



## Investigating diet patterns of highly mobile marine predators using stomach contents, stable isotope, and fatty acid analyses

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Young, T., Pincin, J., Neubauer, P., Ortega-García, S., and Jensen, O. P. Investigating diet patterns of highly mobile marine predators using stomach contents, stable isotope, and fatty acid analyses. – ICES Journal of Marine Science, doi:10.1093/icesjms/fsy025.

Received 7 September 2017; revised 20 February 2018; accepted 23 February 2018.

Determining what animals eat is simultaneously challenging and yet also critical for ecologists, fisheries scientists, and resource managers. The tools of trophic ecology have expanded considerably in the last half century in pursuit of this goal. In this study, we combined stomach contents, stable isotope, and fatty acid analyses to investigate trophic patterns in three species of highly mobile, pelagic predators: striped marlin (*Kajikia audax*), blue marlin (*Makaira nigricans*), and common dolphinfish (*Coryphaena hippurus*). We were particularly interested in examining individual diet specialization among these species. We compared the short-term stomach contents with long-term diet proportions estimated from stable isotope ratios and fatty acid profiles using a Bayesian mixing model. Our results indicate that all three species feed on a mix of prey types. This work furthermore suggests that individuals of all three predator species demonstrate generalist feeding habits, with minimal differences in long-term (weeks, months) diet estimates between individuals with different short-term (hours, days) stomach contents. This novel, three-part analytical approach can elucidate complex and otherwise elusive trophic dynamics.

**Keywords:** apex predator, Baja California Sur, diet consistency, FastinR, fatty acid methyl ester (FAME) analysis, gut content analysis, individual diet specialist, individual variation, Mexico, resource specialization, trophic specialization

### Introduction

Discerning what individual animals eat is a central ecological challenge. Scientists use a wide range of approaches to get at this question. Early work in this field used primarily direct observational approaches, such as behavioral studies (Heinrich, 1976; West, 1986; Estes *et al.*, 2003), and stomach content analysis (Werner and Sherry, 1987; Schindler *et al.*, 1997). In recent decades, stable isotope (SI) analysis has become a common ecological tool, used to illuminate food sources and relative trophic level, integrated over weeks or months (Layman *et al.*, 2012). Researchers have compared the long-term, integrated information from SI signatures with the short-term, “snapshot” data provided by direct observation to elucidate individual diet patterns over time (Beaudoin *et al.*, 1999; Araujo *et al.*, 2009; Newsome *et al.*, 2009).

Fatty acid (FA) analysis has emerged more recently as an additional powerful tool in the trophic ecology toolbox. FAs are the molecular building blocks of lipids, typically 12–24 carbon atoms long, with 0–6 double bonds. Over 70 FAs are commonly found in nature. The composition of longer chain ( $\geq 14$  carbon atoms long) FAs in vertebrate tissues typically reflects dietary FA composition because monogastric vertebrates conduct minimal endogenous long-chain FA biosynthesis or alteration (Dalsgaard *et al.*, 2003; Budge *et al.*, 2006; Iverson, 2008). Like an SI ratio, the FA signature—or proportion of different FA molecules—of vertebrate tissue can be used to infer diet integrated over weeks or months. But while a single SI ratio can typically differentiate between only two dietary groups, multivariate analytical techniques and mixing models (Iverson *et al.*, 2004; Galloway *et al.*, 2015; Neubauer and Jensen, 2015) can use FAs found in consumers and

prey to estimate contributions of multiple prey items to predator diets.

In this study, we used stomach content, SI, and FA analyses in combination to examine diet patterns in three well known, highly mobile, marine fish predator species: striped marlin (*Kajikia audax*), blue marlin (*Makaira nigricans*), and common dolphin-fish (*Coryphaena hippurus*). This study is one of only a few we know of to combine all three of these analytical tools (see Schmidt *et al.*, 2006; Tierney *et al.*, 2008), and as such allowed examination of both short-term and long-term trophic patterns on the level of both the species and the individual organism. It also contributes to the larger body of work on trophic habits of marine top predators (Young *et al.*, 2015).

