



Trophic plasticity of a tropical seabird revealed through DNA metabarcoding and stable isotope analyses

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ARTICLE INFO

Keywords:

Birds
 $\delta^{15}\text{N}$
 $\delta^{13}\text{C}$
 Dietary plasticity
 Feeding strategy
 Gulf of California
 Marine ecology
 Mexican tropical Pacific
 Red-billed tropicbird
 Spatio-temporal dietary divergence

ABSTRACT

DNA metabarcoding and stable isotope analysis have significantly advanced our understanding of marine trophic ecology, aiding systematic research on foraging habits and species conservation. In this study, we employed these methods to analyse faecal and blood samples, respectively, to compare the trophic ecology of two Red-billed Tropicbird (*Phaethon aethereus*; Linnaeus, 1758) colonies on Mexican islands in the Pacific. Trophic patterns among different breeding stages were also examined at both colonies. Dietary analysis reveals a preference for epipelagic fish, cephalopods, and small crustaceans, with variations between colonies and breeding stages. Isotopic values ($\delta^{15}\text{N}$ and $\delta^{13}\text{C}$) align with DNA metabarcoding results, with wider niches during incubation stages. Differences in diet are linked to environmental conditions and trophic plasticity among breeding stages, influenced by changing physiological requirements and prey availability. Variations in dietary profiles reflect contrasting environmental conditions affecting local prey availability.

1. Introduction

The understanding of food webs and species interactions of marine biota can provide insights into feeding strategies, population dynamics and their functional role in species-prey interactions (Hedd et al., 2001; Fauchald et al., 2011; Ceia et al., 2012; Lynam et al., 2017). Seabirds are found at most trophic levels of the marine food web, some of them being among top predators, playing a determining role in the flow of energy in marine environments (Shealer, 2001; Grémillet and Boulinier, 2009; Astarloa et al., 2021). Seabirds have a breeding cycle that lasts several months (Schreiber and Burger 2001; Nelson 2006) during which they need to find food for themselves and their offspring (Ricklefs, 1983; Roby, 1991), causing prey depletion and modifying the trophic structure of the ecosystem (Weber et al., 2021).

The diet composition of seabirds can be influenced by both intrinsic (e.g., energy demands, competition, foraging behaviour) and extrinsic factors (e.g., environmental conditions, prey availability, anthropogenic activities; Masello et al., 2010; Quillfeldt et al., 2013; Dehnhard et al., 2016; Gaglio et al., 2018; Soanes et al., 2021). In particular, extrinsic factors appear to strongly influence foraging decisions of tropical seabirds, as they rely mostly on unpredictable food resources in a highly heterogeneous environment characterized by oligotrophic oceanic waters (Weimerskirch, 2007; Soanes et al., 2021). Variations in local environmental conditions can drive to divergent foraging patterns between conspecific seabird colonies (Dunphy et al., 2020; Soanes et al., 2021; Jacoby et al., 2023). Likewise, seabird prey composition is likely to vary within colonies due to temporal differences in prey abundance, changing nutritional requirements, foraging behaviour, interaction with

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<https://doi.org/10.1016/j.marenvres.2024.106627>

Received 4 May 2024; Received in revised form 19 June 2024; Accepted 28 June 2024

Available online 29 June 2024

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other birds (i.e., competition) and energetic demands (Navarro et al., 2014; González-Medina et al., 2017; et al., 2018; Lerma et al., 2022).

The foraging plasticity in seabirds refers to their ability to adjust feeding strategies in response to changing environmental conditions and resources (Paiva et al., 2010; Dehnhard et al., 2016; Gaglio et al., 2018). These adjustments may include changes in diet, selection of foraging habitats, and diving depth during foraging (Masello et al., 2010; Dehnhard et al., 2016). Understanding foraging plasticity is crucial to assess the adaptability of seabirds in response to environmental changes, such as climate change and fluctuations in food availability (Barrett et al., 2007). Conventional approaches, such as the analysis of the crop, stomach contents and regurgitates, provided valuable insight into the prey spectrum and preferences of focal seabird species (Chiaradia et al., 2003; Barrett et al., 2007). However, some of them (e.g., stomach contents) require an invasive approach for the extraction of sample material (Chiaradia et al., 2003). Therefore, less invasive methods such as stable isotope analysis and DNA metabarcoding, are now frequently used to replace the conventional approaches (Deagle et al., 2007; Inger and Bearhop, 2008).

