.) monthly wind speed (m/s) (A) and Sea Surface Temperature (SST, ºC) (B) during the RFBO breeding season of the two tracking years (2014 and 2015, May 1 through August 31).



	2014 AVG+/-SD	2015
May:	7.3 +/- 1.2	6.8 +/- 2.1
June:	7.9 +/- 1.1	5.6 +/- 1.8
July:	7.4 +/- 1.2	6.6 +/- 1.4
Aug:	7.4 +/- 1.3	6.6 +/- 2.5

	2014 AVG+/-SD	2015
May:	24.8 +/- 0.5	24.0 +/- 0.2
June:	25.5 +/- 0.2	25.0 +/- 0.5
July:	25.3 +/- 0.2	26.2 +/- 0.2
Aug:	25.3 +/- 0.2	26.2 +/- 0.3

Figure 4. Regression of the significant positive relationship between Weighted Average Freshness (WAF, 1: fresh- 3:highly digested) and Total Mass (g) of the diet samples collected from RFBO at MCBH in 2014 and 2015.

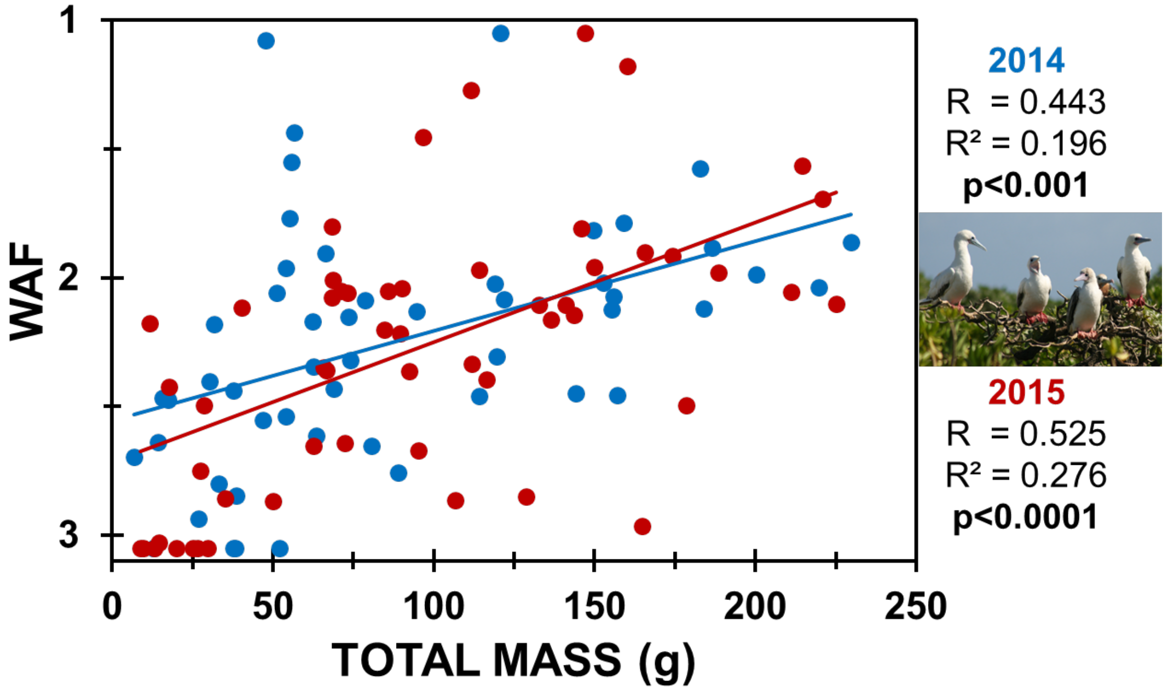


Figure 5. Boxplots of percent of total mass by prey class (fish, squid, mush) consumed by RFBOs in 2014 and 2015.

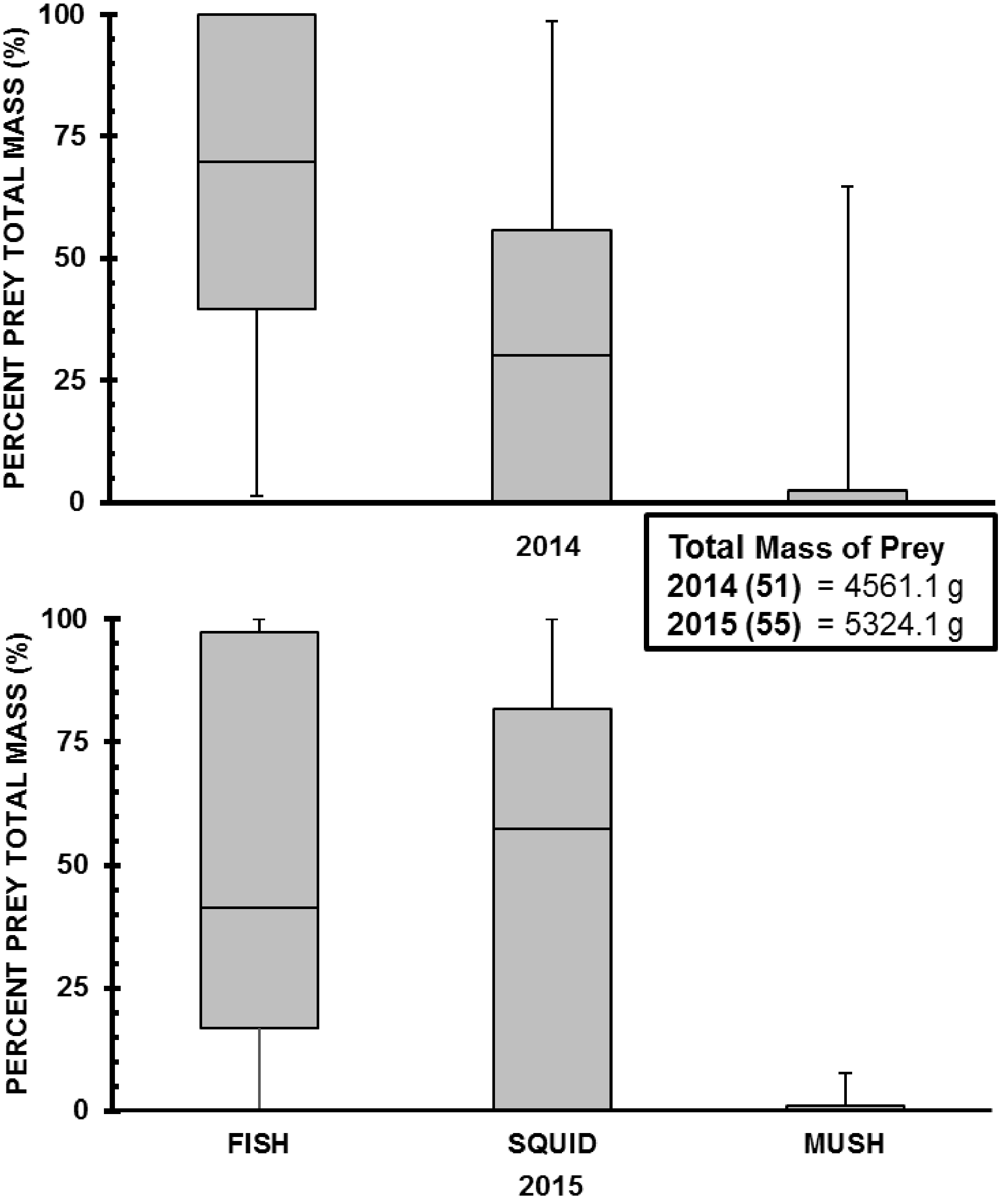


Figure 6. Histogram of squid mantle lengths consumed by RFBO over the two study years (2014 and 2015). Squid were significantly larger in 2015 than in 2014 (ANOVA, F=17.658, df =1, 199 p < 0.0001).

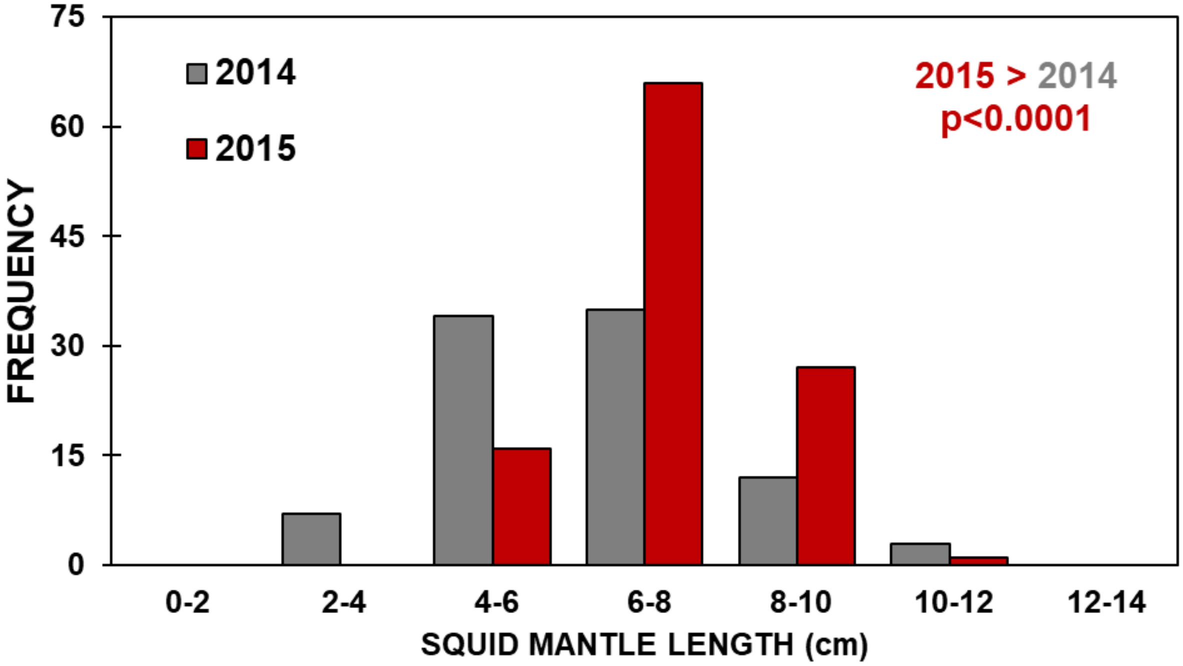
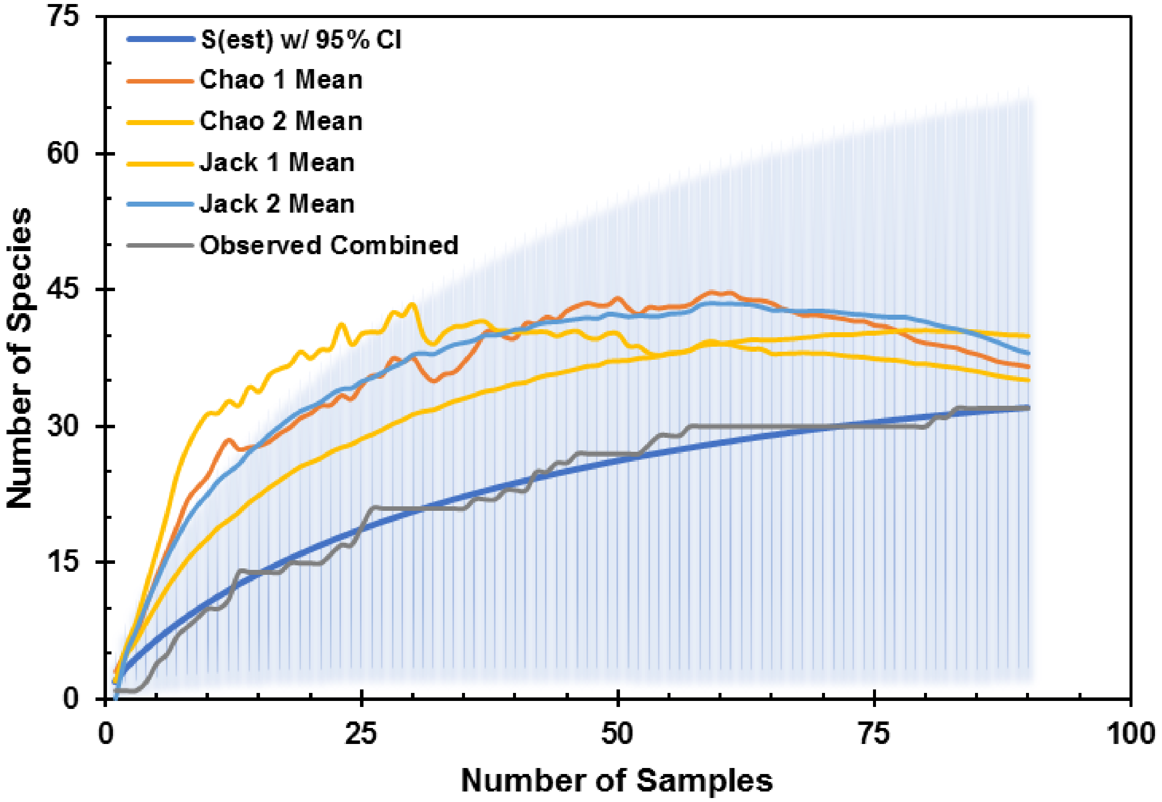


Figure 7. Rarefaction curves derived from 90 identified prey items collected over the two study years (2014 and 2015), showing the combined observed number of species (32), the 95% estimated confidence interval (CI), and four estimates: Chao1, Chao2, Jack1, and Jack2.



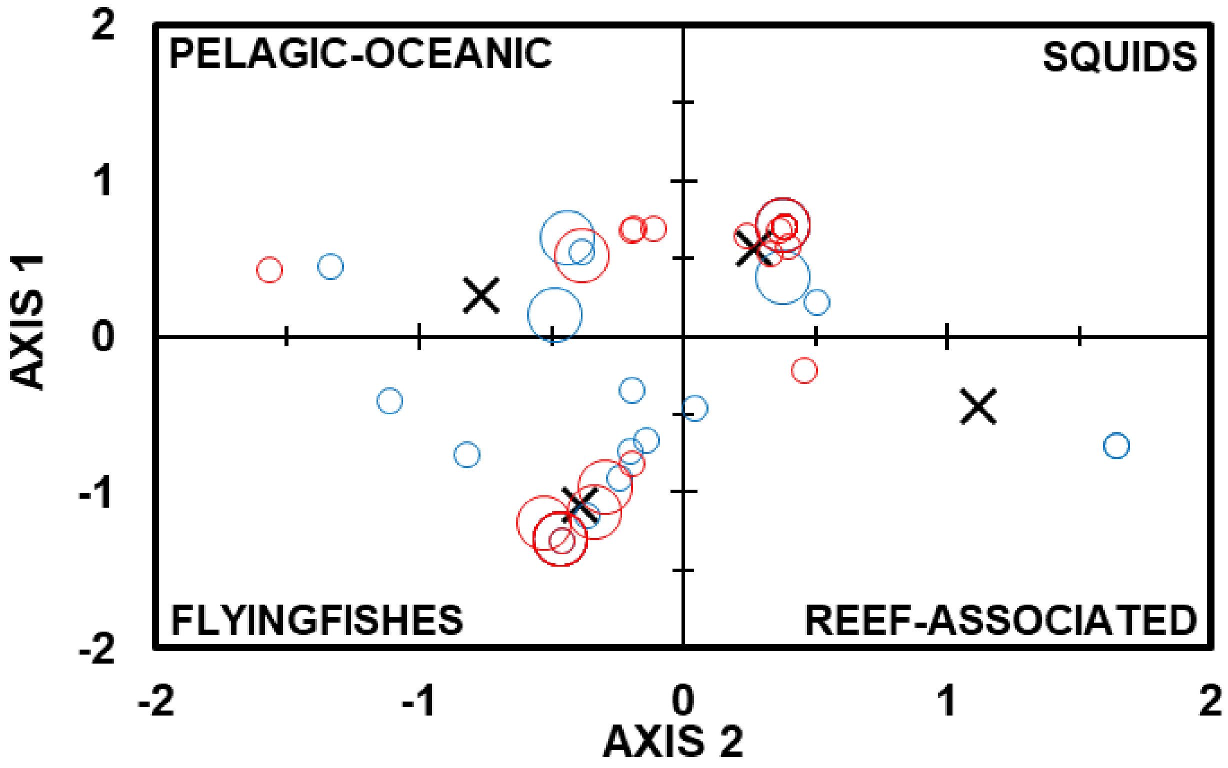
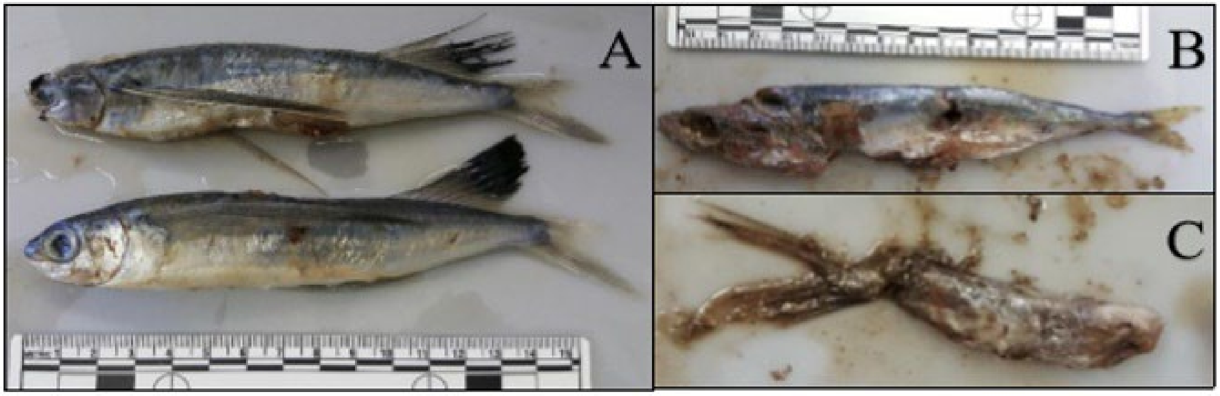


Figure 8. NMDS and MRPP relationships showing the 2-dimensional relationship between prey functional groups where each quadrant resembles one of four prey functional groups (flyingfishes, squids, pelagic-oceanic fishes, reef-associated fishes) where “X’s” are the coordinates of each prey functional group and circles are the individual regurgitations by male (small) and female (big) for the two years, 2014 (blue) and 2015 (red).