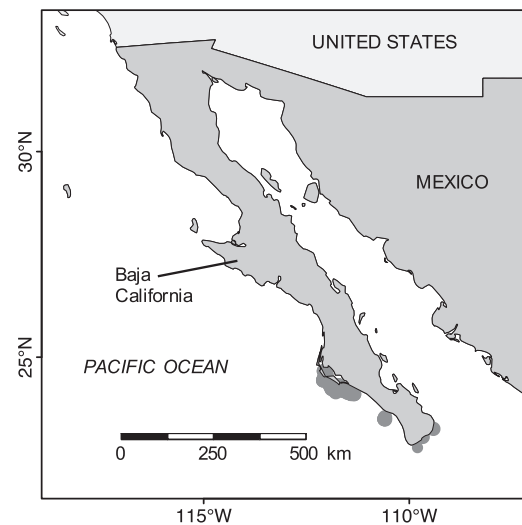
All three of these study species are well established as generalist predators on a wide range of prey types and taxa, including fish, cephalopods, and crustaceans (Evans and Wares, 1972; Abitia-Cardenas *et al.*, 1999; Olson and Galván-Magaña, 2002; Abitia-Cardenas *et al.*, 2011). These kinds of generalist populations may, on the one hand, be comprised of a host of generalist individuals, each of whom consume a wide array of prey types. On the other hand, a generalist population may result from a group of individuals each specializing on a different and narrow set of resources, giving the impression of an overall generalist population (Bolnick *et al.*, 2003). We were interested, therefore, to not only compare diet patterns among the three species, but also to see if these tools could be used to explore individual diet specialization within each species. Density-dependent resource competition is a well-documented ecological driver of individual variation in resource use (Schindler *et al.*, 1997; Bolnick *et al.*, 2007; Tinker *et al.*, 2008; Araujo *et al.*, 2011; Matich *et al.*, 2011). This pattern is generally attributed to increases in foraging efficiency resulting from specialization; consumers that specialize on particular food types can improve foraging efficiency on those foods over time in comparison to more generalist feeders (Werner *et al.*, 1981; Persson, 1985; Lewis, 1986; Sutherland, 1987). Highly mobile species are often not subject to the same classic constraints of density dependence as more stationary or spatially limited species; organisms that range across large areas or migrate long distances are likely to pass through environments with highly patchy resource availability (Parrish, 2000). We hypothesized that these highly mobile species might be better served by flexible generalist feeding strategies rather than specialist ones.

## Methods

### Sample collection

Predator and prey samples were collected from commercial and recreational fishermen fishing in coastal waters of Baja California Sur (BCS), Mexico (Figure 1). The commercial vessels used long-line gear and operated 50 miles or more off the western coast of the peninsula. The recreational fishery used hook-and-line and typically fished within 50 miles of shore around the tip of BCS. This region holds some of the highest abundances in the world for our three study species: striped marlin (*K. audax*), blue marlin (*M. nigricans*), and common dolphinfish (*C. hippurus*) (Squire and Au, 1990; Ortega-García *et al.*, 2003; Jensen *et al.*, 2010).

Samples were collected from 70 predator individuals (42 striped marlin, 16 blue marlin, and 31 dolphinfish) and 359 prey individuals from 36 fish species, 4 cephalopod species, and 1 crustacean species (Supplementary Table S1). All predator samples were collected in 2011. The majority (292 = 81%) of prey samples



**Figure 1.** Map of the study area. Collection locations are shown in dark shading.

were collected in 2011, with a few additional samples collected in 2010 and 2012.

After capture, predators were measured, weighed, sexed, and stomachs removed for analysis. Tissue plugs were collected from fish dorsal muscle, and from cephalopod mantle for chemical analysis. Small prey individuals were collected whole. About half of the prey samples (45%) came from items found in predator stomachs and the remainder (55%) from prey individuals captured live. Samples were kept on ice for < 48 h following collection and then vacuum-sealed and frozen at  $-5^{\circ}\text{C}$  and shipped to the laboratory where they were stored at  $-80^{\circ}\text{C}$  prior to analysis. Prey species were grouped into one of four categories clearly distinguishable based on FA profiles: (1) fish other than sardines, (2) sardines, (3) cephalopods, and (4) pelagic red crab.

### Stomach content analysis

Stomach contents of each individual predator were identified to the lowest identifiable taxonomic group (per Torres-Rojas *et al.*, 2014). Stomach contents were pooled into one of the prey categories mentioned above by percent of total prey weight (%W) (Chipps and Garvey, 2007). Each predator was classified into one of four groups based on its stomach contents: those predators with primarily fish (other than sardines) in their stomachs, those with primarily cephalopods, those with primarily pelagic red crabs, and those with empty stomachs, corresponding to the prey categories above in order to allow comparisons to diet estimates as described below. Although sardines were one of the primary prey categories, there were no predators with primarily sardines in their stomachs, so none were classified as such.