The ratio of nitrogen isotopes (e.g., $\delta^{14}\text{N}/\delta^{15}\text{N}$) has been measured in feathers, blood or other tissue samples to infer the trophic level of the assimilated prey at different time scales (Inger and Bearhop, 2008). On the other hand, DNA metabarcoding is nowadays frequently used in terrestrial and marine ecosystems and has already been successfully implemented in studies involving different animals, including seabirds (Valentini et al., 2009; Crisol-Martínez et al., 2016; McInnes et al., 2017; Kleinschmidt et al., 2019; Masello et al., 2021; Alemany et al., 2023). DNA metabarcoding also allows the detection of soft prey often overlooked in conventional analyses. It supports a higher taxonomic resolution compared to conventional methods (e.g., regurgitates or stomach contents), depending on the availability of suitable primers and complementary gene sequences in data banks (e.g., GenBank) for prey taxa (McInnes et al., 2017). Thus, combining both approaches can improve inferences about seabird trophic ecology (Carreiro et al., 2020; Ceia et al., 2022).

The Red-billed Tropicbird (*Phaethon aethereus*; Linnaeus, 1758) is a pelagic-pantropical seabird that inhabits a range of coastal and oceanic habitats for breeding, with a global population of ~16,000–30,000 mature individuals (Birdlife International, 2024). Despite some spatial variability in prey composition, Exocoetidae (flying fish) and Carangidae (jacks, jack mackerels, etc.) were commonly reported as preferred prey items (Stonehouse, 1962; Castillo-Guerrero et al., 2011; Diop et al., 2018; Madden et al., 2022, 2023). In the Pacific Ocean, prey mainly on flying fish and cephalopods (Nelson, 2006; Almaguer-Hernández, 2016). In contrast to the two congeneric species, little is known about the trophic ecology of the species (see Table S1), including the Eastern Pacific population with globally/regionally important colonies occurring along varied oceanographic conditions along the Mexican Pacific coast (e.g., Peña Blanca and San Pedro Mártir islands; Piña-Ortiz et al., 2018). The study of the trophic ecology of tropical seabirds provides a better understanding of marine ecosystems and the challenges they face (e.g., changes in food availability, pollution, anthropogenic activities), which can improve conservation and management strategies (Gagné et al., 2018a, 2018b; Gatt et al., 2020; Lois et al., 2022). This knowledge is essential for marine conservation, detection of marine environmental changes and sustainable management of fishery resources (Parsons et al., 2008; Lyday et al., 2015).

We used DNA metabarcoding and stable isotope ($\delta^{15}\text{N}$ and $\delta^{13}\text{C}$) analyses to investigate the prey composition of red-billed tropicbirds in the Mexican Pacific, focusing on 1) comparing the diet between San Pedro Mártir and Peña Blanca, two of the most important colonies of the species in the region, located in contrasting oceanic systems in the region (upwelling vs. oligotrophic, respectively); and 2) comparing the diet between the breeding stages for each colony (courtship vs. incubation vs. early chick-rearing vs. late chick-rearing). We hypothesize that dissimilar marine environmental conditions will result in

differences in the diet. We expected that the diet of the San Pedro Mártir colony, being reliant on predictable food resources, would be strongly influenced by temporal changes, especially in response to environmental variations (e.g., El Niño-Southern Oscillation), resulting in significant shifts in productivity and prey availability (Velarde et al., 2004; et al., 2013). In contrast, the Peña Blanca colony in oligotrophic waters will be less affected by the changes in prey abundances, thus expected to show less variability across the breeding season (e.g., Lerma et al., 2020).