APPENDICES

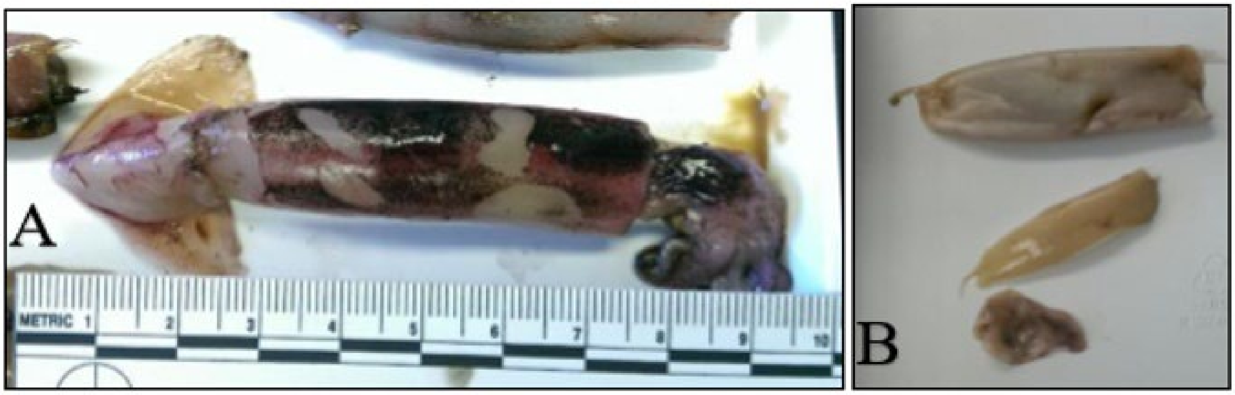
Appendix 1. Sorting and Freshness Methods.

A1a.



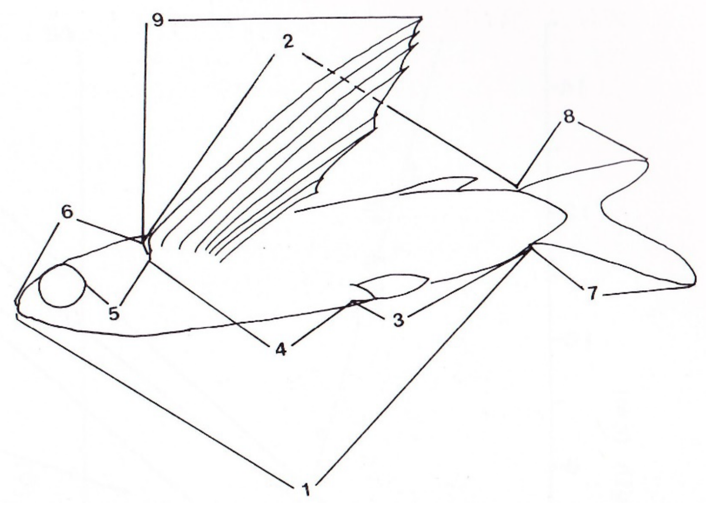
Fish examples of the freshness scale (Ashmole & Ashmole 1967) based on photos taken of prey samples collected from Red-footed boobies nesting at Ulupa`u Crater, Marine Corps Base, Oahu, HI, July 2015. **A:** Top prey item Parexocoetus brachypterus with a freshness rank of 2, Bottom prey item of the same species with a freshness rank of 1. **B:** A more digested representation of a rank of 2. **C.** Fish with the rank of 3.

A1b.



Squid examples of the freshness scale (Ashmole & Ashmole 1967) based on the photos taken of prey items collected from Red-footed boobies nesting at Ulupa`u Crater, Marine Corps Base, Oahu, HI, June 2014. **A:** Sthenoteuthis oualaniensis with a freshness rank of 1. **B:** Top squid mantle is classified as a rank of 2 while the middle and bottom pieces are considered a 3.

A1c.



Morphometric measurements taken from flyingfish: 1 – standard length, 2 – base of pectoral fin to base of lower caudal fin, 3 – base of pelvic fin to base of lower caudal fin, 4 – base of pectoral fin to base of pelvic fin, 5 – shortest length between eye and base of pelvic fin, 7 – length of lower caudal fin, 8 – length of upper caudal fin, 9 – length og pectoral fin (from Diamond 1983).

Appendix 2. Genetic Methods

A2a. Table of possible prey species found around the Main Hawaiian Islands.

FAMILY	Genus species	Common Name	Sequences Available	Location	Seki Species
Ammodytidae	*Bleekeria gillii*	Pyle's sand lance	NA	Eastern Central Pacific: Molakai to the Ladd Seamount, Hawaii.	
Belonidae	*Tylosurus acus melanotus*	Keel-jawed Needlefish	12S	Indo-Pacific: East Africa (Ref. 3130) to the central and south Pacific. Eastern Central Pacific: oceanic islands such as the Revillagigedo, Clipperton and Cocos (Ref. 9279).	
	Tylosurus acus pacificus	Pacific agujon Needlefish	12S, 16S, Cytb	Eastern Pacific: Gulf of California to Cabo Blanco, Peru; including the Galapagos Islands.	
	Strongylura anastomella	Pacific Needlefish	16S, Cytb, Mito	Northwest Pacific: Japan to Taiwan.	
	Tylosurus crocodilus	Hound Needlefish	COI, 12S, 16S, Cytb	Indo-West Pacific: Red Sea and South Africa (Ref. 5317) and Persian Gulf (Ref. 68964) to French Polynesia (Ref.6784), north to Japan (Ref. 559), south to New South Wales, Australia (Ref. 33390). Replaced by Tylosurus crocodilus fodiator in the eastern Pacific. Western Atlantic: New Jersey, USA to Brazil (Ref. 7251). Eastern Atlantic: Fernando Poo, Cameroon, Liberia, and Ascension Island (Ref. 5757); from Senegal and Guinea (Ref.28587); and Cape Verde (Ref. 27000). Recorded from the Mediterranean Sea (Ref. 83387).	
	Ablennes hians	Flat Needlefish	COI, 12S, 16S, Cytb, Mito	Worldwide distribution in tropical and warm temperate waters. Eastern Atlantic: Cape Verde and Dakar to Moçamedes, Angola (Ref. 5757); ranging further south to Namibia (Ref. 12484). Western Atlantic: Chesapeake Bay (USA), Bermuda, and northern Gulf of Mexico to Brazil (Ref. 7251). Throughout the Indian Ocean. Western Pacific: off coasts and islands from southern Japan through the East Indies to Australia (Ref. 9682). Reported from Tuvalu (Ref. 12690). Eastern Pacific: Mexico to Peru. Entire Caribbean, Antilles and South American coast to Rio de Janeiro (Ref. 26938).	
	Tylosurus fodiator	Mexican Needlefish	NA	Eastern Central Pacific: Gulf of California to Panama, including Cocos Island.	
Carangidae	*Selar crumenophthalmus*	Bigeye scad	COI, 12, 16S, Cytb, Mito, halotype 1-61	Circumtropical. Indo-Pacific: East Africa (Ref. 3287) to Rapa, north to southern Japan and the Hawaiian Islands, south to New Caledonia. Eastern Pacific: Mexico to Peru, including the Galapagos Islands (Ref. 5530). Western Atlantic: Nova Scotia, Canada and Bermuda through the Gulf of Mexico and the Caribbean to to São Paulo (Ref.47377), Brazil. Eastern Atlantic: Cape Verde to southern Angola (Ref. 7097).	
	Decapterus maraudsi	Japanese Scad	COI, 12S, 16S, 18S, Cytb, Mito, H1-28	Indo-West Pacific: South China Sea to the Mariana Islands. Reported occurrence in southern Africa could not be confirmed (Ref. 3197).	
	Elagatis bipinnulata	Rainbow runner	COI, 12S, 16S, Cytb	Western Atlantic: Massachusetts, USA and northern Gulf of Mexico to Rio de Janeiro, Brazil (Ref. 57756). Eastern Atlantic: off Genoa, Italy in the Mediterranean (Ref. 4233) and from Côte d'Ivoire to Angola (Ref. 7097). Throughout the Indo-Pacific (Ref. 37816), but rare or absent in the Persian Gulf (Ref. 3287). Eastern Pacific: mouth of Gulf of California to Ecuador; including the Galapagos Islands (Ref. 9283).	
	Seriola dumerili	Greater amberjack	COI, 12S, 16S, Cytb, Mito, Microsat 1-46	Circumglobal. Indo-West Pacific: South Africa, Persian Gulf, southern Japan and the Hawaiian Islands, south to New Caledonia; Mariana and Caroline islands in Micronesia. Western Atlantic: Bermuda (Ref. 26938), Nova Scotia, Canada to Brazil; also from the Gulf of Mexico and the Caribbean Sea (Ref. 9626). Eastern Atlantic: British coast (vagrant) to Morocco and the Mediterranean. Distribution in eastern central Atlantic along the African coast is not well established due to past confusion with Seriola carpenteri (Ref. 7097).	
	Decapterus macarellus	Mackerel Scad	COI, 12S, 16S, Cytb, Mito	Circumglobal. Western Atlantic: Nova Scotia, Canada and Bermuda to approximately Rio de Janeiro, Brazil (Ref.57756). Appears to be absent from the Gulf of Mexico (Ref. 9626). Eastern Atlantic: St. Helena, Ascension, Cape Verde, and Gulf of Guinea (Ref. 7097); Azores and Madeira (Ref. 4233). Indian Ocean: Red Sea, Gulf of Aden, Seychelles, Mascarenes, South Africa, and Sri Lanka (Ref. 3287). Eastern Pacific: Gulf of California and Revillagigedo Island to Ecuador (Ref. 9283).	
	Decapterus macrosoma	Shortfin Scad	COI, 12S, Cytb, Mito	Indo-West Pacific: East Africa (no verified records from the Red Sea) to Malaysia, Indonesia and the Arafura Sea. Eastern Pacific: Gulf of California to Peru, including the Galapagos Islands (Ref. 9283).	
	Decapterus muroadsi	Amberstripe Scad	COI, 12S, 16S, Cytb	Indo-Pacific: Japan and northwestern Hawaiian Is., east and west coast of Australia, Rapa and Easter Is.; and from the Gulf of California to Peru.	
	Decapterus tabl	Roughear Scad	12S, Cytb	Pacific Ocean: Japan, Indonesia (Ref. 3197), Australia and the Hawaiian Islands Western Atlantic: Canada (Ref.5951) to North Carolina, USA to Venezuela (Ref. 7251). Also found in Brazil (Ref. 47377) and in Argentina (Ref.2806). Eastern Atlantic: St. Helena Island (Ref. 7097). Indian Ocean: off Kenya (Ref. 3287) to off East London, South Africa (Ref. 11228).	
	Atule mate	Yellowtail Scad	COI, 12S, 16S, Cytb, Mito	Indo-Pacific: Red Sea and the east coast of Africa to the Hawaiian Islands and Samoa, north to Japan (Ref. 559), south to the Arafura Sea (Ref. 9819) and northern Australia.	
	Trachurus symmetricus	Pacific Jack Mackerel	COI, 12S, 16S, Cytb	Eastern Pacific: southeastern Alaska to southern Baja California, Mexico and the Gulf of California; reported from Acapulco in Mexico and the Galapagos Islands.	
	Seriola lalandi	Yellowtail Amberjack	COI, 12S, 16S, Ctyb, Mito	Circumglobal in subtropical waters: Series of disjunct populations. Indo-Pacific: South Africa, Walter Shoals, Amsterdam Island, Japan, Australia, New Zealand, New Caledonia, Hawaii, Rapa, Pitcairn Island, and Easter Island. Eastern Pacific: British Columbia, Canada to Chile (Ref. 2850), including Desventuradas Is. and Juan Fernández Is. (Ref. 89357). Eastern Atlantic: St. Helena, South Africa (Ref. 7097).	
	Seriola peruana	Fortune Jack	NA	Eastern Pacific: Mexico to Ecuador and the Galapagos Islands.	
	Seriola quinqueradiata	Japanese Amberjack	NA	Northwest Pacific: Japan and the eastern Korean Peninsula to the Hawaiian Islands.	
Chanidae	*Chanos chanos*	Milkfish	COI, 12S, 16S, 18S, Cytb, Mito	Indo-Pacific: along continental shelves and around islands, where temperatures are greater than 20°C. Red Sea and South Africa to Hawaii and the Marquesas, north to Japan, south to Victoria, Australia. Eastern Pacific: San Pedro, California to the Galapagos.	
Coryphaenidae	*Coryphaena hippurus*	Dolphinfish	COI, 12S, 16S, Cytb	Atlantic, Indian and Pacific: in tropical and subtropical waters. Highly migratory species, Annex I of the 1982 Convention on the Law of the Sea (Ref. 26139).	
	Coryphaena equiselis	Pompano Dolphinfish	COI, 12S, 16S, 18S, Cytb	Worldwide in tropical and subtropical seas. Frequently misidentified as juvenile or female of Coryphaena hippurus. Highly migratory species, Annex I of the 1982 Convention on the Law of the Sea (Ref. 26139).	
Engraulidae	*Stolrphorus buccaneeri (Encrasicholina punctifer)*	Buccaneer anchovy	COI, 12S, Cytb, Mito	Indo-Pacific: Red Sea and Persian Gulf south to perhaps Durban in South Africa, Pakistan, India, Sri Lanka, and probably Burma. Also from southern Japan to China, Philippines, Thailand, Indonesia south to Brisbane, Australia, Japan eastward to Hawaii, the Solomon Islands, Fiji, Samoa and Tahiti.	
Exocoetidae	*Exocoetus volitans*	Tropical Two-wing Flyingfish	12S, Cytb, Mito	Widespread in tropical and subtropical zones of all oceans including the western Mediterranean Sea and part of the Caribbean Sea (Ref. 3720), probably absent in the inland seas of southeastern Asia (Sulawesi Sea, Sulu Sea, Flores, Banda, Ceram and Halmahera seas (Ref. 27313)), and the Benguela Current (Ref. 4498). Eastern Pacific: Mexico to central Chile; also the Galapagos and Hawaii. Western Pacific: Japan, Marshall Islands, Philippines, Australia (Ref. 5530), and Tahiti.	
	Exocoetus obtusirostris	Oceanic Two-wing Flyingfish	Cytb	South Pacific: between 10°S and 32°S, from Australia to Ecuador (Ref. 5530) and Peru. Eastern Atlantic: South of Azores and Madeira to Angola, individual stragglers off Portugal and in the Mediterranean (Ref. 6523). Western Atlantic: off New Jersey and Florida to the northern Gulf of Mexico and Brazil (Ref. 7251). Uncommon in the Antilles (Ref. 51183). Conspecificity of the Atlantic and Pacific populations needs confirmation (Ref. 27313).	
	Parexocoetus brachypterus	Sailfin Flyingfish	16S	Indo-Pacific: widespread from East Africa, including the Red Sea (Ref. 26165) to southern Japan, Hawaii, the Marquesas, and Queensland (Australia); separate population in the eastern tropical Pacific (Bay of Panama). Western Atlantic: northeastern Florida, USA and the Bahamas to Brazil (Ref. 7251); common in Caribbean Sea and Lesser Antilles area; less common in the Gulf of Mexico, the Gulf Stream and the western Sargasso Sea (Ref.3720). Eastern Atlantic: Guinea to Angola (Ref. 4498).	
	Cypselurus (Cheilopogon) furcatus	Spotfin Flyingfish	COI	Widespread in tropical zones of all oceans. Western Atlantic: Massachusetts, USA to southern South America (Ref. 7251). Eastern Atlantic: equator eastward to 8°W, Cape Verde. Northwest Atlantic: Canada (Ref. 5951). Indian Ocean: widely distributed but absent from the Red Sea, northern Arabian Sea, and the Persian Gulf (Ref.3395). Western Pacific: absent in inland waters of southeast Asia (Ref. 9839). Eastern Central Pacific. The distinction between Cheilopogon antoncichi and Cheilopogon unicolor awaits clarification.	
	Cypselurus nigricans	Blacksail Flyingfish	COI	Indo-West Pacific: in tropical waters, south to the Mascarenes, Aldabra, and Natal, South Africa (Ref. 33390) and east to Japan, the Caroline and Solomon islands. Western Atlantic: 40°N to off Rio de Janeiro in Brazil. Eastern Atlantic: Gulf of Guinea and adjacent waters.	
	Hirundichthyes rondeletii	Blackwing Flyingfish	COI, 12S	Subtropical waters of all oceans. Eastern Atlantic: occasional to Spain and English Channel, western Mediterranean (a separate population migrates to the southeastern part in winter), Portugal to Mauritania and from south of Namibia; also off the Cape, South Africa (Ref. 2797). Western Atlantic: Massachusetts, USA and Bermuda to southern Brazil (Ref. 7251). Northwest Atlantic: Canada (Ref. 5951).	
	Cypselurus (Cheilopogon) spilonotopterus	Stained Flyingfish	COI, 12S, 16S, Cytb	Indo-Pacific to Eastern Pacific: Widely distributed in tropical waters, including the Ryukyu Islands (Ref. 559), Taiwan (Ref. 5193) and eastern Pacific (Ref. 9300).	
	Fodiator acutus	Sharpchin Flyingfish	COI, 16S	Northeast Pacific and Eastern Atlantic: USA; Dakar, Senegal to Luanda, Angola. Records from the western Atlantic (off Suriname and in the Caribbean Sea) seem doubtful.	
	Fodiator rostratus	Sharpchin Flyingfish	Cytb	Eastern Pacific: Baja California, Mexico and the Gulf of California to Peru, including the Clipperton and Galapagos Islands. Records from the Indo-West Pacific are misidentifications, e.g. of Parexocoetus mento (Ref.27313).	
	Hirundichthyes albimaculatus	Whitespot Flyingfish	Cytb	Pacific Ocean: widely distributed in the central Pacific, but lacking in the inland seas of southeastern Asia, in the Solomon Sea, and in the Coral Sea.	
	Hirundichthyes speculiger	Mirrorwing Flyingfish	Cytb	Circumglobal: all oceans preferably open ocean waters. Atlantic: northward from 37°N in the western part of the ocean to 25° N in the eastern part and southward from 30° S in the western part to 8° S in the eastern part; somewhat broader in the Eastern Atlantic. It is quite rare in the Caribbean (except for the eastern part) and in the Gulf of Mexico. Indian Ocean: Arabian Sea and Bay of Bengal in the north to the southern end of Africa and the western coast of Australia in the south; not found in the Red Sea, Aden, Oman and the Persian Gulfs, as well as in the easternmost part of the Indian Ocean in the waters between the southern Indonesian islands and the northern coast of Australia. Juveniles are distributed throughout the range of the species except in the most southwestern areas from the Mozambique Channel to the False Bay and in the waters off the west coast of Australia; juveniles were found in all seasons. Pacific Ocean: northward from the northwestern tip of the Honshu Island in the west to 20° N in the east, and southward from the east coast of Australia to the waters of Ecuador and Peru. Absent in the East China, Yellow and Japan Seas, quite rare in the South China Sea, none in the inner Indonesian seas.	
	Cypselurus atrisignis (Cheilopogon atrisignis)	Glider Flyingfish	Cytb, Mito	Indo-Pacific: Somalia and Madagascar to the Revillagigedo and Galapagos islands in the eastern Pacific. Absent from the inland seas of Indonesia and from Australian waters (Ref. 27313).	
	Hirundichthyes ilma	Coromandel Flyingfish	NA	Southwest Pacific: west of New Zealand.	
	Hirundichthyes marginatus	Banded Flyingfish	NA	Eastern Pacific: Mexico to Peru.	
	Hirundichthyes oxycephalus	Bony Flyingfish	NA	Indo-West Pacific: Arabian Sea to southern Japan, New Guinea and New South Wales, Australia.	
	Cypselurus spilopterus (Cheilopogon spilopterus)	Manyspotted Flyingfish	NA	Eastern Indian Ocean and Western Pacific: Andaman Sea to Samoa. Reported from the Ryukyu Islands (Ref. 559).	
Gempylidae	*Gempylus serpens*	Snake Mackerel	COI, 12S, 16S, Cytb	Worldwide in tropical and subtropical seas. Adults also often caught in temperate waters. Specimen caught on the Atlantic side of South Africa (33°08'S 16°47'E at 700 m) (Ref. 6193) probably strayed from the Indian Ocean.	
Gonostomatidae	*vinciguerria nimbaria*	Oceanic Lightfish	COI, 12S, 16S	Western Central Atlantic: Florida (USA), Cuba and the Antilles, including the Gulf of Mexico and the Caribbean (Ref. 26340). Eastern Atlantic: 42°N to the tropical region. Northwest Atlantic: Canada (Ref. 5951). Indo-Pacific. Southeast Pacific: Chile (Ref. 9068). South China Sea (Ref.74511).	
	Vinciguerria attenuata	Slender Lightfish	COI	Western Atlantic. Eastern Atlantic: Portugal south to Congo and South Africa, including the Mediterranean. Northwest Atlantic: Canada (Ref. 5951). Indian Ocean: off Inhaca Island, Mozambique (Ref. 3988). Southeast Pacific: Chile (Ref. 9068). South China Sea (Ref.74511)	
	Vinciguerria lucetia	Panama Lightfish	COI, 16S	Western Central Pacific: Papua New Guinea. Eastern Pacific: throughout the California Current region, usually south of Point Conception and seaward of shelf (Ref. 35839); also in Chile (Ref. 9068).	
	Vinciguerria poweriae	Power's Deep-water Bristle-mouth fish	COI, 16S	Circumglobal (Ref. 3988), subtropical waters (Ref.47377). Eastern Atlantic: Portugal to Cape Verde, including the eastern Mediterranean. Western Atlantic: Gulf of Mexico (Ref. 27768). Northwest Atlantic: Canada (Ref. 5951). Western Pacific: Australia (including Western Australia and Tasmania) (Ref. 7300) and New Zealand (Ref. 5755). Eastern Pacific: Seaward edge of the California Current region (Ref. 35839); also found in Chile (Ref. 9068). South China Sea (Ref.74511).	
Hemirhamphidae	*Euleptoramphus viridis*	Ribbon halfbeak	16S, Cytb	Indo-Pacific: Red Sea and East Africa to Hawaii (Ref. 583) and Tonga, north to southern Japan, south to Australia, New Zealand and Kermadec Islands (Ref. 8879). Eastern Pacific: southern California, USA to Ecuador, including the Galapagos Islands (Ref. 2850).	
	Hyporhamphus dussumieri	Dussumier's halfbeak	COI	Indo-Pacific: Seychelles (Ref. 2334) through the East Indies, Borneo, Philippines, and New Guinea north to Hong Kong and Okinawa and eastward as far as Tuamoto Islands.	
	Rhynchorhamphus georgii (Hyporhamphus georgi)	Long billed halfbeak	COI	Indo-West Pacific: the most widespread of the four species of Rhynchorhamphus, found from Persian Gulf through Arabian Sea and Bay of Bengal through the Western Central Pacific north to Taiwan and Hong Kong and east to New Guinea and northern Australia.	
	Hyporhamphus sajori	Japanese halfbeak	COI, 12S, 16S, Cytb, Mito, HYSA1-60	Northwest Pacific: Yellow Sea, Pohai Sea, Japan Sea, and the Pacific coast of Japan, extending north to Sakhalin and to Preobrazheniye Bight and Vladivostok in the Gulf of Peter the Great. Recorded from the lower part of the Yangtze River in China. Occurrence of this species south to the Ryukyu Islands and Taiwan needs verification.	
	Evolantia micropterus (Oxyporhamphus micropterus)	Bigwing halfbeak	COI, 16S, 12S	Indo-Pacific: East Africa to Tonga, north to southern Japan (Ref. 33390). Eastern Pacific: Mexico to Peru (Ref.9306). Replaced in the Atlantic by Oxyporhamphus similis.	
	Hyporhamphus quoyi	Quoy's halfbeak	COI, 16s, Cytb	Indo-West Pacific: in western Pacific, known from Thailand, the East Indies, Borneo, and the Philippines north to China and Nagasaki, Japan, south to New Guinea and northern half of Australia.	
	Hyporhamphus limbatus	Congaturi halfbeak	COI, Cytb	Indo-West Pacific: Persian Gulf to China along the mainland coast of Asia. Replaced in the East Indies, Borneo, and the Philippines by Hyporhamphus neglectus and around northern Australia and southern New Guinea byHyporhamphus neglectissimus.	
	Hyporhamphus intermedius	Asian pencil halfbeak	Mito	Northwest Pacific: Honshu, Japan and the northern part of the Yellow Sea south to southern China, including Taiwan. Reports from Ryukyu Islands do not seem to be supported by specimens and may represent misidentifications. However, all of Gushiken's Okinawa photographs (except the one labeled Hyporhamphus intermedius) seem to be correctly identified.	
	Hyporhamphus acutus	Pacific Halfbeak halfbeak	NA	Pacific Ocean: confined around oceanic islands, ranging from the Marshalls, Gilberts, and Tonga to the Marquesan and Easter islands	
	Hyporhamphus balinensis	Balinese garfish halfbeak	NA	Indo-Pacific: widespread from the Mozambique channel and southern part of the Red Sea through the Western Central Pacific east to Samoa.	
	Hyporhamphus gernaerti		NA	Northwest Pacific: restricted to the temperate zone in coastal waters of continental China (from Peitaho in Liaotung Bay to Hong Kong), Taiwan, and Korea (Fusan).	
	Hyporhamphus gilli	Choelo halfbeak	NA	Eastern Pacific: Mexico to Peru. Particularly common in Gulf of California and Panama Bay (Ref. 9306).	
	Hyporhamphus ihi		NA	Southwest Pacific: restricted to New Zealand, including the Chatham Islands.	
	Hyporhamphus melanopterus		NA	Western Pacific: confined to the Gulf of Thailand, Java Sea and Sulu Sea.	
	Hyporhamphus naos	Pacific silverstripe	NA	Eastern Pacific: from Baja California, with strays north to San Diego, CA, south to Paita, Peru and in the Galapagos Islands. (Named for the Naos Island, Bay of Panama, where the species is common and the site ot the Smithsonian Tropical Research Institute (STRI) Marine Laboratory.	
	Hyporhamphus neglectissimus	Black-tipped garfish	NA	Indo-West Pacific: replaces Hyporhamphus (Hyporhamphus) neglectus in waters around New Guinea and northern Australia.	
	Hyporhamphus pacificus	Acute halfbeak	NA	Eastern Central Pacific: Hawaiian Islands.	
	Hyporhamphus paucirastris		NA	Western Central Pacific: South China Sea basin. The locality (river Svetlaya) for the Viet Nam record (ZIL 8396) needs verification.	
	Hyporhamphus roberti roberti (kronei)	Slender halfbeak	NA	Western Central Atlantic: Venezuela to Brazil. Rhode Island to Florida (USA), Gulf of Mexico. Pacific Ocean.	
	Hyporhamphus rosae (patris)	California halfbeak	NA	Eastern Pacific: Santa Ana in southern California, USA to Costa Rica. Range extends to Panama (Ref. 36576). Reported from Peru and Galapagos Islands (Ref. 5530).	
	Hyporhamphus snyderi	Skipper halfbeak	NA	Eastern Pacific: Guatemala to Peru.	
	Hyporhamphus taiwanensis		NA	Asia: known only from the type specimens from Taiwan.	
	Hyporhamphus yuri		NA	Northwest Pacific: known only from Okinawa and the adjacent island of Ie. Reported from Taiwan (Ref. 5193).	
	Hyporhamphus neglectus		NA	Western Central Pacific: replaces the coastal Hyporhamphus (Hyporhamphus) limbatus in waters around Java, Sumatra, Borneo and the Philippines. Also known from the middle of the eastern coast of Queensland, Arnhem Land, Northern Territory, and the northern part of Western Australia, and from offshore islands (Ref. 10988).	
Holocentridae	*Sargocentron sp.*	Squirrelfishes			
	Sargocentron xantherythrum	Hawaiian squirrelfish	COI, Cytb	Eastern Central Pacific: known only from the Hawaiian Islands and Johnston Atoll.	
Istiophoridae		Marlins			
Kyphosidae	*Kyphosus bigibbus*	Brown chub	COI, 12S	Indo-Pacific: Antitropical; Red Sea, South Africa, western and eastern Australia, Lord Howe and Rapa islands, southern Japan and Ryukyu Islands. Records for countries in the tropical zone needs further confirmation.	
Macrorhamphosidae	*Macrohamphosus gracilis*	Slender snipefish	NA	Indo-Pacific and Western Central Atlantic: Indo-West-Pacific: Africa through the tropical Pacific to Hawaii and Desventuradas Is. and Juan Fernandez Is.	
Molidae	*Ranzania laevis*	Slender sunfish	COI, 12S, 16S, Cytb, Mito	Cosmopolitan. Western Atlantic: Florida (USA), Martinique, Venezuela (Ref. 51183), and Brazil. Eastern Atlantic: Madeira to Scandinavia (Ref. 4711); Dakar, Senegambie, and Sierra Leone (Ref. 6952); South Africa. Eastern Pacific: central California, USA to Chile; rare north of Mexico (Ref. 2850). Indian Ocean: Madagascar (Ref. 33390), Mauritius (Ref. 58078), Reunion (Ref. 53568), Ira, Australia. West Pacific: Japan, China, Taiwan, New Zealand.	
	Masturus lanceolatus	Sharptail mola	COI, 12S, 16S, Cytb, Mito	Circumglobal: In tropical to subtropical waters. Western Atlantic: North Carolina, USA to southeastern Brazi.	
Monacanthidae		Filefish			
Mullidae		Goatfish			
Myctophidae		Myctophids			
Nomeidae	Psenes cyanophrys	Freckled Driftfish	COI, 12S, 16S, Cytb, Mito	Atlantic, Indian and Pacific: tropical and warmer temperate waters. Western Atlantic: Massachusetts, USA and northern Gulf of Mexico to South America. Reported from southern Africa.	
Ommastrephidae		flying squids			
	Eucleoteuthis (Symplectoteuthis) luminosa	Luminous squids	COI, 12S, 16S, 18S	Circumglobal in tropical to temperate waters.	
	Dosidicus gigas	Jumbo/Humbolt squids	COI, 12S, 16S, 18S, Cytb	Eastern Indian Ocean and Eastern Pacific.	
	Ommastrephes bartramii	Neon/Red flying squids	COI, 12S, 16S, 18S, Cytb, Mito	Circumglobal in subtropical and temperate oceanic waters.	
	Sthenoteuthis (Symplectoteuthis) oualaniensis	Purpleback flying squids	COI, 12S, 16S, 18S, Mito	Indo-Pacific. Tropical to subtropical.	
	Todarodes pacificus	Japanese flying squids	COI, 12S, 16S, Cytb, Mito	Northern and Western Pacific and Northeast Atlantic	
	Hyaloteuthis pelagica	Glassy squids	COI, 16S	Indo-Pacific and Atlantic Ocean	
	Ornithoteuthis volatilis	Shiny Bird Squid	COI, 16S	Indo-West Pacific: in warm temperate waters	
	Symplectoteuthis spp.				
Priacanthidae		Bigeyes or Catalufa			
Scomberesocidae	*Cololabis saira*	Pacific Saury	COI, 12S, 16S, Cytb, Mito	North Pacific: Korea (Ref. 37630) and Japan eastward to Gulf of Alaska and southward to Mexico. Highly migratory species, Annex I of the 1982 Convention on the Law of the Sea (Ref. 26139).	
Scombridae	*Rastrelliger brachysoma*	Short mackerel	COI, 12S, 16S, Cytb, Mito	Pacific Ocean: Andaman Sea to Thailand, Indonesia, Papua New Guinea, Philippines, Solomon Islands and Fiji.	
	Katsuwonus pelamis	Skipjack tuna	COI, 12S, 16S, Cytb, MitoUnVer	Cosmopolitan in tropical and warm-temperate waters. Not found in the Black Sea. Highly migratory species	
	Euthynnus affinis	Kawakawa	COI, 12S, 16S, Cytb, Mito	Indo-West Pacific: in warm waters including oceanic islands and archipelagos. A few stray specimens have been collected in the Eastern Central Pacific. Highly migratory species	
	Thunnus sp	Tunas			
Sphyraenidae		Barracudas			
Synodontidae		Lizardfishes			
Tetraodontidae	Lagocephalus lagocephalus	Oceanic puffer	COI, 16S, 18S, Ctyb, Mito	Circumglobal: tropical to temperate seas.	