### SI analysis

Tissue samples for SI analysis were freeze-dried at  $-70^{\circ}\text{C}$  and ground by hand. Bulk  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  analysis was conducted at the University of California Davis (UCD) Stable Isotope Facility on a PDZ Europa 20-20 isotope ratio mass spectrometer. Isotope results are expressed using delta ( $\delta$ ) notation: parts per thousand (‰) from a standard (Vienna PeeDee Belemite for carbon and air for nitrogen) (Peterson and Fry, 1987). Twenty-four duplicate

and five triplicate samples were analysed. Mean standard error for replicated samples was less than 0.6‰ for  $\delta^{13}\text{C}$  and less than 0.4‰ for  $\delta^{15}\text{N}$ . Analytical error (standard deviation of replicate reference material) was less than 0.3‰ for  $\delta^{13}\text{C}$  and less than 0.5‰ for  $\delta^{15}\text{N}$ . A mathematical lipid correction was applied to  $\delta^{13}\text{C}$  values based on Hoffman *et al.* (2015). In order to compare predator and prey SI signatures, trophic fractionation factors of 1.8‰ for  $\delta^{13}\text{C}$  and 1.9‰ for  $\delta^{15}\text{N}$  were used, based on Madigan *et al.* (2012).

### FA analysis

Tissue samples for FA analysis were freeze-dried at  $-70^{\circ}\text{C}$ . Lipids were extracted from each sample based on a modified Folch *et al.* (1957) method and methylated according to Galloway *et al.* (2012). Fatty acid methyl esters (FAMES) were analysed by gas chromatograph (Shimadzu Gas Chromatograph 2010 with a flame ionization detector and a  $60\text{ m} \times 0.25\text{ mm ID} \times 0.25\mu\text{m}$  film TR-FAME column from Thermo Scientific). To identify FAMES, retention times were standardized to the easily identifiable peak of C16: 0 (palmitic acid), and compared with those of FAME standards and FAMES identified in a subset of samples analysed on a gas chromatography mass spectrometer (TRACE Ultra/Polar Q by Thermo) at Dalhousie University.

Sixteen specific FAMES were selected for use in assessing trophic relationships between predators and prey (Supplementary Table S2): nine thought to be dietary only, and seven thought to be either dietary or biosynthetic in origin (Iverson *et al.*, 2004; Budge *et al.*, 2012). Zero values were changed to 0.001: lower than the minimum detection limit (0.002), but not an extreme outlier (Iverson *et al.*, 2002; per 2002). After replacement of zero values, percentage values for the sixteen FAMES were renormalized to 100% before diet estimation and statistical analysis.

### Diet estimation based on SI and FA data using mixing model

Diet proportions based on SI and FA data (not stomach contents) were estimated using the Bayesian mixing model FastInR (Neubauer and Jensen, 2015), for each of the four stomach contents groups for each predator species. For example, diet proportions were estimated for the group of striped marlin whose stomach contents consisted mainly of pelagic red crab, and separate analyses were conducted for the striped marlin whose stomach contents consisted primarily of cephalopods, those with stomach contents of fish other than sardines, and those with empty stomachs. Dolphinfinch were included as potential prey items for striped marlin and blue marlin predators but not for dolphinfinch predators.

Data input into the mixing model included carbon and nitrogen SI ratios as well as data from seven polyunsaturated FA thought to be primarily dietary in origin (Iverson *et al.*, 2004; Budge *et al.*, 2012): C18: 2n-6, C20: 2n-6, C20: 4n-6, C20: 5n-3, C22: 5n-6, C22: 5n-3, and C22: 6n-3. Those seven FA accounted for 99% of the total cumulative separation in the dataset (Supplementary Table S2). Other model input values are listed in Supplementary Tables S2–S4. The model was run with two chains for 1 000 000 iterations (burn-in: 100 000), thinning every 50th iteration. Convergence was assessed using Gelman-Rubin diagnostics and visual inspection of trace plots.