2. Materials and methods

2.1. Study site and sample collection

We conducted the study at two sites (San Pedro Mártir Island [28°22' N 112°19' W] and Peña Blanca Islet [19°06' N, 104°29' W]; Fig. 1) from January to May 2021. San Pedro Mártir Island, located in the Gulf of California, is surrounded by an upwelling system fuelled by nutrient-rich waters that exchange with the Pacific Ocean. This exchange involves deep water inflow (200–600 m) and surface water outflow (0–200 m; Escalante et al., 2013). In contrast, Peña Blanca is a tropical islet located close to the coast of Colima (Mexican Tropical Pacific), which is primarily affected by the open, oligotrophic oceanic waters (Hernández-Vázquez et al., 2018). These two sites are the most important colonies of the species in the region in terms of colony size. San Pedro Mártir hosts 150–190 breeding pairs, while the Peña Blanca colony has 1200–1650 breeding pairs (Tershy and Breese, 1997; Piña-Ortiz et al., 2018). On both islands, we collected faecal samples from adults at different breeding stages: courtship (adults inside crevice but without clutch), incubation, early chick-rearing (chicks ≤ 5 weeks old), and late chick-rearing (≥ 6 weeks old) by inspecting active nests in different areas. Chicks of six weeks of age were assigned to the late chick-rearing stage, based on parental nest attendance rates (Stonehouse, 1962) and the body mass of the chicks, which reached adult weight during that post-natal period (adult mass: 536.85 ± 50.56 g; range: 432.9–664.6 g, $n = 54$; Piña-Ortiz et al., 2023). Nest cavities were labelled, and birds sampled were marked with alphanumeric rings on the tarsus to avoid resampling. In total, we collected 71 samples at San Pedro Mártir (courtship = 25, incubation = 19, early chick-rearing = 10, and late chick-rearing = 17), and 61 samples in Peña Blanca (courtship = 19, incubation = 24, early chick-rearing = 12, and late chick-rearing = 6). All individuals were captured by hand in the nest cavities. Once the birds were captured, they were placed on the legs of a staff member, who had covered his lap with a piece of stretch film or tinfoil to allow the bird to defecate naturally on it. The person in charge of this procedure used a new piece of foil for each individual and took all necessary hygienic measures, such as washing hands including alcohol and wearing latex gloves, to minimise possible contamination of the sample. The handling period for courtship and late chick-rearing individuals was set to a maximum of 60 min, while for incubating and early chick-rearing adults, it was kept to 30 min. All individuals were released immediately post-defecation back to the nest cavity. Subsequent monitoring of breeding success during the season allowed us to determine that no individuals abandoned or failed breeding following the handling of individuals. Faecal samples were collected in 1.5 mL plastic tubes and suspended in absolute ethanol. Additionally, blood samples (about 0.5 mL per bird) were collected from breeding adults during courtship (San Pedro Mártir = 16, Peña Blanca = 11), incubation (San Pedro Mártir = 16, Peña Blanca = 15), early chick-rearing (San Pedro Mártir = 12, Peña Blanca = 18), and late chick-rearing (San Pedro Mártir = 18, Peña Blanca = 9) by brachial vein puncture (3 mL syringe, 23G, 0.5 mm \times 16 mm). Both blood and faecal samples were stored in a portable freezer (-20°C ; GoSun®) in the field and then frozen in the laboratory at -20°C pending preparation for further analysis.