A2b. DNA extraction protocol using Longmire's solution and Chloroform, PCR protocol with cleanup, and Sanger sequencing protocol using Big Dye and Purification using Ethanol .

(A) Tissue Extraction (DMSO & Longmire's): DNA Extraction with Chloroform
Before you begin, figure out which samples you will be extracting and label an additional set of tubes (1.5mL) for each sample.

1. Cut up tissue into small pieces (maximize surface to volume ratio) and place in 1.5mL microcentrifuge tube with 500µl of lysis buffer (i.e. CTAB or Longmire's) and 20µl of Proteinase-K.
2. Incubate in 65ºC water bath for 1-2 hours. Tissue should be completely digested. If pieces of tissue remain, allow incubation for longer or add more Proteinase-K.
3. Add 500µL of Chloroform-Isoamyl (Sevag) to each tube. Close the lid and shake on low in the fume hood for 5 minutes (or vortex for 5-10 seconds). It should look milky.
4. Centrifuge each sample for 5 minutes at 15,000g
5. Remove 400µL supernatant (aqueous/top layer) from each tube and place into a pre-labeled 1.5mL tube.
6. Precipitate DNA:
    a. For each sample, add 200µL of 5M NaCl.
    b. For each sample, add 400µL of isopropanol (in freezer).
    c. Close tubes and invert (mix).
    d. Precipitate at -20 degrees Celsius for at least 1 hour (can be left overnight).
    e. Spin for 10 minutes at 15,000g.
    f. Pour off supernatant and add 150µL of 70% Ethanol (room temperature). Spin for 5 minutes at 15,000g.
    g. Pour off supernatant and add 150µL of 70% Ethanol (room temperature). Spin for 5 minutes at 15,000g.
    h. Pour off supernatant and invert on KimWipes for a mimimum of 10 minutes. Spin in vacuufuge (15 minutes at 45ºC) to dry pellets at the bottom of your tubes. If liquid is still present, allow pellets to air dry at room temperature until liquid is gone.
    i. Re-suspend pellets in 200µL of 1x TE, low EDTA (USB). Allow pellets to rehydrate at room temperature for a minimum of 1 hour before PCR assay (vortexing occasionally) or store in refrigerator.

(B) Sequencing - PCR cleanup Protocol:

1) Remove ExoSAP-IT reagent from -20°C freezer and keep on ice throughout this procedure.
2) Mix 5µl of a post-PCR reaction product with 2µl of ExoSAP-IT reagent for a combined 7µl reaction volume. Note: When treating PCR product volumes greater than 5µl, simply increase the amount of ExoSAP-IT reagent proportionally.
3) Seal plate securely with self-adhesive film; centrifuge sample briefly to make sure everything is at the bottom of the wells.
***use thermocyclers for incubation steps; program saved under *ExoSAP-IT* on the left machine.
4) Incubate at 37°C for 15 minutes to degrade remaining primers and nucleotides.
5) Incubate at 80°C for 15 minutes to inactivate ExoSAP-IT reagent.

6) The PCR product is now ready for use in DNA sequencing, SNP analyses, or other primer-extension applications. Treated PCR products may be stored at -20°C until required.

(C) Sequencing - Big Dye Terminator Cycle Protocol:

1) Combine the following in wells on a 0.1mL 96-well plate

UltraPure Water – 2μl
Sanger_NXT-F primer (sequencing concentration 0.8pmol/μl) - 2μl
DNA (post ExoSAP-IT) – 2μl
Big Dye – 4μl
Total reaction = 10μl

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2) Seal plate securely with self-adhesive film; centrifuge sample briefly to make sure everything is at the bottom of the wells.

3) Perform cycle sequencing in thermocyclers using the following protocol:
***program saved under *Big_Dye_ND* on left machine

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i - 96ºC for 5 minutes
ii – 96ºC for 10 seconds
iii – 50ºC for 5 seconds
iv – 60ºC for 4 minutes
**repeat steps ii-iv an ADDITIONAL 44 times
v – hold at 4ºC indefinitely

(D) Purification by Ethanol Precipitation Protocol:

1) Centrifuge briefly to make sure contents of wells are at the bottom; remove self-adhesive film.
2) Add 5μl of 125mM EDTA (pH 8.0) to each sample. Make sure the EDTA reaches the bottom of the wells.
3) Add 30μl of 100% ethanol to each sample and pipet up and down 5 times to thoroughly mix contents of each well.
4) Incubate at room temperature for 15 minutes. Keep in a dark place (i.e. a drawer) to protect the samples.
***use this time to chill the centrifuge down to 4ºC
5) Centrifuge at max speed (2250g [rcf]) at 4ºC for 30 minutes.
***proceed to next step immediately; if this is not possible, continue centrifuging the plate until you are ready to perform the next step
6) Gently, remove plate from centrifuge, lay down paper towels in centrifuge, and invert plate onto the paper towels. Centrifuge with plate inverted at 180g [rcf] for 1 minute.
7) Add 30μl of 70% ethanol to each sample.
8) Centrifuge plate at 1650g [rcf] for 15 minutes.
9) Gently, remove plate from centrifuge, lay down paper towels in centrifuge, and invert plate onto the paper towels. Centrifuge with plate inverted at 180g [rcf] for 1 minute.
10) Continue to air dry until all visible ethanol has evaporated.
11) Add 11μl formamide to each sample and load on the sequencer!

Chapter 3

Foraging ecology of the Red-footed Booby (*Sula sula*) provisioning chicks at Ulupa`u Crater, O`ahu: linking prey with at-sea distributions

ABSTRACT

Foraging ecology of Red-footed Boobies, (*Sula sula*) provisioning chicks at Ulupa'u Crater, O`ahu, Hawai`i, was examined by relating diet and foraging movements during two years of contrasting oceanographic conditions (2014, 2015) while considering differences between males and females. This was done using 33 birds that yielded a regurgitation upon removal of GPS tags and produced a successfully recorded last trip. Three trip characteristics (duration, range, distance) were positively cross-correlated, revealing that RFBOs that engaged in foraging trips of longer duration, venture farther from the colony, and travel greater distances. Therefore, RFBO may be considered "random foragers", searching unpredictable prey patches rather than commuting to a specific location of predictable prey aggregation. Trip characteristics influenced total mass and freshness of the diet, with freshness and total mass decreasing as duration increased, indicating prey digestion was greater when longer trips were taken. After removing the influence of Julian Day, trip characteristics were significantly different by study year where duration, range and distance were greater in 2014. Moreover, trip duration and distance yielded a significant year*sex interaction, whereby females went farther in 2014 but males farther in 2015. Because foraging trips overlapped (both within and across years), regardless of prey functional groups consumed, I suggest that RFBO do not respond to oceanographic variability by changing where they forage, but instead altering what they eat. Ultimately, prey consumption is likely influenced by prey availability, which in turn, is likely modulated by the encounter rate and abundance of SSPs and mixed-species feeding flocks.