### Assessment of diet specialization over time

Diet specialization was assessed using a permutational, non-parametric, multivariate analysis of variance (PERMANOVA) (Oksanen *et al.*, 2013; Anderson, 2001) to compare SI ratios and FA proportions among groups of predators within each species (i.e. striped marlin with fish in their stomachs vs. striped marlin with cephalopods in their stomachs vs. striped marlin with pelagic red crabs in their stomachs, and so on for each predator species). A PERMANOVA was used because it does not require normal distribution of data; FA data are compositional and not normally distributed. FA data were transformed using a centred log-ratio (clr) transformation prior to analysis because SI and FA data were on different scales. Unless otherwise specified, all analyses were conducted in R (R Core Team, 2013).

## Results

### Overall predator and prey information

Stomach contents, carbon and nitrogen SI ratios, and FA signatures were collected from 70 predator individuals: 42 striped marlin, 16 blue marlin, and 31 dolphinfinch (Table 1). Striped marlin were evenly distributed across all four seasons. Blue marlin were caught primarily in summer and fall, and dolphinfinch were caught primarily in spring, summer, and fall. Five of the 42 striped marlin, six of the 16 blue marlin, and seven of the 31 dolphinfinch had empty stomachs.

### Stomach content analysis

Stomach content analysis suggested that all three predator species had recently eaten mostly fish other than sardines (Figure 2a). Most of the striped marlin (30/37) and dolphinfinch (21/24) individuals with prey in their stomachs, and all of the blue marlin individuals with prey in their stomachs, had recently eaten predominantly (56–100%) fish. Six striped marlin and two dolphinfinch had predominantly (54–100%) cephalopods in their stomachs. One striped marlin and one dolphinfinch had mostly (50–83%) pelagic red crabs in their stomachs. No predators had primarily sardines in their stomachs. The two dolphinfinch with cephalopod stomach contents were significantly longer than the dolphinfinch with fish in their stomachs (Welch's *t* test:  $t = 4.90$ ,  $df = 2.41$ ,  $p = 0.03$ ). Stomach contents are reported in detail in Supplementary Table S1.

### SI analysis

Carbon and nitrogen SI ratios did not resolve prey groups with any clarity except for pelagic red crab, which had significantly lower  $\delta^{15}\text{N}$  than the other prey items (Welch's *t* test:  $t = -6.24$ ,  $df = 17.05$ ,  $p < 0.001$ ) (Figure 2c). The only pelagic red crab collected in 2010 (fall) had an unusually enriched  $\delta^{13}\text{C}$  value, and the only sardine collected in the winter (2011) had an unusually enriched  $\delta^{15}\text{N}$  value, but in both cases those outlier points had no effect on the resolution of the prey groups.

The three predator species had comparable  $\delta^{13}\text{C}$  values. Striped marlin had significantly higher  $\delta^{15}\text{N}$  values than did blue marlin and dolphinfinch (Welch's *t* test:  $t = 9.90$ ,  $df = 86.82$ ,  $p < 0.001$ ) (Figure 2c). No obvious relationships between length and carbon or nitrogen isotope signature were apparent for any of the three predator species (Supplementary Figure S1).

Striped marlin caught in winter demonstrated significantly more depleted  $\delta^{13}\text{C}$  ratios (Welch's *t* test:  $t = -2.99$ ,  $df = 17.32$ ,  $p = 0.008$ ) and higher and narrower  $\delta^{15}\text{N}$  ratios (Welch's *t* test:

**Table 1.** Sample sizes, season caught, and size range of predators analysed for stomach contents, SI ratios, and FA profiles.

Predator	Scientific name	N	Season caught				Fork length range (cm)
			Spring	Summer	Fall	Winter	
Striped marlin	<i>Kajikia audax</i>	42 [5]	10	10	9	13	151–221
Blue marlin	<i>Makaira nigricans</i>	16 [6]	0	4	12	0	204–270
Dolphinfish	<i>Coryphaena hippurus</i>	31 [7]	9	9	12	1	58–143

Numbers in brackets indicate individuals with empty stomachs.

$t = 3.67$ ,  $df = 33.09$ ,  $p < 0.001$ ) than those caught in other seasons (Supplementary Figure S2). The single dolphinfish individual caught in winter also demonstrated enriched  $\delta^{15}\text{N}$  and depleted  $\delta^{13}\text{C}$  values. None of the blue marlin were caught in winter. There were no significant isotopic trends by sex. SI values by prey species are reported in Supplementary Table S5.