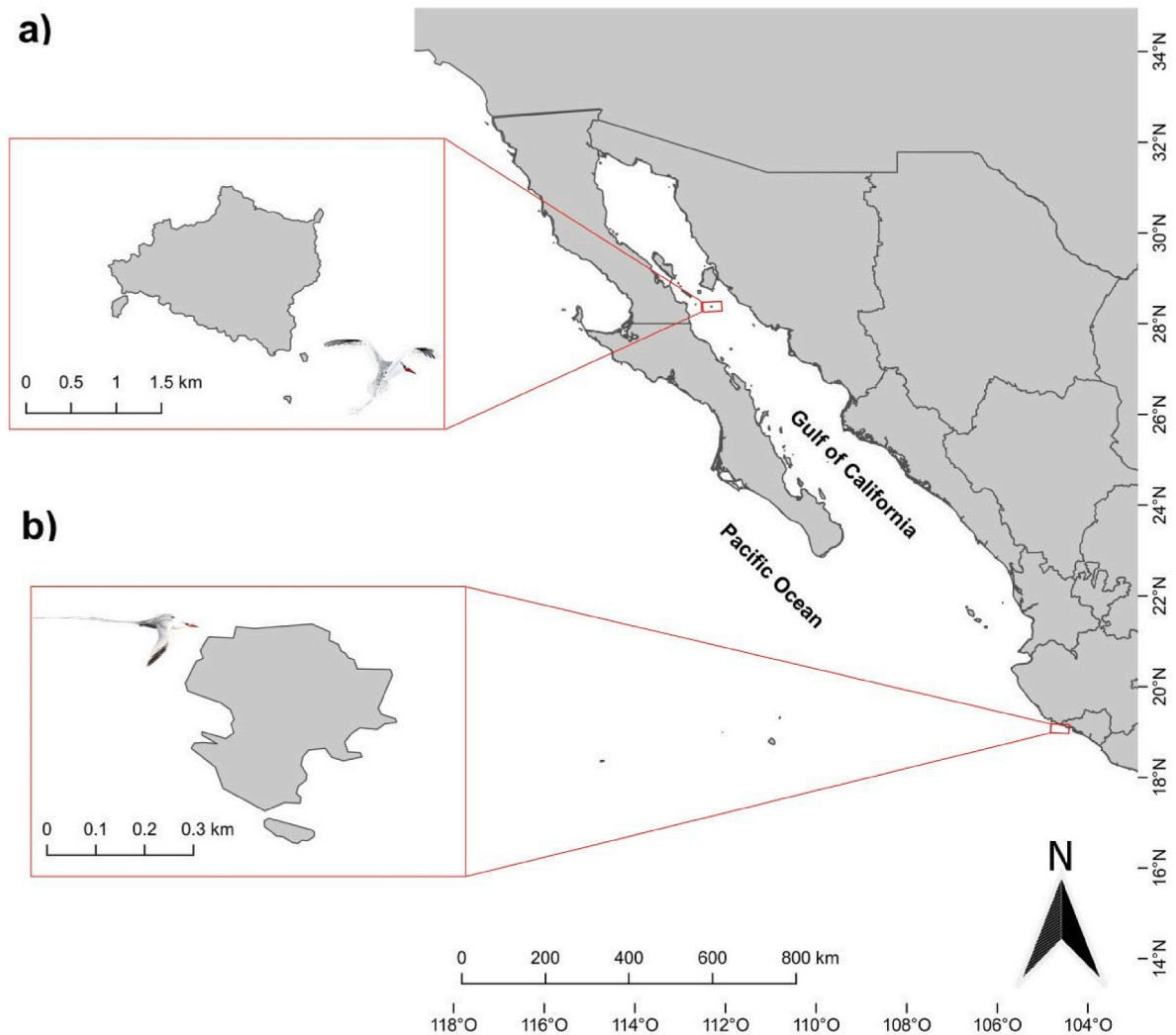


Fig. 1. Geographic locations of Red-billed Tropicbird (*Phaethon aethereus*) colonies sampled in this study. a) San Pedro Mártir Island and, b) Peña Blanca Islet.

2.2. DNA isolation and library preparation

We performed DNA extractions following the manufacturer's instructions for the Qiagen Fast DNA Stool Mini Kit (QIAGEN GmbH, Germany). For PCR amplifications, we used a Metazoa COI primer set to identify prey at family level. Based on our prior knowledge of the diet of the focal study species (Table S1), two more specific 16S rDNA primer pairs were used to identify the two main prey categories (fish and cephalopods; Table S2). For PCR amplifications, a 20 μ L reaction volume was prepared, containing 10 μ L Qiagen Multiplex PCR Buffer, 5.1 μ L double-sterilized water, 0.1 μ L BSA, 0.4 μ L forward primer (10 μ M), and 0.4 μ L reverse primer (10 μ M), along with 4 μ L or 6 μ L of the DNA template. PCRs were run following the protocol for the Qiagen Multiplex PCR Buffer (for annealing temperatures, see Table S2). For the fish and cephalopod primers, a touchdown PCR reaction was used, where the annealing temperature was decreased after each cycling step by $\Delta t = -1$ $^{\circ}$ C to optimize amplification. The adapter PCR products were inspected using the QIAxcel Advanced-System sequencer (QIAGEN), with products showing DNA concentrations below 0.5 ng/ μ L being repeated with more DNA template.

Amplicons resulting from our adapter PCR reactions underwent purification using the illustra™ ExoProStar™ 1-STEP kit (Cytiva, Amersham, UK), and we combined the amplicons of each samples following Swift et al. (2018). Subsequently, we prepared the illumina library using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA).

Index PCR amplifications were carried with a 30 μ L PCR reaction volume, including 7.5 μ L Qiagen Multiplex PCR buffer, 13.3 μ L double-sterilized water, 10 μ M primer (2.1 μ L forward and 2.1 μ L reverse primer for all three specific primers), and 5 μ L of the DNA template. PCRs were run following the protocol for the Qiagen Multiplex PCR Buffer (with annealing temperature 56 $^{\circ}$ C). Subsequently, amplicons were purified using a SequalPrep™ Normalization kit (Invitrogen™, Massachusetts, USA). The library was sequenced using 250-bp paired-end reads on a MiSeq desktop sequencer (Illumina) at SEQ-IT (SEQ-IT GmbH and Co.KG, Kaiserslautern, Germany).