INTRODUCTION

The tropical oceans are characterized by low productivity and patchy prey distributions (Ashmole 1971, Longhurst & Pauly 1987), which vary spatially and temporally in response to atmospheric and oceanographic forcing operating at inter-annual and longer-term scales (Hyrenbach & Veit 2003; Fielder & Tally 2006; Surman & Nicholson 2009; Velarde et al. 2015). Oligotrophic conditions are caused by permanent stratification of the thermocline due to constant solar radiation and little vertical mixing of the water column, leading to reduced primary production, and low food availability for upper-trophic level predators, compared with temperate and polar regions (Wyrtki 1964; Longhurst 1976; Ballance et al. 1997; Fielder & Tally 2006). Nevertheless, the tropical ocean is an important global biome, with a vast geographic extent, and large populations of commercially-valuable predatory fishes (Ballance and Pitman 1999; Le Manach et al. 2016). Evidence of changing community composition of commercial longline fishery catches (Polovina et al. 2009) and seabird trophic position (Gagne et al. 2018), underscore the potential for long-term changes in the community structure and food webs in the central Pacific. Therefore, novel approaches including integrating information on the distribution, movements, and diet of upper-trophic predators are needed to monitor changes in the structure and productivity of the tropical ocean (Lyday et al. 2015; Donahue Chapter 2).**E**Tropical seabirds display key adaptation to thrive in prey-scarce environments. For example, many tropical seabirds range widely and often forage with subsurface predators (SSPs) that drive prey to the surface, making them more readily accessible to seabirds (Au & Pitman 1986, Harrison 1990, Spear & Ainley 1998, Ballance & Pitman 1999). Some prey families, such as flyingfishes (Exocoetidae), halfbeaks (Hemiramphidae), and flying squids (Ommastrephidae) have evolved to be volant to evade predation by larger fishes and marine mammals. Volant prey however, become prime targets for seabirds that partake in aerial pursuit, such as Red-footed Boobies (*Sula sula*, RFBO).**E**The RFBO is a non-migratory, pan-tropical seabird, with a year-round presence at their breeding colonies. Because RFBO forage primarily by day and often return to the colony at dusk, their diurnal foraging pattern restricts foraging trips to a maximum distance of 150 – 276 km from the colony (Weimerskirch et al. 2005; Lormee et al. 2005; Friedlander et al. 2009; Young et al. 2010). RFBO can capture prey both in the water and in the air while foraging. Based on 15 observations made onboard research cruises taken throughout the Pacific, RFBOs forage by using two distinct methods: plunge diving (60% of observations) and aerial pursuit (40% of observations) (Ainley & Boekelheide 1983). RFBOs often forage in association with a variety of SSPs, as evidenced by 40 mixed-species feeding events documented around O`ahu, which involved five different SSPs (Hebshi et al. 2008): large predatory fishes (Skipjack Tuna *Katsuwonus pelamis,* Mahimahi *Corypahena hippurus* Yellowfin tuna *Thunnus albacares*) and marine mammals (spotted dolphin *Stenella attenuata* and false killer whale *Pseudorca crassidens*). Overall, there were no significant species-specific associations between RFBO and these five SSP species, underscoring their generalist foraging associations (Hebshi et al. 2008). **E**Whereas observations of the foraging methods and SSP associations of RFBOs provide insights into the mechanisms by which they find and capture prey, additional information can be obtained from studies of their diet and movements at sea. However, few integrative studies using prey species identification have been completed (Weimerskirch et al. 2005; 2006; Young et al. 2010). All RFBO diet studies report the dominance of flyingfishes (Exocoetidae) and flying squid (Ommastrephidae) (Ashmole & Ashmole 1967; Nelson 1978;

Harrison et al. 1983; Seki & Harrison 1989; Harrison 1990; Schreiber et al. 1996; Weimerskirch et al 2005; 2006; Young et al. 2010). A recent study (Donahue, Chapter 2) described RFBO diet on O`ahu, HI, through genetic analysis of 106 regurgitations from 81 birds, and found that Exocoetidae contributed to majority of the diet (35% by mass), followed by ommastrephid squids (22% by mass), with the remaining diet a combination of pelagic and near-shore fish species, belonging to eight families. Although observed dietary breadth by family underscored the generalist nature of the RFBO diet, the prey analysis by species revealed much specialization, with the Purpleback flying squid *Sthenoteuthis oualaniensis*, dominating the diet, and contributing ~22% (by mass) and ~60% (by number). Despite great importance of Purpleback flying squid in the RFBO diet, it is not targeted by fisheries in the central north Pacific and there is little information about its abundance and distribution (Young & Hirota 1998; Xinjun et al. 2006).**E**Donahue (Chapter 2) also described a RFBO diet shift from fish dominated prey during a "normal" environmental year (2014) to greater squid consumption during the onset of an El Niño (2015). Based on these results, Donahue advocated the use of RFBO as an indicator of food web changes within the local epi-pelagic marine environment surrounding O`ahu. **E**The goal of this study is to characterize the foraging ecology of RFBO provisioning chicks, by relating their diet to their movements during two years with contrasting oceanographic conditions (2014, 2015). Therefore, these analyses focus on a subsample of 33 RFBO of the 56 sexually identified birds analyzed previously in Chapter 2. These individuals were selected because they had a regurgitation collected in association with a complete last foraging trip tracked with telemetry. **E**Herein, I explore the value of the RFBO as an indicator of ocean variability around O`ahu. This study (1) tested the relationships among three foraging trip characteristics (duration, range, distance) and two diet sample characteristics (freshness and total mass), using linear regressions, for the purpose of including these covariates in subsequent Analysis of Variance (ANOVA) tests; (2) assessed the effect of study year and sex among trips: RFBO diet varied significantly by year and sex, the tracking analysis also investigated the influence of these two categorical variables; and (3) investigated associations between foraging trips and four prey functional groups (squids, flyingfishes, pelagic-oceanic fishes, and reef-associated fishes). Based on previous dietary analyses, I expected males and females to have similar tracks in 2014, with males and females foraging in different areas in 2015, when diet differed by sex (i.e., disproportionately more squid taken by males and flyingfish by females).**EMETHODSEStudy Area**

The RFBO colony is located on the eastern crest of Ulupa`u Crater (21.46° N; 157.72° W), within the Marine Corps Base Hawai`i (MCBH), on the island of O`ahu, Hawai`i. Here breeding pairs build their nests in the available Kiawe trees (*Prosopis pallida*) and Haole Koa shrubs (*Leucaena leucocephala*). The colony size estimates range from a minimum of 337 nesters in 2008, to a maximum of 2,380 in 1969 (Russell & Vanderwerf 2010). Currently, the RFBO breeding population is estimated at a minimum of 200 individuals (Unpublished data, Russell; Pyle & Pyle 2017).

Tagging

We tagged 80 birds total during two periods in the summers of 2014 and 2015 and explored the birds' at-sea distribution and diet with the use of GPS (Geographic Positioning System) archival tags and the opportunistic collection of regurgitation during tag retrieval, respectively.

During each deployment period, we haphazardly selected 20 chick-rearing RFBOs based on chick growth stage and nest accessibility. No adults were incubating or had featherless chicks.

As a control, all nests were chosen that contained similar sized chicks, between C4 and C6B growth stages (Table 1). For comparison of chick stages across years and to meet the sample size requirements of the Chi-squared test, we classified chick stages were classified as “Early” (downy chick < 50% of adult’s size; C4) and “Late” (downy chick ≥ 50% of adult’s size or primaries present; C5, C6A, C6B) (Similar to Adams et al. unpublished).

Starting at 19:00 HST, we captured birds at their nests and tagged them until all tags were deployed. We attached modified i-gotU GT-120 GPS loggers (Mobile Action Technologies, New Taipei City, Taiwan) to RFBOs. Specifically, we stripped the GT-120 loggers from their factory housings and sealed them within 2:1 low-shrink-temperature, polyolefin heat-shrink tubing (AMS-DTL-23053/5-310, BuyHeatShrink.com, Deerfield Beach, Florida) to create a light-weight, waterproof housing. With the original 380-mAh battery, the repackaged GPS logger weighed 17 grams. We attached the modified GT-120s near the base of the 3–4 central tail feathers (retrices 6 through 9) using 3-4, 1 cm wide strips of Tesa® tape (Tesa® 4651, Norderstedt, Germany). We programed GPS loggers to collect location data every 2 minutes continuously to achieve a maximum expected duration of approximately 10–14 days. To assist in their identification at the colony, we marked equipped RFBOs with blue sharpie on their forehead. We also provided non-banded birds with metal leg bands to identify individuals over time.

Tagged RFBOs were recaptured between 4-19 days after deployment between the hours 19:00 – 23:00 HST. We measured body morphometrics (body mass, culmen length, wing chord), and took blood and feathers for equipped birds during tag retrieval periods. The blood and feathers were used to determine sex using genetic methods.

Diet Analysis

The diet information incorporated into the present study was previously described for this colony by Donahue (Chapter 2) as a prelude to this tagging analysis. For specific information about the RFBO diet or methods of diet analysis, not covered in this study, please refer to Chapter 2 of this thesis.

Tracking

USGS created the RFBO tracking database and "cleaned" all the tracking data acquired at MCBH during this study. This study analyzed data from a subset of 33 of the 80 RFBO tagged at MCBH, by selecting those birds that provided a diet sample during retrieval of their GPS units and yielded a complete and successfully recorded last foraging trip. I used geospatial analysis to relate an individual birds' stomach contents to their foraging movements, using three metrics: trip duration (hr), maximum distance from the colony (range; km), and overall distance traveled (km) from the colony (Table 2, Figures 1, 2, 3). I calculated the "overall distance traveled" and "range" variables for each trip by USGS using the R package geosphere (Adams et al. unpublished; Hijmans et al. 2016). Trip lengths were divided into "short", "long" and "multi-day" based on a bimodal distribution, using the following definitions: short trips (< 12 hr), long (> 12 hr and < 24 hr) (Adams et al. unpublished, Figure 1). Only one multi-day trip, lasting 38.4 hr (1.6 days) was observed (Table 2, Figure 1). I used these variables were used to interpret the variability in the diet of individual birds by sex and between years.

Merging of Diet and Tracking Data

By mapping the last tracks and evaluating the birds' foraging distribution using ArcGIS 10.3, an individuals' diet was matched to the characteristics of its foraging location (e.g., Weimerskirch 2005; Young 2010). By combining the diet and the tracking data, I determined whether the foraging areas searched during a foraging trip and the prey returned to the colony were related. I used bathymetry data obtained from the School of Ocean and Earth Science Technology at the University of Hawai'i, Manoa (http://www.soest.hawaii.edu/HMRG/Multibeam/bathymetry.php) to determine features that RFBOs were most commonly found associated with while foraging around O`ahu.

Statistical Analyses

I performed all single variate statistical tests using the IBM SPSS Statistics software (Version 25, Albany, NY) and assessed significance using *alpha* 0.05. Normality was determined using the Shapiro – Wilk test with homogeneity of variances determined using the Levene's test. To ensure normality, the foraging variables were log_{10} transformed (trip duration, range, distance) and the prey functional group proportions were *arcsine* transformed (Zar 1984).

Hypotheses Testing: Assumptions of Covariance

The assumptions of possible influences of the tracking variables on the diet sample mass (g) and freshness (Weighted Average Freshness, WAF; Donahue, Chapter 2) were tested using linear regression; significant factors were included as co-variates in the subsequent Two-way Analysis of Variance ANOVA) used to test changes in the mass and freshness of the regurgitated samples as a function of year or sex.

Hypothesis Testing: Year and Sex Differences Among Trips

I conducted two separate ANOVA tests to compare two dependent variables (prey mass and WAF) as a function of two independent variables (year and sex) and their interaction. The sample sizes of these two-way ANOVA tests were unbalanced, because a different number of males and females provided a diet sample with their associated foraging trip: 16 birds (12 Male, 4 Female) in 2014, and 17 birds (14 Male, 3 Female) in 2015.

Exploratory Analyses: Trip & Diet Effects

The association of RFBO foraging patterns and diet were explored with multivariate statistics using PC-ORD 6 software (PC-Ord v.6; McCune & Mefford 2006). Specifically, I used Non-Metric Dimensional Scaling (NMDS) to compare non-parametric relationships between the importance of the prey functional groups and RFBO foraging trip characteristics.

The Relative Sorensen distance measure was used to relativize the proportional composition of each sample, by species (Kruskal and Wish 1978). The ordination distance matrix (Matrix 1) involved 33 samples (birds) as rows and 6 variables (trip characteristics: duration, range, distance; and % prey functional groups: flyingfishes, squids, pelagic-oceanic fishes, reef-associated fishes) as columns (McCune & Grace 2002). The ancillary matrix (Matrix 2) involved 33 samples (birds) as rows, and 5 variables (centroid azimuth, greatest azimuth, Julian Day, WAF, diet total mass) as columns. I assessed statistical significance of the ordination axes with the Matrix 1 and Matrix 2 variables with Kendall Tau Correlations, calculated at two significance levels: basic (alpha 0.05) and high (alpha 0.001).

For the NMDS, I used random starting coordinates for 500 iterations with the real data and 999 runs with the randomized data. The maximum of 6 axes (the number of variables in matrix 1 minus 1) was selected to explore the ordination and correlations with the axes. The stability criterion for the tests were set to 0.000001. To facilitate comparisons between tests, I used the same matrix set-up as the NMDS for the Multi-Response Permutation Procedure (MRPP). Additionally, I used the categorical grouping variable “Sex by Year” within the second matrix, to assign birds into one of four groups: Males 2014, Females 2014, Males 2015, and Females 2015.

RESULTS

The RFBO foraging range encompassed 22,211.4 km^2, spanning 119.3 km North-westward 163.8 km North, 76.9 km East, and 83.7 km South from the colony. The depth range was from 0 m to a little over 6000 m, the greatest depth around O`ahu. The proportion of depths within the foraging range are as follows: 0 to -200 m, 6.9%; -200 to -1000 m, 14.2%; -1000 to -3000 m, 17.3%; and -3000 to -6000 m, 61.5%.

Testing of Correlations between Foraging Metrics and their Influence on Diet Samples

When regressing the foraging trip characteristics, duration was the independent variable with range and distance as the dependent variables. When exploring the relationship between regurgitations and trip characteristics, regurgitation freshness and total mass were the dependent variables with trip duration, range and distance as independent variables. Regurgitation freshness and total mass were regressed to all three foraging metrics to determine if the digestion of a sample is soley determined by duration of a trip or if range and distance traveled from the colony also play a role.

The duration of a foraging trip was positively correlated to the total distance traveled (Pearson Correlation: $r = 0.931$, df = 1,32, $p < 0.0001$) and to the foraging range (Pearson Correlation: $r = 0.840$, df = 1,32, $p < 0.0001$). Birds that went on longer duration trips, ranged farther and coverd more distance.

The freshness of the regurgitated prey items (WAF) was positively correlated to the distance (Pearson Correlation: $r = 0.369$, df = 1,32, $p < 0.034$), duration (Peason Correlation: $r = 0.400$, df = 1,32, $p < 0.021$) and range (Pearson Correlation: $r = 0.349$, df = 1,32, $p < 0.046$). WAF was mostly described by trip duration (Regression: WAF = (1.4 +/- 0.3SE) + (0.718 +/- 0.3SE(Duration)); Shapiro-Wilk Test of Residuals: 0.976, df = 33, $p < 0.674$); the farther the bird traveled, the more digested its diet sample.

The total mass of a diet sample was negatively related to the duration of the trip (Regression: Total Mass = (219 +/- 43SE) + (-111 +/- 44SE(Duration)), R = -0.415, $R^2 = 0.172$, df = 1,32, $p<0.016$; Shapiro-Wilk Test of Residuals: 0.950, df = 33, $p < 0.134$) and not correlated to trip distance (Pearson Correlation: r = -0.259, df = 1,32, $p < 0.145$) or range (Pearson Correlation: r = -0.185, df = 1,32, $p < 0.302$). Distance does not affect the total mass of a regurgitation, but the amount of time it takes to complete the trip does; the longer the trip, the smaller the sample may be due to digestion.

Inter-annual and Sex Differences in Forging Metrics

The three range metrics were pooled together and averaged from the last trips of both males and females during both study years (2014, 2015) (Table 3), and compared using three two-way ANOVA tests. There were no significant differences by year or sex and no significant year×sex interaction, for the three foraging metrics: Trip Duration ($R^2 = 0.056$, $F_{3,29} = 0.568$, $p < 0.640$; Shapiro-Wilk: SW = 0.960, df = 33, $p < 0.255$), Range ($R^2 = 0.118$, $F_{3,29} = 1.294$, $p < 0.294$; Shapiro-Wilk: SW = 0.970, df = 33, $p < 0.476$), and Distance ($R^2 = 0.096$, $F_{3,29} = 1.025$, $p < 0.396$; Shapiro-Wilk: SW = 0.985, df = 33, $p < 0.924$).

Ordination of Foraging Trip & Diet

NMDS revealed a 2-dimensional solution with an instability of zero after 69 iterations that explained 96.4% (R^2) of the observed variance in the three foraging trip descriptors and the four prey functional groups. The low final stress (6.98) was indicative of a “good” ordination that accurately captured the distances in variable space (Clark 1993; McCune & Grace 2002). The orthogonality of the two NMDS axes was 98.6%, indicative of independent axes.

Moreover, the two NMDS axes were significantly correlated with variables in matrix 1 and matrix 2. Trip range, distance, WAF, reef-associated prey and pelagic-oceanic prey were negatively correlated to axis 1; pelagic-oceanic prey were highly correlated ($p < 0.001$) (Table 4). Flyingfishes and Julian Day were the only two variables correlated with axis 1 (Table 4). For axis 2, the squids (negative) and flyingfishes (positive), were strongly correlated with axis 2 (Table 4). The greatest Azimuth was the only matrix 2 variable significantly correlated with axis 2 (Table 4).

Group Comparisons of Foraging Trip & Diet

Following the exploratory NMDS analysis, I used MRPP to test for differences in the foraging parameters and diet of birds of different sex (males, females) and during the two study years (2014, 2015). MRPP compared four groups: 2014 females, 2015 females, 2014 males, 2015 males. The metric of within-group heterogeneity was greater than expected ($A = 0.164$) and there was an overall significant result when comparing all four groups ($p < 0.002$). Pairwise post-hoc comparisons revealed that half (3 of 6) of the pair-wise comparisons were significant: 2014 males versus 2015 females ($A = 0.240$, $p < 0.004$), 2014 females versus 2015 females ($A = 0.459$, $p < 0.011$) and 2015 males versus 2015 females ($A = 0.265$, $p < 0.001$). The other three pair-wise comparisons were not significant: male to female comparison within 2014 ($A = -0.008$, $p < 0.452$), males between both years ($A = 0.013$, $p < 0.222$) and 2014 females versus 2015 males ($A = -0.015$, $p < 0.599$).