### FA analysis

The four prey groups (sardines, non-sardine fish, cephalopods, and pelagic red crabs) were chosen because they separated out clearly based on their FA profiles (Figure 2d). The seven FAMES which contributed to 99% of the variation between the prey groups in the non-metric dimensional scaling (NMDS) analysis were all long-chain, unsaturated FAMES thought to be dietary rather than dietary or biosynthetic (Dalsgaard *et al.*, 2003; Iverson *et al.*, 2004; Budge *et al.*, 2012) (Supplementary Table S2). The two FAMES which contributed the most to the separation were docosahexaenoic acid (DHA, C22: 6n-3), and eicosapentaenoic acid (EPA, C20: 5n-3) (Supplementary Table S2). FAME percentages by prey species are reported in Supplementary Table S6.

### Diet estimation

Estimated diet proportions from the mixing model differed clearly among the three predator species, but not among stomach content groups within each predator species. For example, dolphinfish demonstrated higher estimates of (non-sardine) fish than either marlin species did (Figure 3). But none of the predators with (non-sardine) fish in their stomachs showed higher diet estimates of (non-sardine) fish (Figure 2a), nor did predators with cephalopods (Figure 2b) or pelagic red crabs (Figure 2c) in their stomachs have noticeably higher contributions of those items to their diets according to the results of the mixing model.

### Assessment of diet specialization over time

The PERMANOVA supported the diet estimates, indicating that the FA and SI data were not significantly between different stomach content groups for any of the predator species (striped marlin with fish, cephalopod, or crab in stomachs ( $F_{3, 38} = 1.12$ ,  $p = 0.34$ ); blue marlin with fish or cephalopod in stomachs ( $F_{2, 13} = 0.71$ ,  $p = 0.69$ ); dolphinfish with fish, cephalopod, or crab in stomachs ( $F_{3, 27} = 0.71$ ,  $p = 0.71$ ).

### Discussion

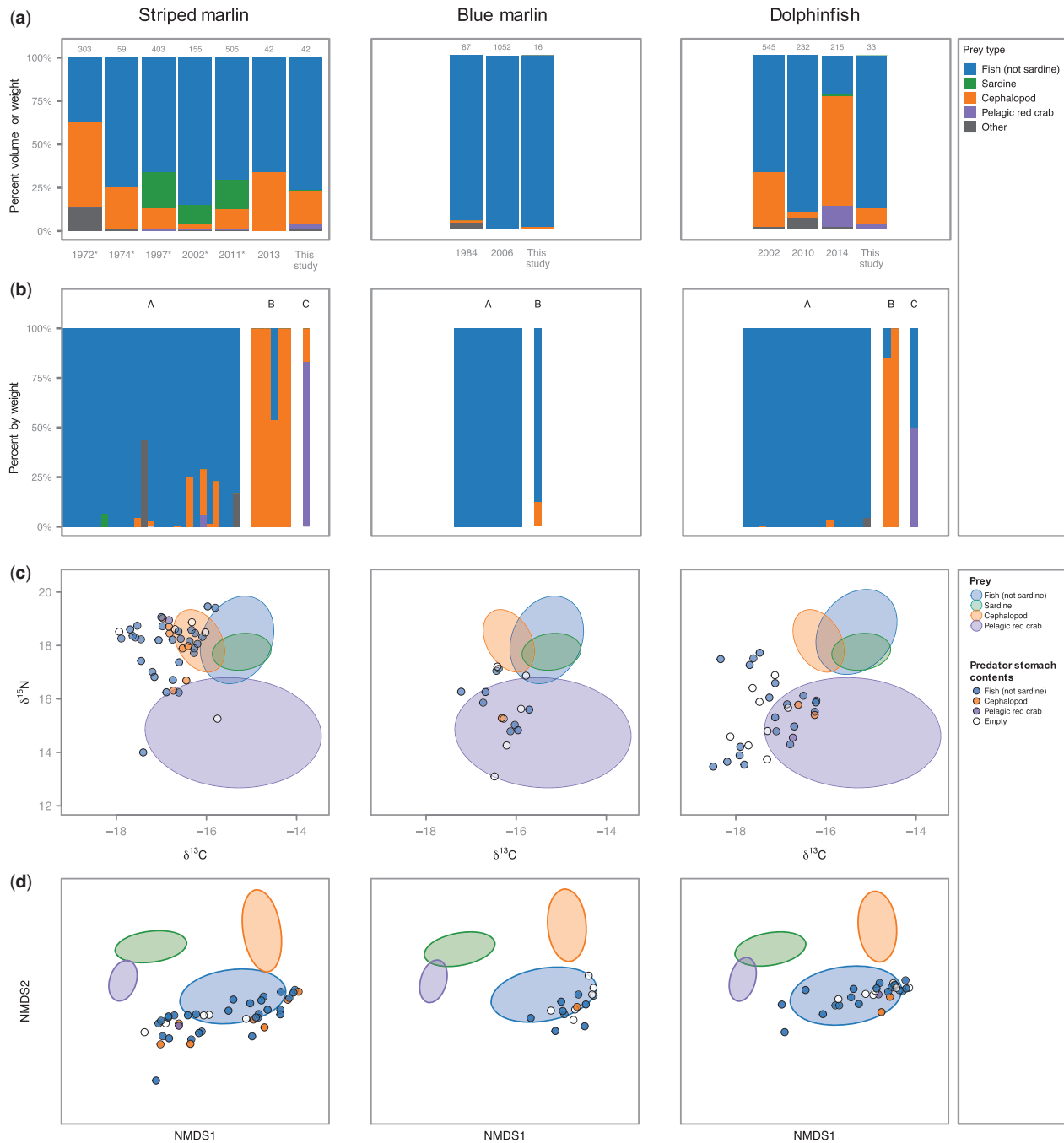
The combination of stomach content, SI, and FA data employed here enabled a higher resolution analysis of long-term trophic patterns that would have been possible with any one or two of the data sources. Stomach contents are a rich and descriptive data source, but can provide only a snapshot of what an organism has just eaten, with no historical perspective. SI have deepened