2.3. Bioinformatic analysis

To obtain a list of molecular operational taxonomic units (MOTUs), we ran a custom workflow (Masello et al., 2021) in GALAXY (The Galaxy Community, 2022). The workflow included the following steps: 1) assessing sequence quality with FASTQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>) (accessed on Sep 5, 2023), 2) adapter and quality trimming of the paired-end reads with TRIMMOMATIC (minimum quality score of 20 over a sliding window of 4 bp; Bolger et al., 2014), 3) merging of the overlapping paired-end read pairs using FLASH (Magoč and Salzberg, 2011), 4) transforming sequence files to FASTA with the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) (accessed on Sep 5, 2023), 5) extracting amplicons from the FASTA files in MOTHUR (Schloss et al., 2009), 6) removing identical

replicates (dereplicate, plus strand), 7) detecting and removing chimeric sequences (de novo, minimal abundance ratio of parent vs. chimera 2, 'no' vote pseudo count 1.4, 'no' vote weight 8, minimum number of differences in segment 3, minimum divergence from closest parent 0.8, minimum score 0.28), and 8) clustering sequences into MOTUs, rejecting if identity was lower than 0.97, with VSEARCH (Rognes et al., 2016). Finally, using the BLASTn algorithm (Altschul et al., 1990), we matched MOTU sequences to reference sequences in the National Center for Biotechnology Information (NCBI) GenBank nucleotide database, employing a cut-off of 90% minimum sequence identity and a maximum e-value of 0.00001. For the taxonomic assignments, we used the percentage similarity of the query and the reference sequences, retaining a BLASTn assignment match greater than 98%, and a minimum sequence length of 190 bp since short fragments are less expected to contain trustworthy taxonomic information (Deagle et al., 2009; Vesterinen et al., 2013). We allocated MOTUs to the species level only in cases when all retained hits of a MOTU, with the same quality criteria (sequence identity, sequence length, e-value), corresponded to the same species. Otherwise, we assigned the MOTU to the lowest shared taxonomic level, (e.g., genus or family; Kleinschmidt et al., 2019). The raw data set included a wide range of unspecific, contaminant DNA (e.g., human, bacteria) that could be excluded as potential prey taxa based on previous literature (Stonehouse, 1962; Nelson, 2006; Castillo-Guerrero et al., 2011; Almáguer-Hernández, 2016; Diop et al., 2018; Madden et al., 2022, 2023). Non-prey MOTUs included various taxa of the orders Insecta, Reptilia and Aves, which were omitted during the validation steps, as they were ecologically irrelevant or very distant distribution ranges. As in Masello et al. (2021), records with <10 reads or in singular MOTUs, where the read number accounted <1% of the maximum count were excluded from the analysis.

In order to restrict secondary prey identifications, we applied two approaches previously employed in other studies using DNA metabarcoding (see Hardy et al., 2017; Ando et al., 2020; Nimz et al., 2022). Firstly, we assumed that fish and squid were the primary prey based on previously published studies on the diet of the species, while other matches such as Gastropoda, Copepoda, Branchiopoda, Bivalvia, and Isopoda were probably due to secondary predation, accidental ingestion during foraging, prey parasites or even contamination of samples. Secondly, using prey diet information, we examined potential cases of secondary predation by considering both the co-occurrence of prey items and their distributions.

For the three main prey groups—fish, cephalopods, and crustaceans—we calculated the frequency of occurrence (FO; Formula 1) and the relative read abundance (RRA; Formula 2). We utilized the RRA to enhance our interpretation of FO, as recommended by previous studies (Barrett et al., 2007; McInnes et al., 2017; Young et al., 2020). In this context, FO is defined as:

$$FO = (n / t) * 100 \quad (1)$$

where n represents the number of samples in which we detected prey DNA, and t is the total number of samples in which DNA from the considered prey group was present. We defined the RRA as:

$$RRA = (\text{reads} / \text{total number of reads}) * 100 \quad (2)$$

representing the percentage ratio of reads in relation to the total number of reads recorded for the respective MOTU.

Moreover, while DNA metabarcoding is a powerful tool for obtaining comprehensive insights into a species' diet using a non-invasive approach and small sample sizes, several disadvantages are associated with the method (Ando et al., 2020). Sample contamination, whether from the laboratory, field environment, or secondary prey, poses a significant issue. Additionally, the preselection and use of DNA barcoding markers can affect taxonomic resolution and detectability (Ando et al., 2020). Technical biases such as inappropriate PCR settings, DNA host amplification, and PCR inhibition, as well as the selection of

bioinformatic scripts and the steps to compile the reference database, are also crucial considerations (Ando et al., 2020).