I conducted several analyses to investigate potential drivers of these patterns, including changes in the timing of the tracking period between years and sexes and changes in the development stages of the chicks. Because Julian Day was significantly correlated with Axis 1 of the NMDS (Table 4), I performed a Two-way ANOVA to test for differences between years, sexes and year×sex interaction. Julian Day was significantly different by year ($F_{1,28}$=177.6, $p < 0.0001$), with a significant year×sex interaction: $F_{1,28}$=5.384, $p < 0.028$). However, Julian Day did not vary significantly by sex ($F_{1,28}$=4.047, p<0.054). This indicated the two years were influenced by different sampling periods which could explain variation in the foraging range characteristics between years**.** However, there was no difference in the chick development stages among affiliated adults that were tagged during the two years (χ^2 Fisher's Exact Test, df = 1, p < 0.603) Therefore, I concluded that it was appropriate to compare foraging parameters between years.

Effect of Julian Day: Inter-annual and Sex Differences in Forging Metrics

I used Julian Day as a covariate within ANCOVA to see if there was an effect on the trip characteristics when controlling for Julian Day. The ANCOVA used the three-range metrics from the two sexes (males, females) during both study years (2014, 2015) (Table 5), and are comparable to the three two-way ANOVA tests used previously to describe the trips.

Julian Day was found to have a significant effect on all three trip characteristics and when removed (Table 5), year was significant for all three characteristics (ANCOVAs - Duration: R^2 = 0.240, $F_{4,28}$ = 0.8.212, p < 0.008; Shapiro-Wilk: SW = 0.981, df = 33, p < 0.818; Range: R^2 = 0.253, $F_{4,28}$ = 8.142, p < 0.008; Shapiro-Wilk: SW = 0.968, df = 33, p < 0.428; Distance: R^2 = 0.253, $F_{4,28}$ = 0.8.712, p < 0.006; Shapiro-Wilk: SW = 0.964, df = 33, p < 0.331) with year×sex interaction being significant for trip distance ($F_{4,28}$ = 4.608, p < 0.041) and duration ($F_{4,28}$ = 4.809, p < 0.037); Shapiro-Wilk: SW = 0.960, df = 33, p < 0.255) (Table 5, Figure 4). Sex was still not significant for any characteristic.

DISCUSSION

The overarching goal of this study was to describe the foraging ecology of provisioning RFBO, by relating their diet to their movements during two years with contrasting oceanographic conditions (2014, 2015), and contrasting males and females. To this end, foraging trips were characterized using three metrics, derived from 33 birds: duration, range, and distance covered. Regurgitation samples collected at the end of these foraging trips were characterized in terms of the relative mass of four functional groups: squid, epipelagic flyingfishes, other pelagic fishes, and reef-associated fishes.

The three foraging trip characteristics were positively cross-correlated, revealing that RFBOs that engaged in foraging trips of longer duration, venture farther from the colony and travel greater distances. In the absence of examining individual foraging site fidelity, this implies that RFBO are random foragers and can commute far distances in search of randomly occurring prey patches rather than commuting to a specific location where prey may be predictable. The freshness of a sample decreased (smaller WAF) and the total mass decreased as the foraging trip duration increased. Together, these results indicate the effect of prey digestion is greater, leading to lower prey mass and freshness, when the RFBO take longer foraging trips.

Interestingly, no significant differences were found between years and sexes, and there was no significant year×sex interaction, when the three foraging trip characteristics were compared (Figure 6). When the effect of Julian Day was removed from the analysis, year became significant with 2014 having farther, longer trips than 2015 with interactions between the females between years, where they had shorter trips in 2015 (Figure 4, 8). Although not significantly different, female trips at MCBH (Duration: 7.1 hr ± 0.6 SD; Distance: 128.0 km ± 19.4 SD) were on average shorter than the male trips (Duration: 11.1 hr +/- 8.7 SD; Distance: 201.6 km +/- 123.6 SD) in 2015 (Figure 4, 7, 8). Based on the RFBO reverse sexual dimorphism (females being larger than males), our results are consistent with Young et al. (2010) who found that females travel shorter distances than males due to greater energetic cost of flying. Nevertheless, the unbalanced sample sizes, with only 3 females and 14 males tracked (4 and 12, respectively, in 2014), may have inhibited the significant characterization of sexually segregated foraging trips (Figure 7 and 8).

There were significant differences in the diet composition of the males and females, where 2014 males versus 2015 females, 2014 females versus 2015 females and 2015 males versus 2015 females were found to be significantly different from each other. The same differences between 2014 males versus 2015 females and 2015 females and males were also seen during the larger diet analysis (Donahue Chapter 2) and indicate that the dietary differences hold even with smaller sample size comparisons. I suggest that diet is an important driver of patterns between year and sex interactions (Figure 10). The only dietary relationship that changed between the large dietary analysis (Donahue Chapter 2) and the present analysis was that males of both years were different from each other (males eating less squid in 2014) to females of both years being different from each other (females eating less fish in 2014), respectively. This is possibly due to the small number of diet samples from females, especially in 2015 (n=3; n=4, 2014). Nevertheless, the significant differences in the prey functional groups consumed by females and males do not appear to be related to their foraging trip dynamics, because both sexes have similar ranging patterns and their trips overlap spatially in both 2014 and 2015.

Additionally, the change in the tracking period during the two study years, from 5 June – 8 June (JD 156 – JD 159, 2014) to 23 June – 8 July (JD 174 – JD189, 2015), could be another potentially confounding factor, that influenced the documented inter-annual patterns. The significance of Julian Day across years indicated that the date of the sampled foraging trips was delayed by approximately three weeks. Therefore, this result must be taken with caution, if the timing of sampling is driving the observed prey differences, rather than sexual dimorphism or temporal changes in the oceanic environment. With Julian Day having a significant effect, this could influence the foraging trip characteristics by what prey may be present during the two years or how the sexes may forage at different times of the breeding season. It is possible that Skipjack tunas have mostly squid in their diet and since they are more abundant during ENSO events in the Central and Eastern Pacific (Senina et al. 2008), Skipjack tuna may have been more abundant off O`ahu in July of 2015 (the warmest month on record) and could have been a reason for why more squid was present in the RFBO diet if the birds were foraging with Skipjack tunas.

Nevertheless, the lack of significant differences in the chick development stages across the two study years, suggests that the chick phenology and energetic demands were comparable in both study years. Thus, despite the delay in the tracking period in 2015, the chicks were not more developed than in 2014. I suggest two possible explanations: that there may have been a small delay in breeding in 2015, or chick development may have been slower in 2015.

Adams et al. (Unpublished) and Felis et al. (2015) described how RFBO chick phenology of the MHI affected trip lengths, with incubating RFBO taking longer trips, RFBO with small chicks taking short trips and RFBO with larger chicks having a mixture of short and long trips. Even by using a smaller subset of birds from our complete tracking records at MCBH, the same mixture of short and long trips was present. This was true even with the smaller subsample of MCBH birds selected, with the birds who provided diet having similar sized chicks.

When the trips were mapped out over each other, males and females traveled to similar areas but caught two different prey functional groups (Figure 6, 8). The greatest azimuth was significantly correlated with the squid – flyingfish dichotomy; birds that traveled north to northeast returned with a greater proportion of flyingfish in their diet, whereas squid was dominant (by mass) in the rest of the samples, following trips which varied from as far west as Ka`ena Ridge, south to Penguin Banks, east to Moloka`i and northeast to the debris field from the O`ahu - Moloka`i slides (Figure 7, 8, 9). In 2015, female trip distances were shorter than male trip distances, but so few females were tracked that this significant result may not be representative to the pattern for the entire colony. However, the analysis of prey functional groups across sexes, involving a larger sample size, revealed significant differences. Thus, these two results indicate likely sexual differences in prey selection.

Likely because RFBOs can adjust to changing ocean conditions, differences in RFBO foraging characteristics were not significantly different and the foraging trips overlapped (both within and across years) regardless of the prey functional groups taken. In particular, I suggest that RFBO do not respond by changing where they forage, but instead alter what they eat. Ultimately, the prey consumption is likely influenced by the prey availability, which, in turn, is likely modulated by the encounter rate and abundance of SSPs and mixed-species feeding flocks.

In 2014, during the relatively normal year, the species diversity of prey was greater and driven by the numerous pelagic fish species found that year (Donahue Chapter 2). The trips for 2014 were also wide-spread throughout the foraging range but focused mostly north and south of the colony. In 2015, the birds travelled more to the east and west, with less prey species diversity, and a greater reliance on squids (by mass).

Whenever reef-associated fish species are found within the diet, it raises the question of where and how the RFBOs captured them. Do they forage close to shore where reef-associated fish are typically present or are reef-associated fish present farther from shore than we realize? Interestingly, tunas and mahi-mahi, caught within 100km from shore, occasionally ingest reef-associated fish and crustaceans advected offshore or individuals associated with drifting debris (Allain et al. 2002; 2012). Similar mechanisms may explain how RFBOs find and capture reef fish.

The increase in squid in the diet during 2015 could be related to an increase in their abundance or availability to foraging RFBO due to the increase in SST, from June to July. Purpleback flying squids, *Sthenoteuthis oualaniensis,* which contributed 99% of the squid within the diet, have been observed to spawn sooner and grow faster in warmer conditions in the Indian Ocean (Xinjun et al. 2006). This same environmental response was invoked off the windward (Eastern) side of O`ahu, where majority of the larger fertile adult male squids were found in late spring and early summer (Young & Hirota 1998). Female squids around eastern O`ahu were observed to be mature around 160 to 205 mm in mantel length and males around 130 to 175 mm mantel length (Young & Hirota 1998). All squids found within the RFBO diet were juveniles since they were all smaller than 103 mm mantel length (Donahue Chapter 2). Nevertheless, more concurrent squid spawning and RFBO diet studies are needed, to fully understand the phenology and drivers of *Sthenoteuthis oualaniensis* spawning and development. In particular, this would require performing annual spring - summer squid and flyingfish surveys over a few years of contrasting oceanographic conditions, in conjunction with colony-based studies of RFBO diet and productivity.

And so, from evidence provided from within this study and Donahue (Chapter 2), RFBOs are a uniquely helpful indicator species of ocean environment and can be used as a monitor for local oceanographic environmental change and the accompanying prey shifts around O`ahu.

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TABLES & FIGURES

Table 1. Chick growth stages for boobies (Adams et al. Unpublished).

Chick Growth Stages for Boobies

1) Naked
2) Down appearing on back
3) Down beginning to cover whole body
4) All downy approximately 1/2 adult size
5) All downy approximately adult size
6A) Primaries and rectrices visible; no scapulars
6B) Scapulars visible; down still present on back
6C) Scapulars unite in midline; down still on wings
7) Wings and back almost clear of down; thick down remaining on head, neck, flanks, belly and breast variable
8) Fully feathered but not flying; thin or wispy down may persist on radio-ulna, neck or flanks
9) Fully feathered

Table 2. Summary of the 33 tagged Red-footed Boobies from Marine Corps Base, Hawai`i (MCBH) that provided a diet sample and after a complete foraging trip, arranged according to the duration of their foraging trip (Short, Long, Multi-day), year (2014, 2015) and sex (Male, Female). Trip duration, range and distance are summarized using the mean +/- S.D.

Trip Length	Year	#Trips(Birds)	#Male/Female	Duration (hr)	Range (km)	Distance (km)
Short	2014	8	6/2	6.9 +/- 2.2	60.8 +/- 21.9	152.0 +/- 52.2
	2015	12	9/3	7.0 +/- 2.3	53.4 +/- 25.0	137.5 +/- 59.5
Long	2014	8	6/2	13.9 +/- 1.0	108.7 +/- 29.3	297.7 +/- 68.8
	2015	4	4/0	13.7 +/- 1.0	88.8 +/- 16.5	254.5 +/- 34.2
Multi-day*	2015	1	1/0	38.4	139.3	537.3

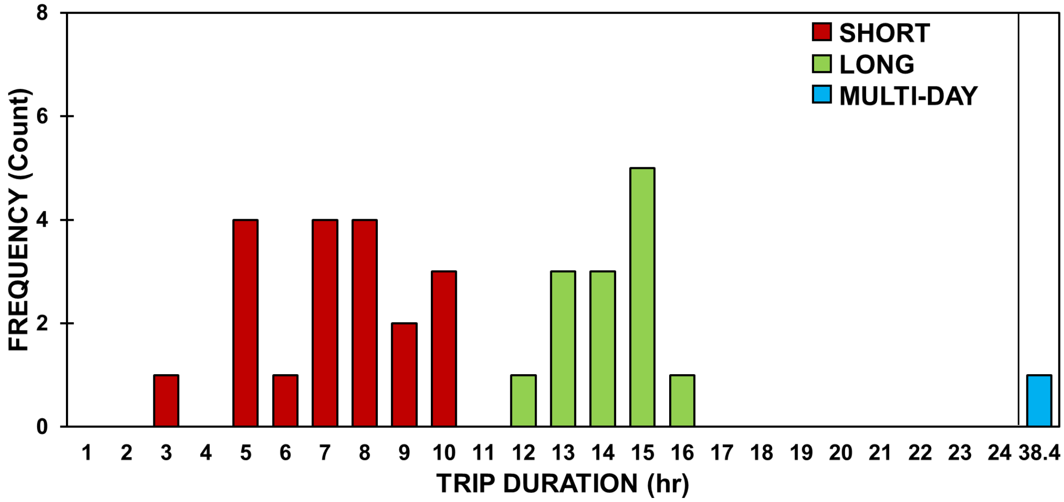


Figure 1. Hourly distribution of 33 Red-footed Booby foraging trip durations (hour, hr) within a 24-hour day. A single 38.4 hr multi-day trip was recorded.

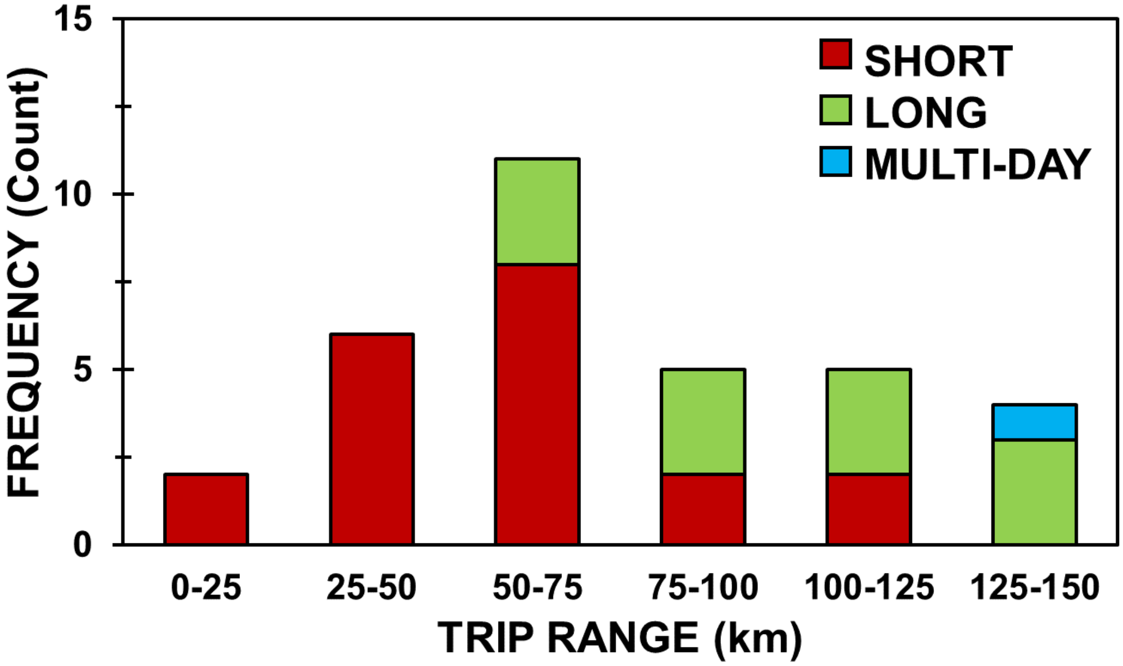


Figure 2. Distribution of 33 Red-footed Booby foraging trip ranges (km) for different trip durations (Short, Long, Multi-day).

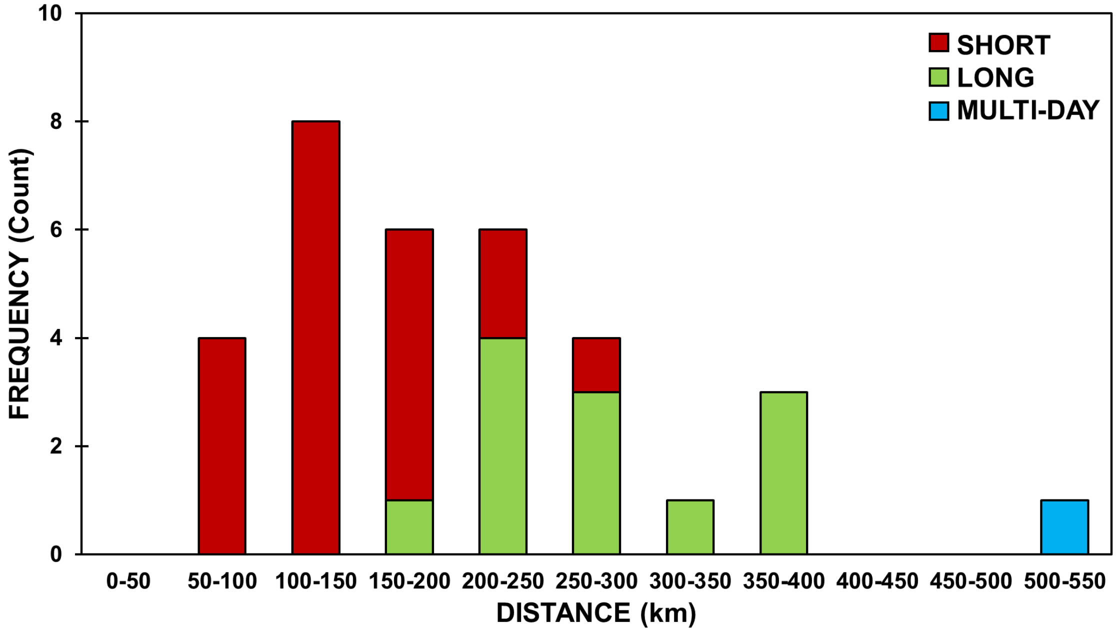


Figure 3. Distribution of 33 Red-footed Booby foraging total distance traveled (km) for different trip durations (Short, Long, Multi-day).

Table 3. Summary of foraging trip characteristics and diet from the 33 Red-footed Boobies that provided a regurgitation sample after complete foraging trip. Trip duration, range, distance, regurgitation freshness and regurgitation total mass are summarized using the mean +/- S.D., the median and the range.