possible trophic analyses enormously, but our results underscore limited utility of carbon and nitrogen SI analysis alone in distinguishing among a diverse group of prey species in this pelagic context. Without a strong littoral influence, most of the prey had similar  $\delta^{13}\text{C}$  ranges and trophic levels, and were not resolvable through carbon and nitrogen SI signatures (Figure 2c). In contrast, the FA profiles were able to resolve four separate prey groups with clarity, providing a temporal contrast to the stomach content “snapshot” (Figure 2d).

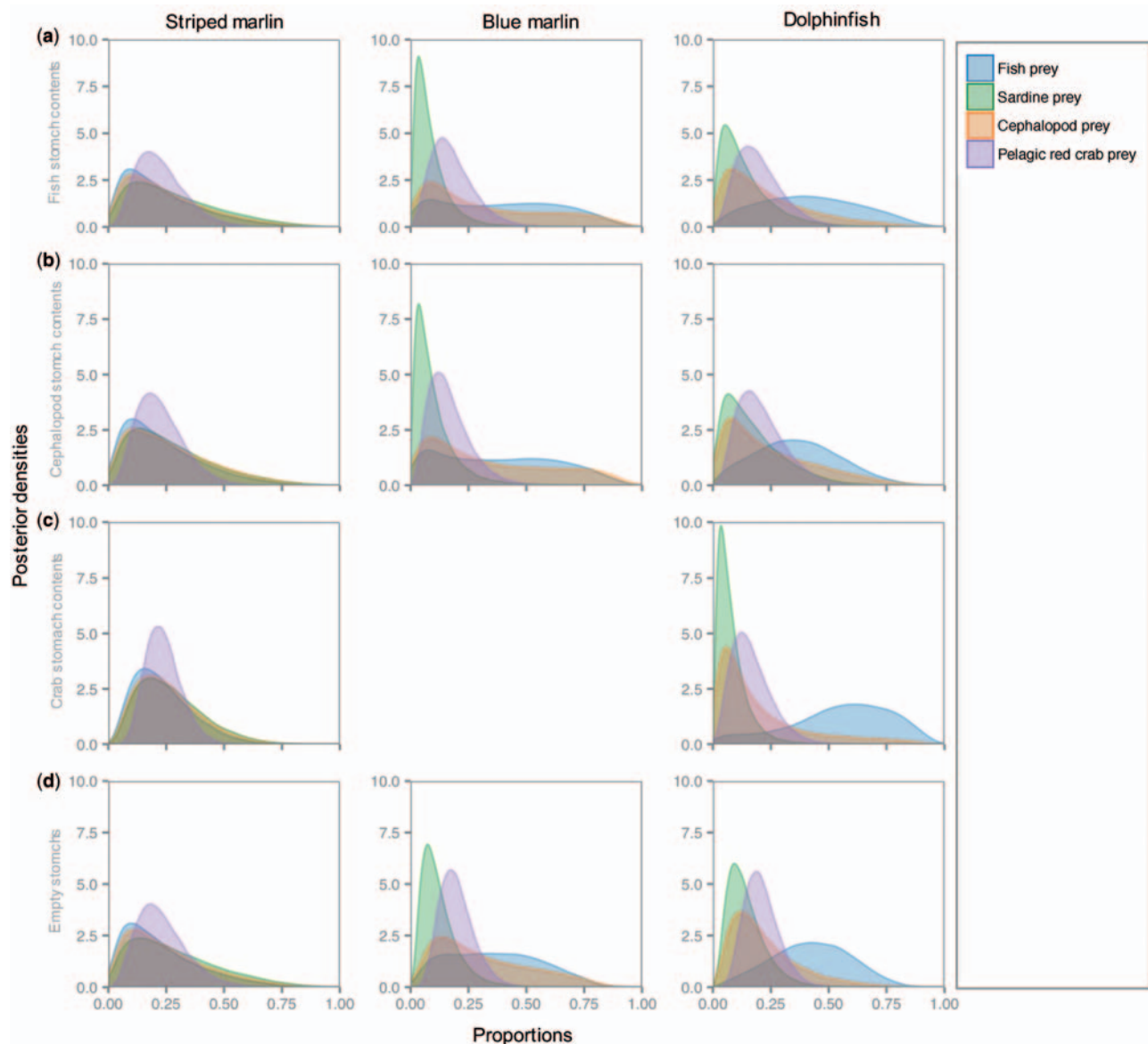
Our analysis suggests some differences in diets among the three predator species. For example, the diet estimates based on the combined SI and FA data suggest that the striped and blue marlin were eating a fairly even mix of fish and cephalopods, while dolphinfish were eating more (non-sardine) fish than other prey items. These results are in line with earlier stomach content analyses for these species (Figure 2a) (striped marlin: Evans and Wares, 1972; Eldridge and Wares, 1974; Abitia-Cardenas *et al.*, 1997, 2002, 2011; Torres-Rojas *et al.*, 2014; blue marlin: Brock, 1984; Shimose *et al.*, 2006; dolphinfish: Olson and Galván-Magaña, 2002; Tripp-Valdez *et al.*, 2010; Torres-Rojas *et al.*, 2014). A number of studies have found that cephalopods comprise a larger portion of the diet for larger dolphinfish individuals than for smaller ones (Massutí *et al.*, 1998; Olson and Galván-Magaña, 2002; Varghese *et al.*, 2013). Interestingly, in this study, the two dolphinfish with cephalopods in their stomachs were longer than the others, but the mixing model indicated that cephalopods did not comprise a significant long-term fraction of the prey for those individuals, compared to other diet items or compared to individuals with stomach contents other than cephalopods.

This analysis also suggests that the striped marlin, blue marlin, and dolphinfish individuals sampled here all appear to be more generalist predators rather than specialist ones. These generalist diet patterns are especially interesting given well-documented and extensive individual variation in habitat use among these species. For example, tagging work suggests that certain individual striped marlin remain in a fairly limited spatial range, while others traverse longer distances (Holts and Bedford, 1990). In tagging studies of blue marlin, some individual fish have been shown to spend substantially more time below the thermocline than their tagged peers (Block *et al.*, 1992; Graves *et al.*, 2002), and individual variation in depth preference has also been documented in striped marlin (Holts and Bedford, 1990). But such individualized patterns in habitat use may not result in long-term differences in food consumption. These results suggest that highly mobile predators may employ generalist rather than specialist feeding strategies at the individual level; this feeding pattern may be an adaptation to a foraging environment in which patchy resource availability presents a greater constraint than competition.

Our results about generalist feeding patterns in these predator species are far from definitive. Our sample sizes were limited (especially for blue marlin,  $n = 16$ ), and our study was able to capture