2.4. Stable isotope analyses

Blood reflects a dietary integration period of 2–4 weeks prior to sampling for carbon and nitrogen isotopic analyses (Bearhop et al., 2002). Therefore, blood samples obtained from adults in our study are expected to represent the diet consumed during each respective stage. We oven-dried (50 °C) the blood samples and then we finely ground them. We packed subsamples (0.3–0.5 mg) in tin capsules to be analysed for %N, %C, $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ using a Flash 2000 elemental analyser (Thermo Scientific, Milan, Italy) coupled with a Delta V Plus isotope ratio mass spectrometer with a ConFlo IV interface (Thermo Scientific, Bremen, Germany). We carried out the analyses at the 'Littoral, Environnement and Sociétés (LIENSs)' Joint Research Unit stable isotope facility (CNRS – La Rochelle Université, France). Results are expressed as δ (‰) for $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$, calibrated against the international isotopic references (atmospheric nitrogen for $\delta^{15}\text{N}$ and Vienna-Pee Dee Belemnite for $\delta^{13}\text{C}$). The analytical precision was ± 0.15 ‰ for $\delta^{15}\text{N}$ and ± 0.10 ‰ for $\delta^{13}\text{C}$ based on internal standards USGS-61 and USGS-62 inserted every ten measurements. All samples had a low C:N mass ratio (<4.0), indicating low lipid content, so we did not perform any lipid extraction (Cherel et al., 2005).

2.5. Statistical analyses

We assessed species diversity using rarefaction curves and determined the percentage of samples covering the four breeding stages at each study site, confirming the adequacy of our sample sizes. This analysis was performed using the 'iNEXT' package (Hsieh et al., 2016) within R v4.1.8 (R Core Team). We compared prey composition between sites and breeding stages employing a Permutational Analysis of Variance (PERMANOVA) test with the 'VEGAN' package (Oksanen et al., 2018). We integrated various effects into the Adonis base model, including sites, collection date (Julian calendar), and chick age, ensuring a clear distinction between early and late chick-rearing stages. Interaction effects in the model (e.g., stage*collection date, stage*age) were also explored. We selected the optimal model based on our significance level and further evaluated it using the Akaike Information Criterion (AIC). The model with the lowest score was deemed optimal for our specific data set. We employed a non-metric multidimensional scaling (NMDS) with Bray-Curtis dissimilarity for binary data to illustrate the dissimilarity in prey composition between the stages and islands. We estimated the stress level in both study sites using the 'VEGAN' package (Oksanen et al., 2018). A stress level <0.05 is considered as an excellent agreement, a stress level below 0.1 very good, and below 0.2 as good for representing the data set (Masello et al., 2023). In our models, the stress was 0.07–0.08. A hierarchical cluster analysis was employed to assess dissimilarity among prey MOTUs, utilizing Ward's cluster method with a Manhattan distance measure. The frequencies of the respective taxonomic class (i.e., species, genus or family) to which the readings were assigned were visualised in a heat map using the corresponding add-on in OriginPro Lab (Version, 2023; OriginLab Corporation, Northampton, MA, USA).

To examine differences in adult $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ isotope ratios we conducted general linear models (GLM) considering sites (San Pedro Mártir and Peña Blanca) and breeding stages (courtship, incubation, early chick-rearing, and late chick-rearing) as categorical factors, and the sample collection date as a continuous predictor. GLMs were based on complete initial models that included all variables and interactions. To compare differences between adults in isotopic values during the breeding stages, we employed t-tests with Bonferroni correction ($\alpha = 0.05$, 1 comparison, and $\alpha = 0.017$, 3 comparisons for site and breeding stage, respectively). Moreover, we assessed niche breadth among breeding stages within and across sites by calculating two-dimensional

isotopic niches using standard ellipses areas, as implemented in the 'SIBER' package (Jackson et al., 2011). Specifically, for the quantification of niche breadth and comparisons across stages and sites, we employed the standard ellipse area corrected for small sample sizes (SEA_C). Simultaneously, Bayesian standard ellipses (SEA_B) were employed to evaluate the proportion of overlapping area among stages and sites (Jackson et al., 2011). For the rest of analyses, we conducted all statistical tests at a significance level of $\alpha = 0.05$, and the results are presented as the mean \pm standard deviation.