Variable	Year	Sex	Avg +/- SD	Median	Range
Trip Duration (hr)	2014	M	9.8 +/- 4.3	10.2	4.5-15.1
		F	12.2 +/- 2.7	12.1	9.8-14.8
	2015	M	11.1 +/- 8.7	8.7	2.3-38.4
		F	7.1 +/- 0.6	7.0	6.7-7.7
Trip Range (km)	2014	M	79.7 +/- 35.3	67.7	38.2-131.9
		F	99.6 +/- 34.8	89.4	71.6-148.2
	2015	M	70.0 +/- 35.0	64.5	23.0-139.3
		F	51.6 +/- 11.4	50.5	40.8-63.6
Distance Traveled (km)	2014	M	215.4 +/- 103.3	199.8	94.0-384.6
		F	253.5 +/- 71.5	228.1	199.0-358.6
	2015	M	201.5 +/- 123.6	188.1	51.0-537.3
		F	128.0 +/- 19.4	122.3	112.1-149.6
Regurgitation Freshness (WAF, 1-3)	2014	M	2.2 +/- 0.4	2.2	1.5-2.6
		F	2.3 +/- 0.5	2.2	1.8-2.8
	2015	M	2.0 +/- 0.5	2.0	1.1-2.8
		F	2.0 +/- 0.0	2.0	2.0
Regurgitation Total Mass (g)	2014	M14	110.1 +/- 53.4	118.0	14.3-182.9
		F14	129.5 +/- 93.0	125.0	38.6-229.6
	2015	M15	109.6 +/- 61.2	104.2	17.6-220.9
		F15	118.5 +/- 80.3	73.2	71.1-211.1

Table 4. Summary of Non-Metric Dimensional Scaling (NMDS) ordination results of Red-footed Booby trip characteristics and prey functional groups (Matrix 1), showing the Kendall Tau correlations for the 2-Dimensional outcome with the ordination variables (Matrix 1) and other ancillary variables used to characterize foraging trips and regurgitation samples (Matrix 2). Significant correlations are bolded, with asterisks indicating two significance levels: significant (***p < 0.05)**, and highly-significant (****p < 0.001)**.

Variable	**Axis**	
Matrix 1	**1**	**2**
Trip Duration	-0.144	-0.083
Trip Range	**-0.258***	0.038
Distance	**-0.250***	0.015
Squids	0.070	**-0.823****
Pelagic-oceanic	**-0.635****	0.132
Reef-associated	**-0.299***	0.063
Flyingfishes	**0.216***	**0.740****
Matrix 2	**1**	**2**
Centroid Azimuth	0.057	0.148
Farthest Azimuth	-0.091	**0.212***
Julian Day	**0.292***	0.175
Total Mass	-0.127	0.165
WAF	**-0.212***	-0.015

Table 5. Analysis of Covariance results with using Julian Day as the covariate in order to remove the effect of Julian Day to see how significant the Julian Day Kendall Tau correlation was within the NMDS result.

ANCOVA (df = 4,33)	**JULIAN DAY COVARIATE**		**YEAR FACTOR**		**SEX FACTOR**		**YEAR*SEX INTERACTION**		**RESIDUAL NORMALITY**
Trip Characteristics	**Coefficient +/- S.E.**	**F - test, p value**	**Result**	**F - test, p value**	**Result**	**F - test, p value**	**Result**	**F - test, p value**	**SW test (statistic, p value)**
Distance (F = 2.371, p < 0.077)	-0.002 +/- 0.003	F = 5.888, **p < 0.022**	**2014 > 2015**	F = 8.712, **p < 0.006**	-	F = 0.890, p < 0.353	**1 < 2; 3 > 4**	F = 4.608, **p < 0.041**	SW = 0.964, p < 0.331
Range (F = 2.375, p < 0.076)	-0.003 +/- 0.003	F = 5.062, **p < 0.033**	**2014 > 2015**	F = 8.142, **p < 0.008**	-	F = 0.333, p < 0.568	-	F = 3.942, p < 0.057	SW = 0.968, p < 0.428
Duration (F = 2.209, p < 0.094)	0.001 +/- 0.003	F = 6.790, **p < 0.015**	**2014 > 2015**	F = 8.212, **p < 0.008**	-	F = 0.572, p < 0.456	**1 < 2; 3 > 4**	F = 4.809, **p < 0.037**	SW = 0.981, p < 0.818

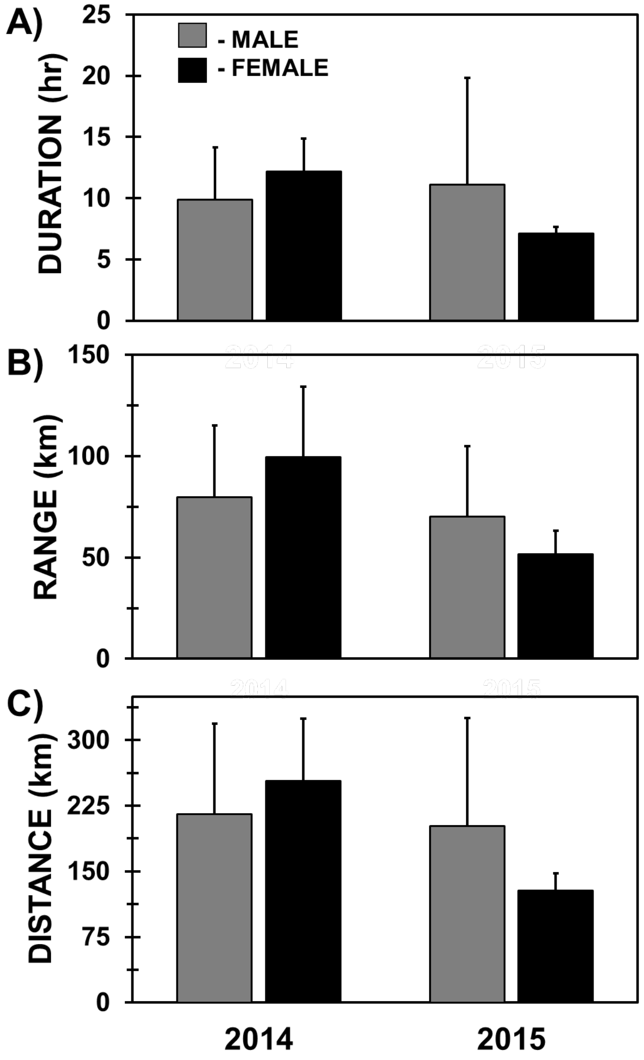


Figure 4. Average with Standard Deviation of the trip characteristics, duration (hours), range (kilometers) and distance (kilometers), from 33 RFBO that provided diet samples and sex identification from MCBH in 2014 and 2015.

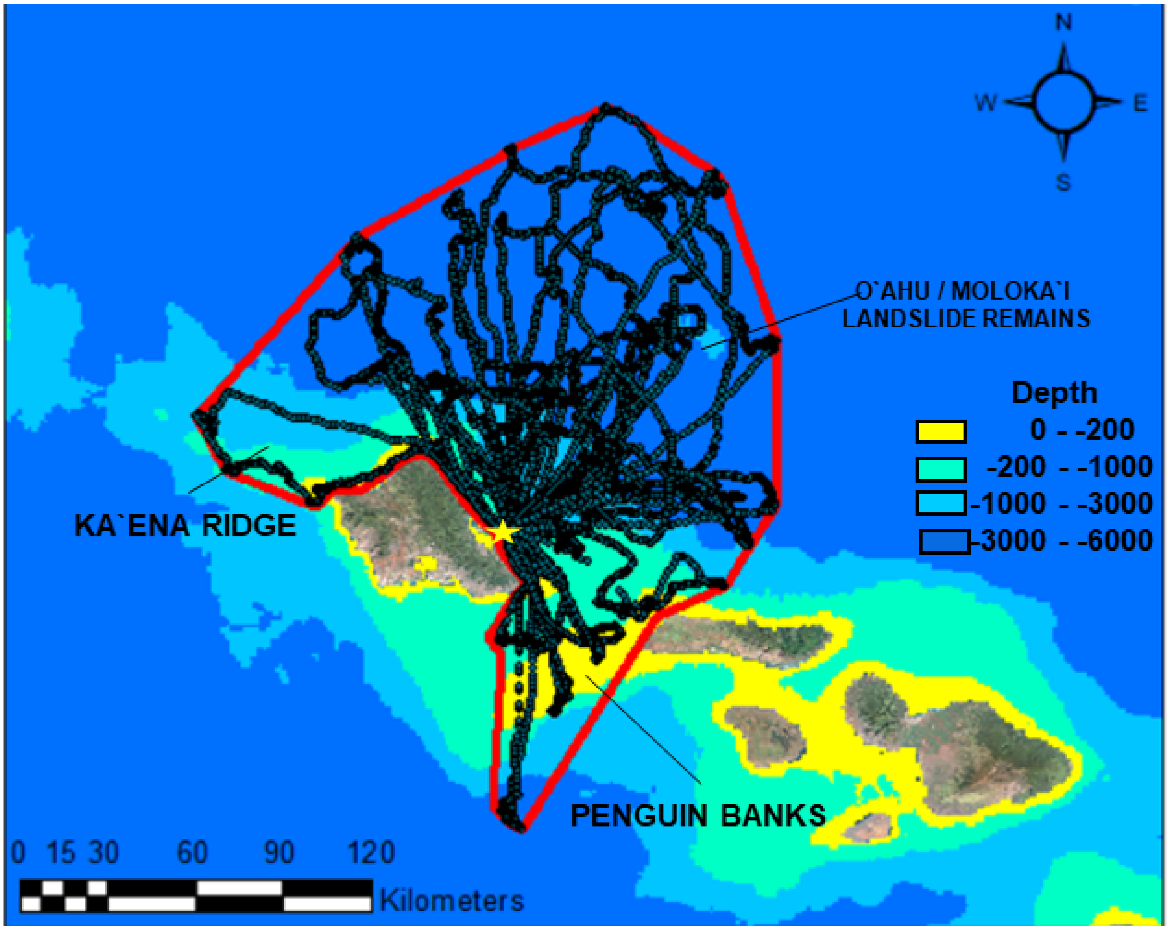


Figure 5. Map of the foraging trips of 33 Red-footed Boobies from MCBH that provided a regurgitation sample. The RFBO "footprint" was derived from these data, using a minimum convex polygon (MCP) and used for extracting environmental information of the RFBO's foraging area.

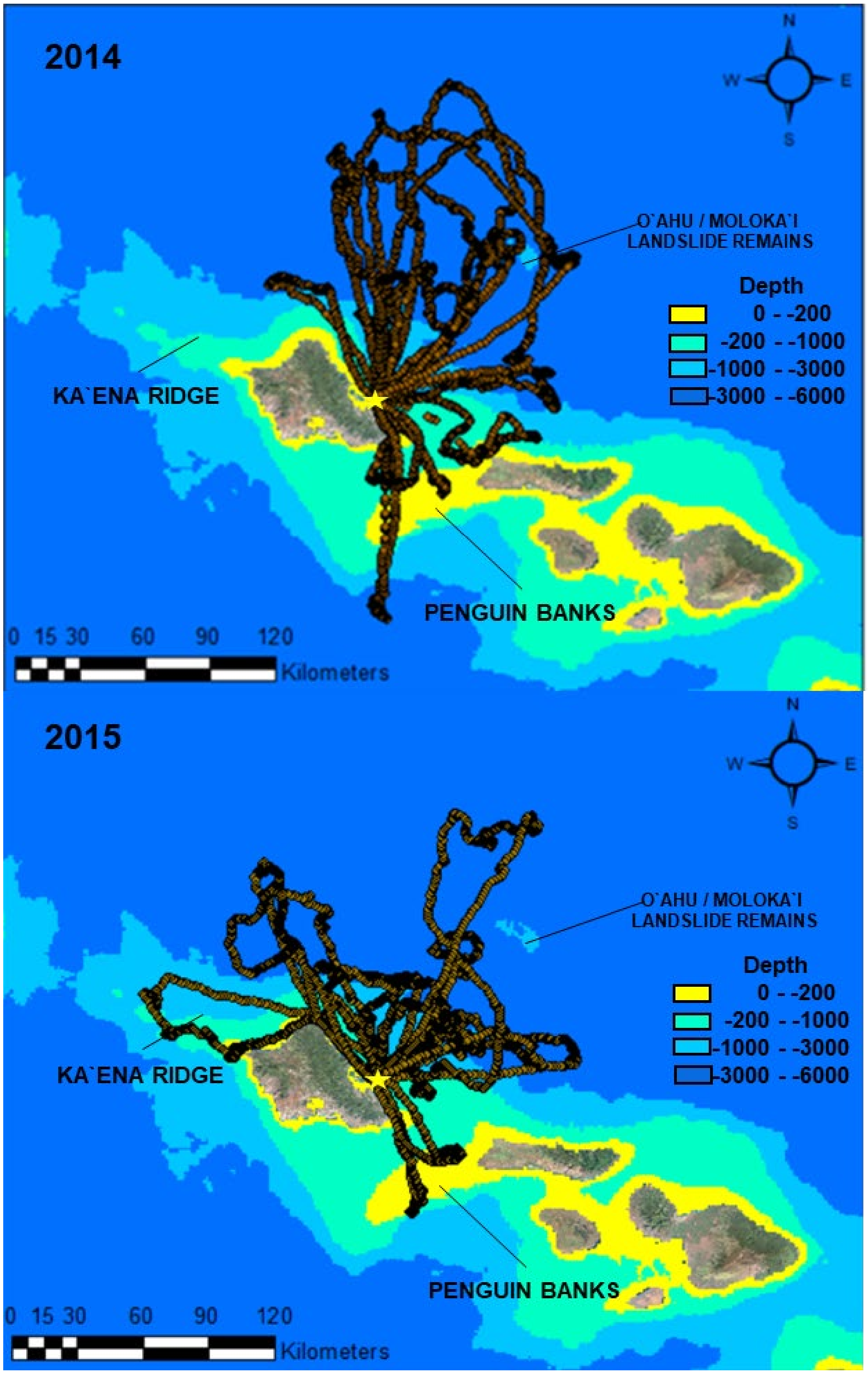


Figure 7. Map of 2014 and 2015 Red-footed Booby tracks.

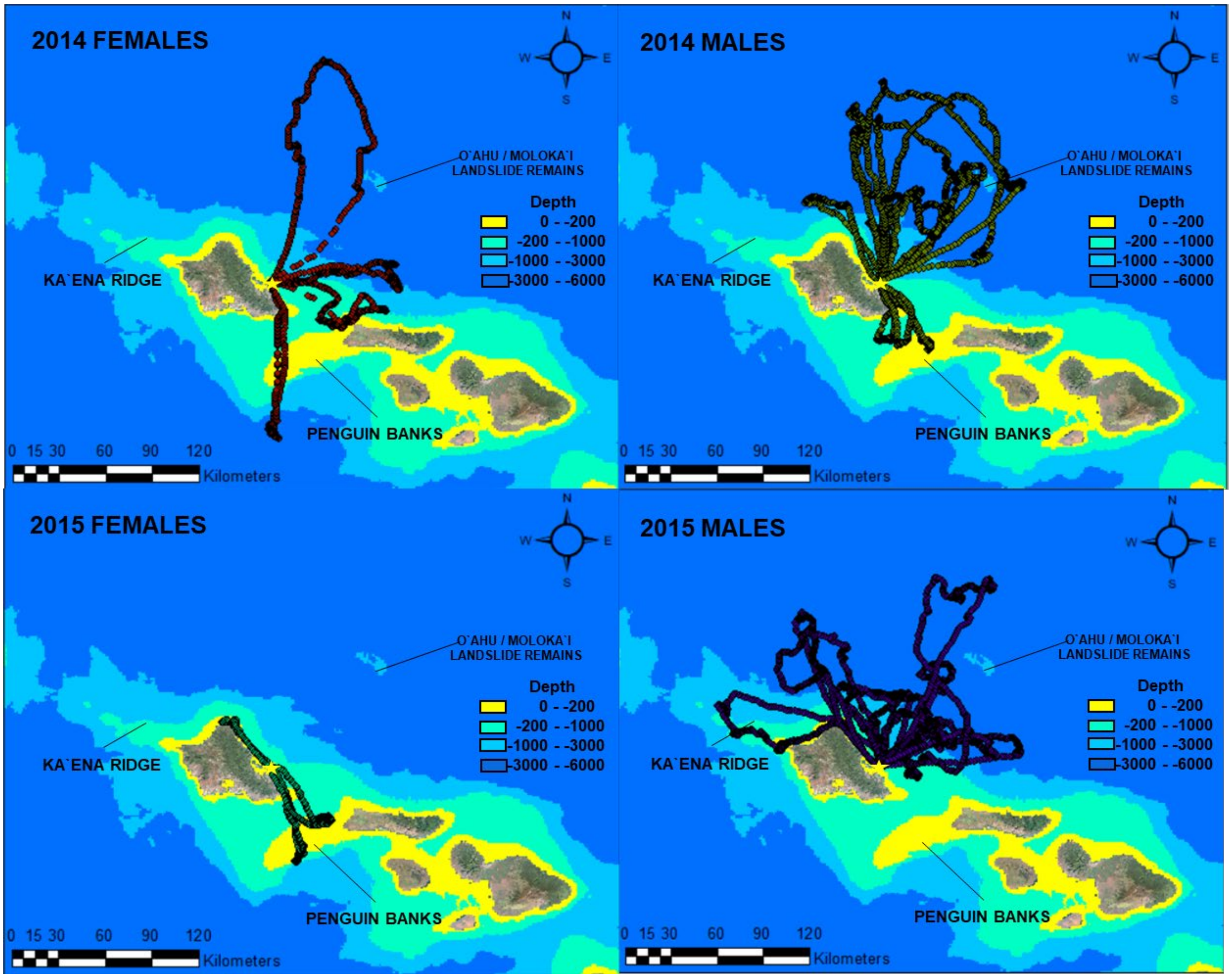


Figure 8. Maps of Red-footed Booby foraging trips by year (2014 & 2015) and sex (Male & Female).