**Figure 2.** Diet data for striped marlin, blue marlin, and dolphinfish, including: (a) overall stomach contents data from this study in comparison to published data, (b) Stomach contents by individual predator, (c) carbon and nitrogen SI data, and (d) results from NMDS on 18 dietary FAs. In (a), numbers below bars indicate year of study (\* indicates percent volume; other studies are percent weight), and numbers above bars indicate sample sizes (including empty stomachs). Previously published stomach contents data from: striped marlin: Evans and Wares, 1972; Eldridge and Wares, 1974; Abitia-Cardenas *et al.*, 1997; 2002; 2011; Torres Rojas *et al.*, 2013; blue marlin: Brock, 1984; Shimose *et al.*, 2006; and dolphinfish: Olson and Galván-Magaña, 2002; Tripp-Valdez *et al.*, 2010; Torres-Rojas *et al.*, 2014. All previously published studies included here were conducted near our study region, with the exception of the blue marlin data which came from Hawaii and Japan, respectively, because there were no percent volume or percent weight data available from the eastern Pacific. In (b), predators are grouped by their stomach contents, for example, “fish eaters” or “cephalopod eaters.” In (c) and (d), small circles indicate individual predators, with colors indicating stomach contents (e.g., orange small circles are predators who had primarily cephalopods in their stomachs, with white circles denoting empty stomachs), and shaded ellipses indicate prey ranges. Prey ellipses are standard ellipses, indicating approximately 40% of points (Batschelet, 1981). Prey fish ellipses for striped and blue marlin predators include dolphinfish prey; prey fish ellipses for dolphinfish predators do not include dolphinfish prey.



**Figure 3.** Posterior densities for estimated diet proportions for: (a) predators with fish in stomachs, (b) predators with cephalopods in stomachs, (c) predators with pelagic red crabs in stomachs, and (d) predators with empty stomachs.

neither geographic variation in prey nor temporal or seasonal variation in foraging. We collected prey items only from one geographic region in the eastern Pacific, and the high degree of mobility in these predators suggests that they could consume prey from a wide area of the central Pacific. Although our prey selection does not necessarily include all of the possible prey, the predators nonetheless did not demonstrate specialization on any of the prey groups represented. This approach also cannot capture temporally variable individual specialization. As Bolnick *et al.* (2003) point out, individual specialization may be a short-term strategy, especially for mobile predators depending on seasonally or spatially available resources. Rather, this study highlights the potential power of combining stomach content, SI, and FA analyses, and suggests that this combination of tools could be used to further investigate this and other important questions in trophic ecology and beyond.

Another important factor to consider when using chemical tracers to infer diet is how best to estimate trophic efficiency factors for

both SI (fractionation rates) and FA (calibration coefficients). SI fractionation rates are well known to vary based on a range of factors, including species, and diet content itself (Caut *et al.*, 2009). Such rates have not been directly assessed for our study species, so we used laboratory-assessed isotope fractionation values from bluefin tuna (Madigan *et al.*, 2012), but cannot verify that those rates are accurate for these three predator species in question. However, even if we were to have used different isotopic fractionation rates, all predators would have shifted as a group in one direction or another, which would not have led to predators with different stomach contents separating out more clearly.

FA calibration coefficients have been published for many fewer species than the stable isotopic fractionation rates. We used values calculated from juvenile salmon (Budge *et al.*, 2012), as the best available data. Budge *et al.* (2012) were able to generate diet estimates within 10% of actual values in the laboratory but predicted larger errors in wild fish because of greater variation in FA

in prey consumed in the wild compared to that in the laboratory. Using FA data effectively in the future will require laboratory determination of correlation coefficients for more species. Nonetheless, we believe that the broad agreement between our FA diet estimates and previous work discussed above suggests that the FA diet estimates are not biased by incorrect coefficients.

In summary, this three-part approach—combining stomach contents, carbon and nitrogen SI analysis, and FA analysis—can be a powerful tool in elucidating complex and otherwise elusive trophic and ecological dynamics. This combination of old and new tools has allowed us to examine feeding habits in an unprecedented manner in highly mobile, pelagic predators.

## Acknowledgements

This work was supported by a grant from The Billfish Foundation to SOG and OJ, a Graduate Research Fellowship from the National Science Foundation (DGE: 0937373) to TY, and a COFAA fellowship to SOG. We thank A Vastano, M Hernandez, H Kouan, P Falkowski, K Wyman, S Yeung, M Brett, J Fiorendino, E Kwitny, S Piotrowski, A Strauch, MK Swenarton, and R Weinberg for laboratory assistance; the Cocio brothers, E Camacho-Bareño, and the crew of the Kelsie Lee for field assistance; the Jensen lab, especially C Free, P Lopez-Duarte, M McCann, and M Provost; S Budge, J Fodrie, and J Hoffman, and one anonymous reviewer. In memoriam: Dr Russell Nelson and Paxson Offield for their dedication to billfish conservation and support of this work.

## Supplementary data

Supplementary material is available at the *ICESJMS* online version of the manuscript.

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Handling editor: Marta Coll