3. Results

3.1. Diversity and total identified taxa

From a total of 131 samples, 97 (74%) successfully amplified with the Metazoa primers, 47 (36%) with the Fish primers and 11 (8%) with the Cephalopoda primers (Fig. S1). We identified 20 different MOTUs (6 species, 9 genus, and 13 families) for the San Pedro Mártir data set and 22 different MOTUs (4 species, 9 genus, 13 families) for the Peña Blanca data set, with the highest MOTUs numbers recorded for courtship individuals in San Pedro Mártir and incubating birds in Peña Blanca (Table S3). In San Pedro Mártir, the average number of taxa detected for

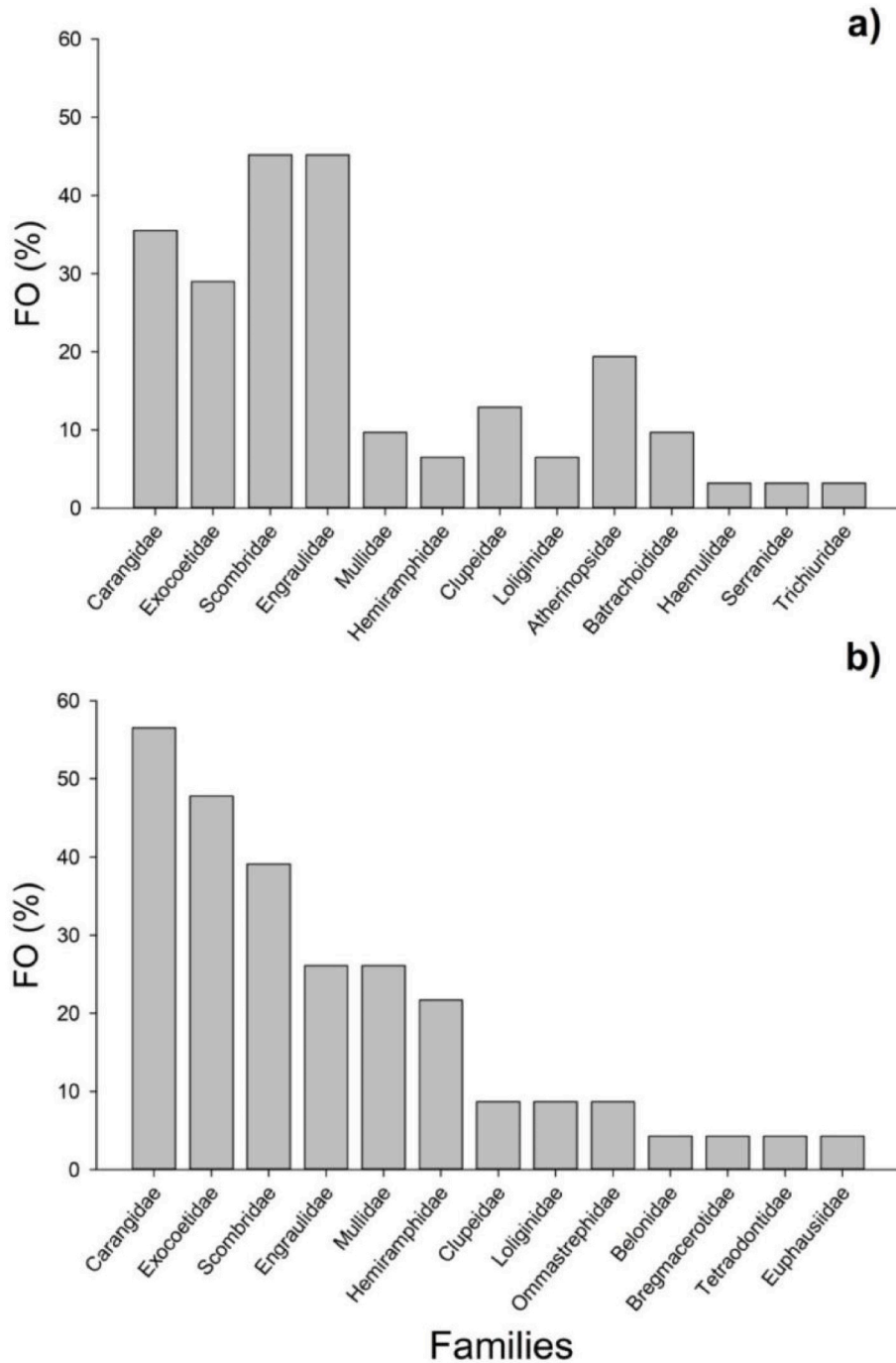


Fig. 2. Bar charts showing the frequency of occurrence (% of samples; FO) of the predominant prey families in the diet of the Red-billed Tropicbird (*Phaethon aethereus*) in **a)** San Pedro Mártir Island and **b)** Peña Blanca Islet estimated by metabarcoding.

each breeding stage was 2.8 ± 1.2 ($n = 13$) for individuals at courtship, 2.8 ± 1.8 ($n = 5$) at incubation, 2.5 ± 1.0 ($n = 6$) at early chick-rearing, and 2.9 ± 1.2 ($n = 7$) at late chick-rearing stage. For Peña Blanca, the numbers of taxa were 2.3 ± 1.4 ($n = 8$) at courtship, 3.2 ± 1.5 ($n = 6$) at incubation, 3.5 ± 1.3 ($n = 6$) at early chick-rearing, and 4.7 ± 2.3 ($n = 3$) at late chick-rearing (Fig. S1). Rarefaction curves indicated that sufficient species coverage was obtained with the sample sizes for most breeding stages at both sites (species coverage between 73 and 85%), except for the incubation stage, which explained only around 55% and 45% of the variation for San Pedro Mártir and Peña Blanca, respectively (Fig. S2). Hence, we encourage readers to take and use the results of this stage with due discretion.