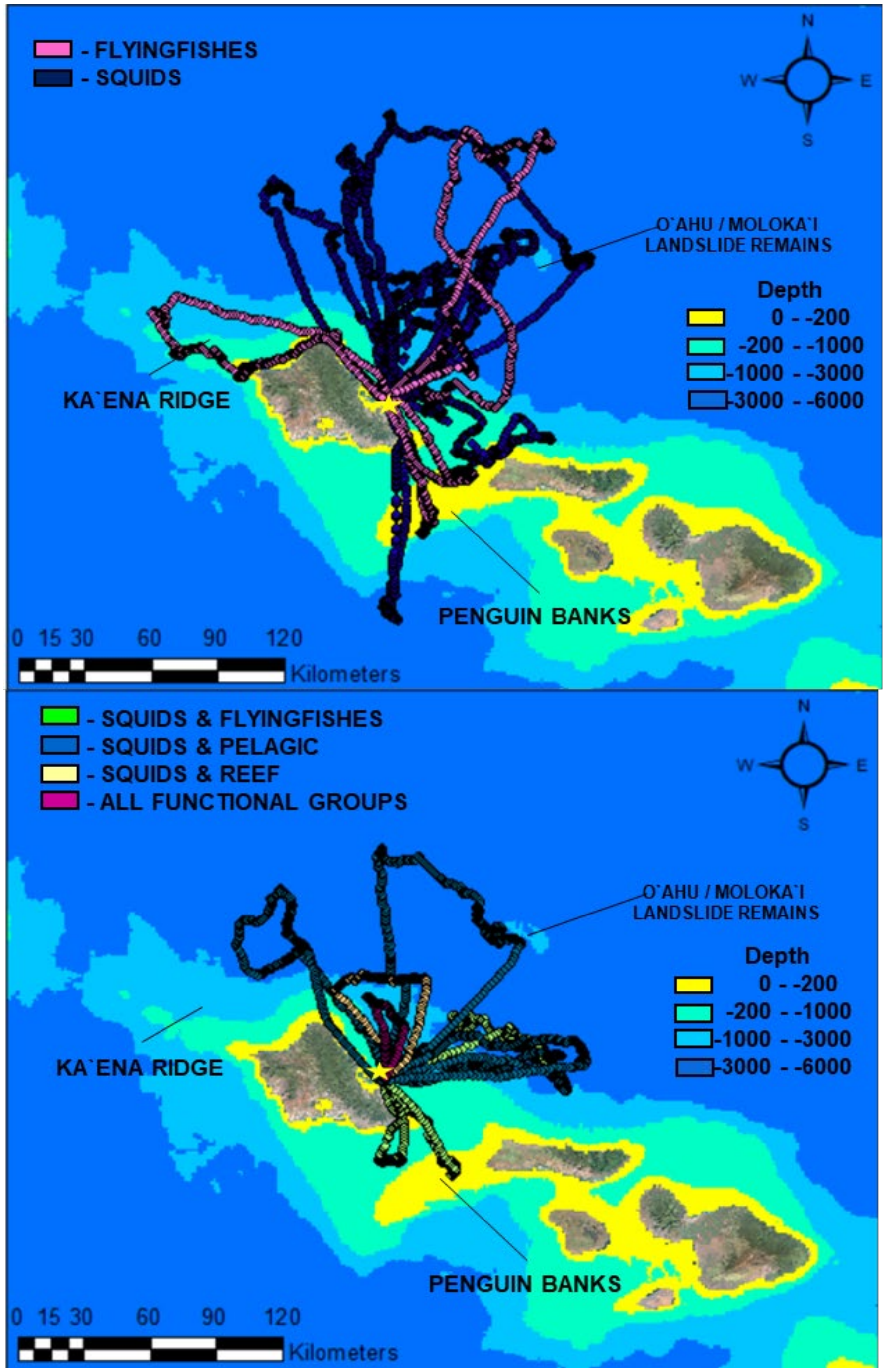
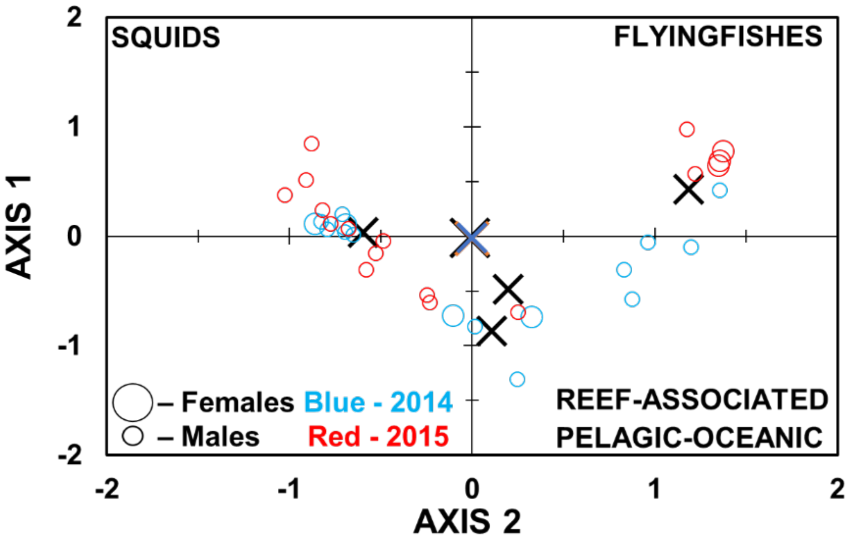


Figure 9. Maps of Red-footed Booby (*Sula sula*) foraging trips based on the occurrence of prey functional groups in the regurgitation samples. The top map shows prey samples that only contained either flyingfish or squids, and the bottom map shows instances where squids were present together with other functional groups. Note that a single trip had all functional groups present.



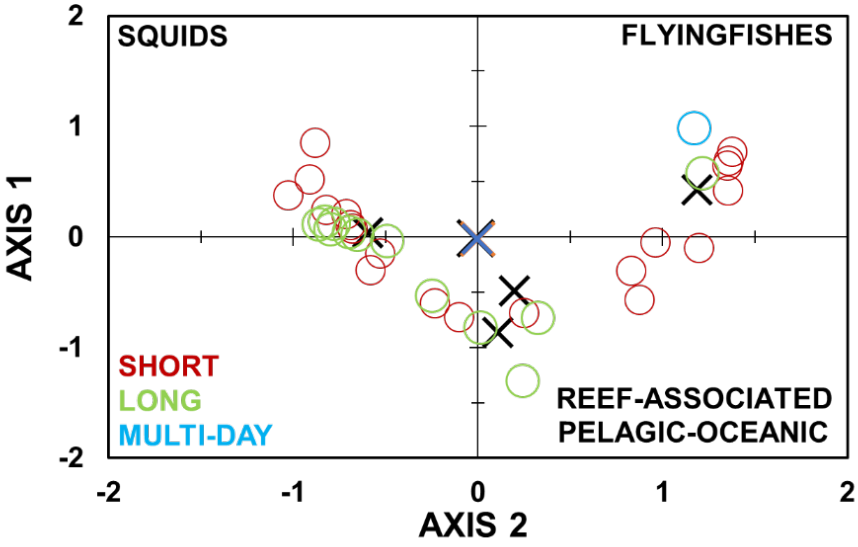


Figure 10. NMDS and MRPP relationships showing the 2-dimensional relationship between trip characteristics and prey functional groups, where distance and duration ("X's" on 0,0) were correlated with Axis 1 and the flyingfish – squids dichotomy was correlated with Axis 2. The top figure shows the 33 track relations by male – female for the two years, 2014 and 2015. The bottom figure shows the 33 track relations by length of trip, short, long and multi-day.

Chapter 4

The Nest Steps:
A summary of results, scientific contributions and future direction

From the results resented in the previous chapters, we can conclude that the diet and foraging behavior of chick-provisioning RFBO changes during years of contrasting oceanographic conditions. In this final chapter, I discuss the broader implications of the results of this thesis, by relating these findings to historical observations from this colony and discussing steps for implementing a long-term effort to monitor RFBO responses to environmental variability using diet and tracking data from the Ulupa'u Crater colony on O\`ahu, Hawai\`i.

Chapter 2 Summary

The purpose of chapter 2 was to develop a comprehensive understanding of the diet of RFBOs during the chick rearing season, using standardized methods augmented with genetic analyses, during two years of contrasting oceanographic conditions (2014, 2015), characterized by changes in basin-wide conditions (MEI and PDO) and regional environmental parameters (SST and WSPD).

A prey shift from fish to ommastrephid squid occurred during the warm-water year leading into an ENSO event. In particular, the Purpleback flying squid (*Sthenoteuthis oualaniensis)* was the most abundant prey species, by mass, identified across the two study years, 22.0% in 2014 and 20.3% in 2015. Based on the mantle lengths of complete squids, these were juveniles spawned in the spring of the same year they were collected (Young & Hirota 1998).

While RFBO ingested other reef-associated and epipelagic / mesopelagic fish species, flyingfish and flying squid were the main prey functional groups in the diet. Nevertheless, the large number of ingested species (29 fish and 3 squid), resulted in a highly diverse diet, suggesting that RFBO foraging behavior is flexible, and that the birds take prey of different sizes and habits.

RFBO also exhibited reverse sexual dimorphism, with larger females than males, similar to other RFBO around the world. When the diets of males and females were compared, the larger-bodied females yielded significantly heavier regurgitation samples. Nevertheless, the relative composition of the diet was not different between males and females during 2014, the year of average environmental conditions. However, there was a significant sexual-segregation in 2015, when females ate more fish and males more squid.

Chapter 3 Summary

The purpose of chapter 3 was to describe the foraging ecology of provisioning RFBO, by relating their diet to their movements during two years of contrasting oceanographic conditions (2014, 2015), while considering differences between males and females.

The three foraging trip characteristics (trip duration, trip range, distance covered) were positively cross-correlated, revealing that RFBOs that engaged in foraging trips of longer duration, venture farther from the colony, and travel larger distances. Therefore, RFBO may be considered "random foragers", which search unpredictable prey patches, rather than commuting to a specific location of predictable prey aggregation (e.g., seamounts, eddies).

These foraging trip characteristics also influenced the total mass and the freshness of the diet samples retrieved at the colony, with the freshness decreasing (higher WAF) and the total mass decreasing as the foraging trip duration increased. Together, these results suggest that the impact of prey digestion is larger, leading to lower prey mass and freshness, when chick-provisioning RFBO take longer foraging trips.

After removing the effect of Julian Day, which lead to longer trips later in the breeding season, the trip characteristics were significantly different by study year. Trip duration, range and distance were larger in 2014, when RFBO took longer foraging trips. Moreover, the trip duration and distance yielded a significant year*sex interaction, whereby females went farther in 2014 and males went farther in 2015.

Scientific Contributions

The augmented diet analysis incorporated Ashmole & Ashmole's (1967) freshness scale, and genetic analysis through DNA extraction and sequencing for identification through the cytochrome oxidase I gene code sequence derived from mitochondrion DNA. Genetic analysis has been recently applied to the analysis of seabird prey, though mainly for quantifying the presence / absence of species by sampling guano, intestinal fluid, and stomach / gizzard contents. These augmented methods were designed to facilitate future comparable and standardized diet studies.

The genetic identification of the prey allowed an in-depth analysis of the diversity of the diet. The Simpson's Diversity Index (SDI) has never been used to describe the diet of tropical seabirds, due to the inability to identify all the prey items to species level. Rather, these analyses usually focus on higher-level taxonomic levels (e.g., families) or address prey functional groups. In chapter 2, I characterize the diversity of the RFBO diet using the SDI and species richness to investigate the degree to which RFBO can be used to sample epipelagic prey communities.

Next research steps will involve the stable isotopic analysis of the prey items that have been genetically identified, to determine their trophic position in relation to the analysis of blood samples from the tracked RFBO. Additionally, these isotopic values will contribute to a prey library of tissues of seabirds, predatory fishes, and prey species. This prey library will also include complete fish bones, correct COI and possibly complete gnome sequences, and morphometric data. This prey library will facilitate future diet studies for the Main Hawaiian Islands and the Central Pacific.

In order to update Genbank with sequences from the central Pacific, I plan to cross reference prey tissue samples from this study to other prey samples, to ensure the accuracy of the Genbank sequences. In particular, identifying flyingfish was difficult because there are few sequences for central Pacific species. The Genbank sequences originate from Japan, the Indian Ocean, or the Atlantic Ocean. This validation may require additional samples from the Main Hawaiian Islands and the central North Pacific, collected by working wither other fishery scientists and seabird ecologists across the North Pacific.

Linking Past, Present and Future

Historical Diet Comparison

The only historical RFBO data from the Ulupa`u breeding site were notes published from five 1963 colony visits over a two-month period (April 1st and June 5th), where they opportunistically collected 12 regurgitations (Ashmole & Ashmole 1967a). Therefore, the methods used in chapter 2 were comparable to those of the Ashmole's, in order to compare the recent diet of this colony (2014, 2015) to the information collected 55 years ago (1963).

These 12 diet samples collected in 1963 contained 108 complete prey items with an average of 9 items per sample (Table 1). The number of prey items within a sample ranged from 1 to 33. Squid was present in 75.0% (9 out of 12) of the samples making up 36% of the diet by number and 39% by volume (Figure 1). All squids were described to be all of the family Ommastrephidae with them all most likely being Purpleback flying squids (Then: *Symplectoteuthis sp*.; Now: *Sthenoteuthis oualaniensis*). The squids ranged from 4 – 8 cm in mantle length and fish 4 – 20 cm standard length (Table 1). As for the fish, Gempylidae (Snake Mackerel) were the most abundant by number but were only present in 5 samples and contributed little to the diet by volume (Table 2). Exocoetidae (flyingfishes) were less numerous but had a higher frequency of occurrence (7 samples) with a larger contribution to the diet by volume (Table 2).

During the current study, I opportunistically collected 106 regurgitations during two tagging periods from June-July 2014 (51 diet samples) and 2015 (55 diet samples) (Chapter 2). From the total 1007 prey items sorted, 259 were complete (Table 1). The average number of prey items was 8.1 +/- 5.7, ranging from 1-24 items within a sample in 2014, and 10.6 +/- 8.4, ranging from 1-34 within in 2015. Prey analysis methods were similar to those of the Ashmole's, except mass (g) was used instead of volume (ml), sizes were not estimated for partially digested prey and species identification used genetic methods. While the current frequency of occurrence of squid (72.5% and 74.6% for 2014 and 2015) was similar to the historical data, squid were more abundant, by both number and mass, than in the past (2014: 45.8% N, 29.8 % M; 2015: 46.2% N, 42.1% M) (Table 2, Figure 1).

Through genetic identification, a total of 32 species (29 fish and 3 squid) were identified, including 9 fish families and 2 squid families. Three of the 11 families (two fish: Acanthuridae, Molidae, and one squid: Onychoteuthidae) were found only in 2014 (Table 2). On a family level, flyingfishes (Exocoetidae) and flying squid (Ommastrephidae) had the highest importance in 2014 (15.6% and 10.8%, respectively) and 2015 (15.0% and 10.6%, respectively) (Chapter 2). Tropical two-winged flyingfish (9.4%, 2014) and glider flyingfish (8.3%, 2015) were the most important fish in the diet. The majority of the identified squid (98.7%; 232 of 235) were Purpleback flying squids, which was the most important species in the diet (10.7% in 2014, 10.5% in 2015). The remaining three squids were identified as two Neon flying squids *Ommastrephes batramii* (one from each year) and one *Onykia species* from 2014.

Fish size frequencies were not compared due to small sample sizes (<20 prey items per sample year). The mantle lengths of the squid sampled during the two study years were compared using a single-factor ANOVA. Squid from 2015 were significantly larger than in 2014 and 1963 (ANOVA F=17.658, df=2,34, p<0.0001) (Figure 2). While the 2015 size distribution involved medium-size individuals, with 96.9% (31/32) ranging from 4 – 8 cm and 60% falling between 6-8 cm, the 2014 size distribution involved a wider range (2 – 10 cm), with 75.8% of squid falling between 4-8 cm. In 1963, the squid mantle lengths ranged from 2 - 8 cm, with 89.5% falling between 4-6 cm. This result highlights the year-to-year variability in squid sizes, likely due to changes in the phenology of spawning and the growth rates in the recent years (2014, 2015). Unfortunately, the observed interannual variability and the lack of more samples from the 1960s, inhibits the interpretation of any long-term changes between historical and the current squid size distributions.

Though there was a significant difference between the squid lengths over the years, this pattern is likely the result of differences in the timing of sample collection and the accompanying environmental conditions. From the current study we have learned that RFBO only eat juvenile squids. In particular, Purpleback flying squid spawn off Eastern O`ahu around March (where the birds are foraging) and are smaller than 11 cm. Since regurgitations were collected from April to June in 1963, this study likely sampled smaller (younger) squids available to seabirds earlier in the year. Alternatively, 2014 and 2015 were characterized by slightly larger squids than 1963 because juveniles grow faster in warmer water.

Nevertheless, despite the 50-year separation between these two diet studies (1960s and 2010s), these analyses concluded that both Ommastrephid squid and flyingfish are of great importance to RFBO rearing chicks at the Ulupa'u Crater colony in O`ahu, Hawai`i, reinforcing the findings from other breeding sites in the central Pacific (Ashmole & Ashmole 1967b; Harrison & Seki 1983; Harrison et al. 1990; Lewis et al. 2005; Young et al. 2010). In particular, flying squid are present in roughly 75% of the diet samples, no matter when they were collected, underscoring the ecological importance of these poorly-studied epipelagic squid.

RFBO "Foraging Footprint"

Because the basin-wide Multivariate ENSO Index (MEI) and the Pacific Decadal Oscillation (PDO) do not capture the changing SST and SSH around Hawai`i (Wolter & Timlin 1993; Mantua et al. 1997), other local indices are needed to characterize local oceanographic conditions within the RFBO foraging "footprint" (Mantua 2005). To this end, the tracking data can be used to delineate the foraging range of the chick-rearing RFBO, a key step for matching local environment conditions with RFBO responses (diet, reproductive success) over time.

The minimum convex polygon (MCP) created, excluded land and incorporated the maximum recorded RFBO foraging area using the perimeter of all 33 tracks, was used to extract SST and bathymetry data from the foraging range. The size of the MCP encompassed 22,211.4

km^2, spanning 119.3 km North-westward 163.8 km North, 76.9 km East, and 83.7 km South from the colony. The depth ranged from 0 m to a little over 6000 m, with the following relative proportion of depth domains within the foraging range: 0 to 200 m, 6.9%; 200 to 1000 m, 14.2%; 1000 to 3000 m, 17.3%; and 3000 to 6000 m, 61.5% (see Figure 5 from Chapter 3).

SST data spanning 26 years, 1990 to 2015, were taken from the GOES-POES monthly dataset (Ocean Watch, LAS through ERDDAP, http://oceanwatch.pifsc.noaa.gov/las/) (Figure 3, 4). This time series provided a long-term context for the two recent study years (2014, 2015), and the foundation for developing additional baselines of local oceanographic environmental conditions for future studies of RFBO productivity and diet, as the local oceanic and atmospheric conditions continues to change.

I compared monthly observations from the MCP **(**22.800 N, 20.500 N, -158.700 W, -156.87 W) during the breeding season (June and July) of both study years (2014, 2015), using paired T-tests. For the paired T-tests, there were 196 observations of SST for each year with 98 observations for each month; therefore, the yearly and monthly SST have the same degrees of freedom. The mean SST of June and July from the same year (Paired T-test; 2014: T= -30.617, df=196, $p<0.0001$; 2015: T= -86.149, df=196, $p<0.0001$) and across years (T-test; June: T= 7.075, df=196, $p<0.001$; July: T= -34.032, df=196; $p<0.0001$) were significantly different with 2015 being significantly warmer, especially in July (Figure 3, 4). In fact, July 2015 was the warmest July, and the warmest month, within the last 26 years.

The analysis of June and July SST within the MCP, spanning the last 26 years, suggested a seasonal warming trend, with ENSO years (1998 and 2015) starting with unusually cooler Junes followed by a spike of at least 1ºC in July (Figure 3). 2015 was the warmest July in the time series (1990 – 2015). This drastic change from a relatively normal June to the hottest July on record could have been responsible for the prey functional group dichotomy of males eating more flying squids and females more flyingfish. To determine this, it would require performing annual spring - summer squid and flyingfish surveys over a few years of contrasting oceanographic conditions, in conjunction with colony-based studies of RFBO diet and productivity. The RFBO "footprint" provides a starting point for linking the local marine ecosystem around O`ahu with changes in RFBO diet and productivity.

Given that the prey shift occurred with the onset of an El Niño event, which are associated with anomalously warm-water conditions in the MHI, a key research question involves determining whether El Niño negatively affect RFBO productivity and reproductive success. On the one hand, the increased abundance of squid in the diet, both in number and by mass, and the larger sizes of the juvenile squids, suggest that this prey was more available to foraging RFBO in 2015. On the other hand, the lower relative importance of fish in the prey during 2015 could impact the RFBO, if these were the highest energy prey. Ultimately, this requires an understanding of the prey preferences of the RFBO, an answer which will involve information on the availability, dispersion, and energetic cost / reward of capturing the different prey types.

A particularly intriguing result is the differentiation of the diet between males and females in 2015, suggesting that males and females targeted different prey resources to minimize competition during a year of poor foraging conditions. Alternatively, the differential sizes of males and females may have modulated their ability to exploit changing squid and fish prey stocks, given the underlying changes in their size distributions.

Thus, this research has opened many unanswered questions, concerning how RFBO respond to changes in the relative abundance, dispersion, and size distributions of their prey.

Therefore, long-term monitoring efforts are needed to answer these intriguing questions. While switching from a fish to a squid diet may affect the energetic balance of RFBO, given the higher water content and lower fat content of squid, there is no data on the energy density of Purpleback flying squids and the various flyingfish species taken by RFBO. Circumstantial diet observations collected during this study suggest there are differences in prey aggregation, which may further influence RFBO foraging ecology. Namely, while those birds that are squid contained many prey items, those birds that ate fish either contained a couple of large flyingfish or many small juvenile reef-associated / pelagic fish (~3-5cm). This observation suggests that the aggregation of the different prey functional groups may also play a key role.

Currently, it is unclear whether the documented prey shifts and changing environmental conditions impact the RFBO colony at Ulupa'u Crater. Considering the breeding population has declined from roughly 1000 nests in the late 1960's to about 200 nests today, there seems to be a long-term decline in the RFBO population at the colony (Russell & Vanderwerf 2010; Pyle & Pyle 2017). Given the large interannual changes of the number of individuals documented during past censuses (1960's to today), it is possible that there have been major reproductive success failures over time, which have gone unnoticed due to the lack of regular monitoring. Thus, a management priority would entail establishing a regular monitoring program, that would allow wildlife managers to better understand the drivers of the RFBO population dynamics at this site, the southern-most breeding colony for the species in the MHI (Russell & Vanderwerf 2010).

During the RFBO breeding and chick rearing season, March to September, the skipjack tuna is the most common predatory fish on the windward side of O`ahu (Senina et al 2008). Skipjack tuna are associated with the warm surface waters, like the Pacific Warm Pool, and shift their distributions during ENSO events, moving eastwards with the warmer unproductive waters

(Lehodey 1997; 1998). This is a common response in many tropical predatory fishes (Lehodey et al. 1997; 1998; Bertrand et al. 2002; Lyday et al. 2015) and seabirds (Ashmole 1971; Ballance et al. 1997; Reid and Croxall 2001, Durant et al. 2009), which respond to changes in oceanographic conditions and prey dispersion by shifting their distributions and diets.

Though RFBOs are considered generalist foragers, foraging with a wide range of SSPs, during the breeding season they forage most often with Skipjack tunas (Hebshi et al. 2008). Thus, understanding how prey shift during changing environmental conditions, we may be able to help managers to anticipate how commercially-valuable fishery species, like the Skipjack tuna, will respond to these oceanographic forcing if these two predators are affected by the same prey shifts and environmental patterns. This ecosystem-level approach can rely on sampling the diet and foraging ecology of seabirds, like the RFBO, that are also easier to study consistently at breeding colonies and at sea while looking into fisheries diet and abundances to determine the association between predators and their environment.

In particular, because seabirds can be easily studied at breeding colonies over time, making them great indicators of long-term environmental changes (Ashmole & Ashmole 1968; Ballance et al. 1997; Surman & Nicholson 2009; Velarde et al. 2015), understanding how and where seabirds forage (through diet and telemetry studies) and how they interact with other marine predators (marine mammals and predatory fishes) can help characterize pelagic food webs and provide a broader ecosystem-level context for the conservation of species within a particular system (Wiemerskirch et al 2005; Bost et al. 2009; Votier et al. 2010; Young et al. 2010; Raymond et al 2014; Mendez et al 2016).

Conclusions

In conclusion, based on all the data and results of this thesis research, I contend that the RFBO is an ideal indicator of the local ocean environment around O`ahu and can be used to sample epi-pelagic prey that are otherwise difficult to sample and study. Future research needs entail collecting colony-based data on bird counts during the breeding season, reproductive phenology, reproductive success, and regurgitation samples for diet studies. Additionally, tracking of the birds during the non-breeding season would be needed to characterize the RFBO habitat use patterns throughout the year. Together, these efforts would allow managers to elucidate the influence of oceanographic variability and changing prey distributions on RFBO productivity and abundance. Furthermore, the integration of these RFBO studies with similar research on other upper-trophic marine predators (tunas, dolphinfish) would help to determine if the RFBO-environment interactions can provide insights into broader ecosystem-level shifts affecting other commercially-valuable species targeted by local commercial and recreational fishers.

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TABLES & FIGURES

Table 1. Size distributions of regurgitated fish and squid, from Red-footed Boobies (*Sula sula*) nesting at Ulupa`u Crater, O`ahu in 1963 and two recent years (2014 and 2015).

Fish Standard Length (cm)

	0 – 4	4 – 8	8 – 12	12 – 16	16 – 20	Total
1963	3	20	34	6	6	69(w/estimations)
2014	0	6	2	7	2	17
2015	0	15	0	3	1 (20.5)	19

Squid Mantle Length (cm)

	2 – 4	4 – 6	6 – 8	8 – 10	10 – 12	Total
1963	1	18	16	3	0	38 (w/estimations)
2014	7	34	35	12	3	91
2015	0	16	66	27	1	110

Table 2. Percent number (N) and mass or volume (M / V) for prey families of Red-footed Booby (*Sula sula*) regurgitations collected from 1963, 2014 and 2015.

	1963		2014		2015	
	%N	%V	%N	%M	%N	%M
Acanthuridae	-	-	0.5	0.2	-	-
Carangidae	-	-	1.4	0.1	4.4	1.2
Exocoetidae	14	38	22.0	35.0	19.7	31.4
Gempylidae	30	9	3.2	2.1	0.5	<0.05
Hemiramphidae	-	-	1.8	2.6	3.8	2.9
Molidae	-	-	0.5	1.3	-	-
Mullidae	-	-	10.6	0.9	6.0	0.4
Nomeidae	-	-	0.5	0.1	3.8	0.5
Scombridae	-	-	2.3	4.6	1.1	0.1
Ommastrephidae	36	39	56.9	22.1	60.7	20.4
Onychoteuthidae	-	-	0.5	<0.05	-	-
Coryphaenidae	3	5	-	-	-	-
Unidentified fish	18	10	-	-	-	-

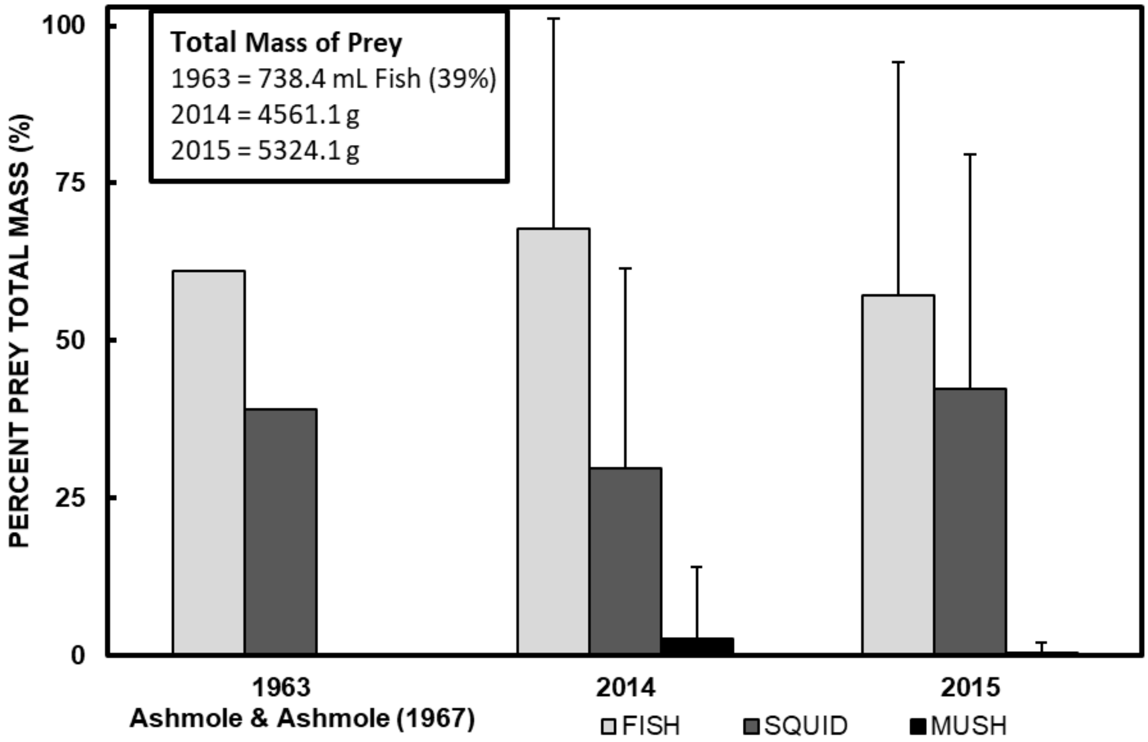

Figure 1. Average (+/- S.D.) percentage of total mass by prey classes (Fish, Squid, Mush) from RFBO (*Sula sula*) regurgiations during this study (2014, 2015), compared to historical (1963) (Ashmole & Ashmole 1967).

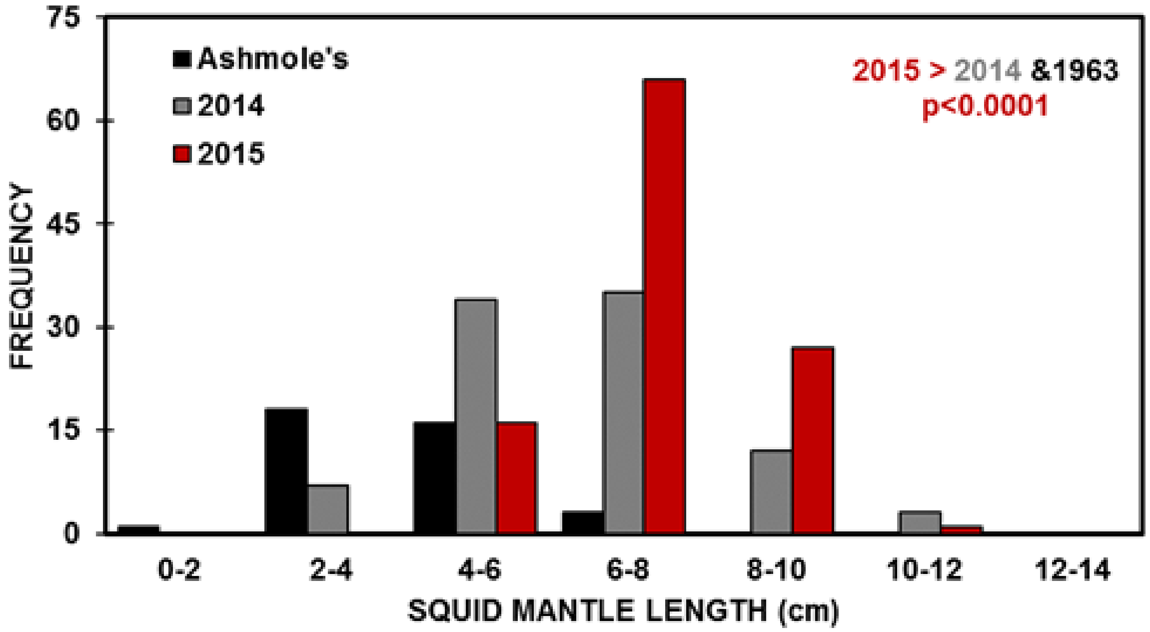

Figure 2. Histogram of squid mantle lengths consumed by RFBO (*Sula sula*) over two recent years (2014 and 2015), compared to historical data 1963 (Ashmole & Ashmole 1967). Squid from 2015 were significantly larger than in 2014 and 1963 (ANOVA $F_{2,238} = 17.658$, $p < 0.0001$).

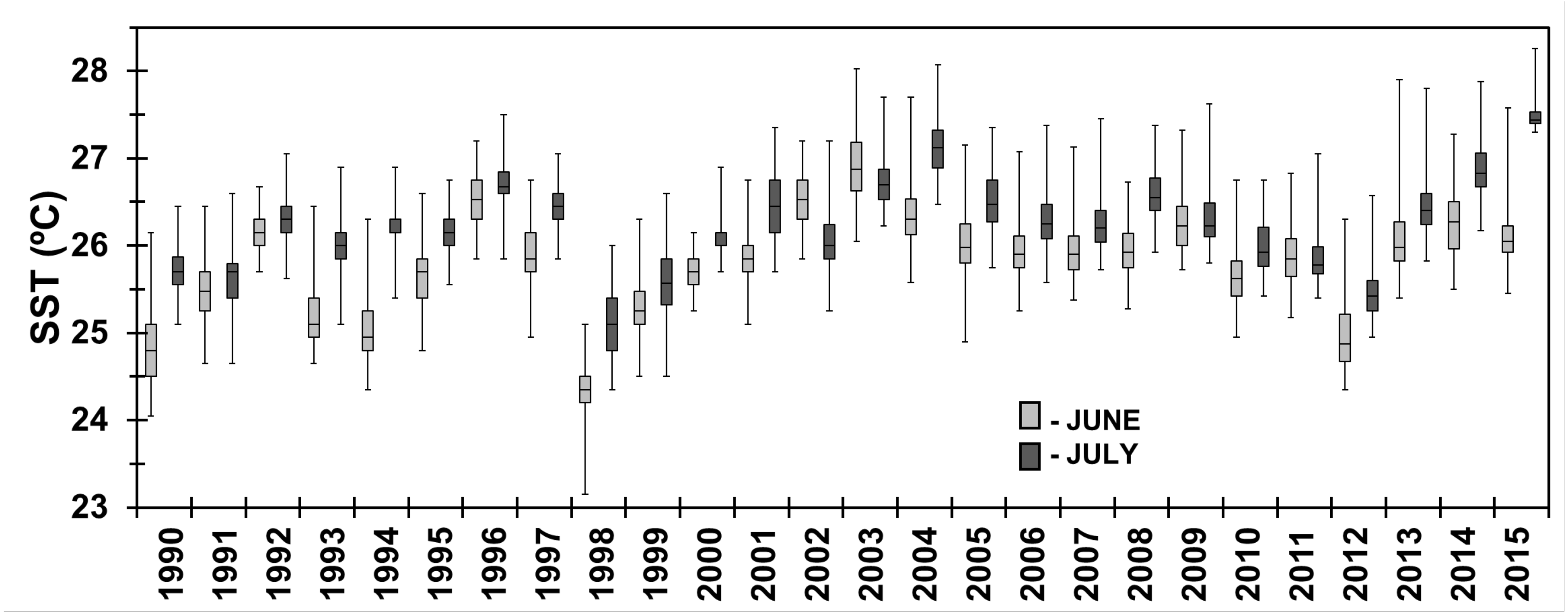

Figure 3. Box plots (5%, 25%, 50%, 75%, 95%) of monthly sea surface temperature (SST, ºC) within the "footprint" of the tracked MCBH Red-footed Boobies (*Sula sula*), calculated during the sampling months of this study, (June and July), for the period 1990 - 2015.

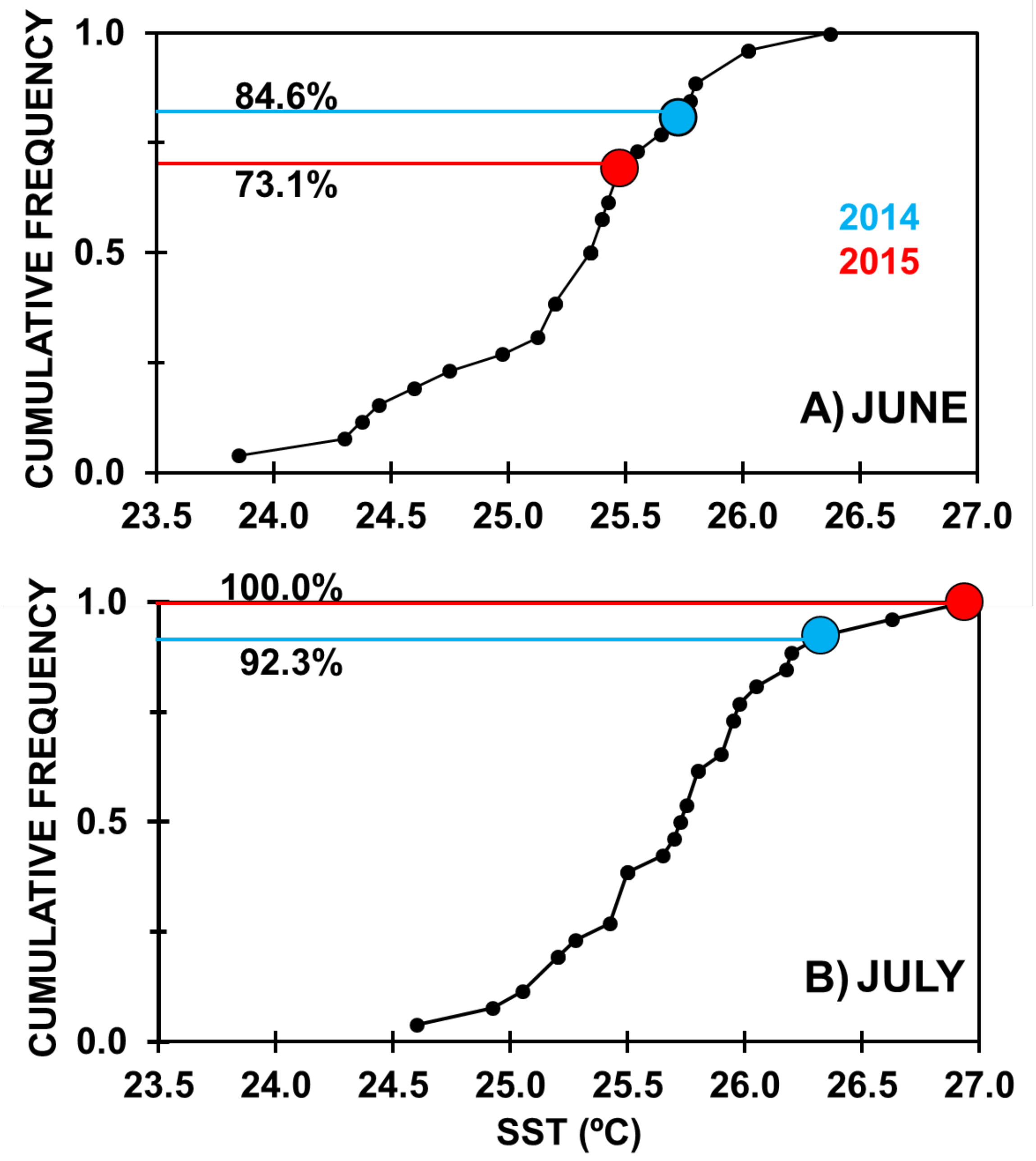

Figure 4. Cumulative frequency distribution of the median sea surface temperature (SST, ºC) within the "footprint" of the tracked MCBH Red-footed Boobies (*Sula sula*) from 1990 to 2015, during the two sampling months of this study, June and July. For each month, the rank of the 2014 and the 2015 values is highlighted.

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