The most common prey items at family level for both study sites were Carangidae (San Pedro Mártir: 35.5%, Peña Blanca: 56.5% of samples), Scombridae (San Pedro Mártir: 45.2%, Peña Blanca: 39.1%), Exocoetidae (San Pedro Mártir: 29%, Peña Blanca: 47.8%), and Engraulidae (San Pedro Mártir: 45.2%, Peña Blanca: 26.1%; Fig. 2). For San Pedro Mártir, the Californian anchovy (*Engraulis mordax*) was the most frequent prey for courtship individuals (Table 1). The topsmelt silverside (*Atherinops affinis*) was the most frequently consumed prey at incubation and early chick-rearing stages, while *Scomber* sp. and the Californian anchovy formed the most frequently observed prey of late chick-rearing adults (Table 1, Fig. 3; for RRA values see Table S4). On the other hand, Carangidae and Engraulidae represented concurrently the most frequent prey of Peña Blanca courtship individuals. The Bullet tuna (*Auxis rochei*), and *Scomber* sp. were the predominant prey for incubation individuals, while *Cypselurus* sp. was the main prey for early chick-rearing individuals (Table 1). For late chick-rearing adults *Caranx* sp., an unidentified Carangidae, and an unidentified Engraulidae were the most frequent prey (Table 1; Fig. 3). Crustaceans were only recorded as prey from Peña Blanca courtship individuals, and cephalopods were omnipresent in courtship and early chick-rearing adults in San Pedro Mártir and in all breeding stages, except for the courtship at Peña Blanca, reaching a low FO (8–33%) throughout the data set (Table 1).

3.2. Multivariate analysis of spatio-temporal divergence in prey selection

The multivariate analysis of prey composition revealed spatial divergence between sites ($AIC_{perm} = -60.93$, Permanova test, $pseudoF_1 = 4.16$, $p = 0.005$; Table 2). Likewise, differences in the diet between the breeding stages were present in the Peña Blanca population (Permanova test, $pseudoF_{1,3} = 3.06$, $p = 0.003$), and from San Pedro Mártir population, however for the latter the prey composition between breeding stages varied in interaction with the collection date (Permanova test, $pseudoF_{1,26} = 3.88$, $p = 0.002$; Table 2). The dietary composition for the early and late chick-rearing stages showed significant differences between breeding adults in Peña Blanca (Permanova test, $pseudoF_{1,7} = 3.06$, $p = 0.05$). The cluster analysis comparing the FO highlighted the formation of two clusters between the breeding stages for the San Pedro Mártir data set, with the highest dissimilarity being estimated between courtship and late chick-rearing individuals and incubation and early chick-rearing birds (Fig. 3a). In contrast, for Peña Blanca, only the individuals in incubation and late-chick rearing had a notable overlap between the prey incidences (Fig. 3b). The NMDS analysis highlighted similar divergence between the stages in San Pedro Mártir (stress level = 0.076), indicating a certain degree of overlap between courtship and late chick-rearing individuals, as well as between incubation and early chick-rearing individuals, while in Peña Blanca (stress level = 0.076) there was an overlap in prey composition between most breeding stages, with the exception of the incubation and early chick-rearing stages (Fig. 4a and b).

3.3. Stable isotopes

The $\delta^{15}N$ and $\delta^{13}C$ values differed significantly between sites (GLM, $\delta^{15}N$: $F_{1,106} = 525.76$, $p < 0.001$; $\delta^{13}C$: $F_{1,106} = 24.0$, $p < 0.001$), and

breeding stages ($\delta^{15}N$: $F_{3,106} = 6.50$, $p < 0.001$; $\delta^{13}C$: $F_{3,106} = 8.61$, $p < 0.001$), and the interaction between these factors was non-significant (Table 3). In general, individuals from San Pedro Mártir had significantly higher $\delta^{15}N$ and $\delta^{13}C$ than adults from Peña Blanca (Fig. 5). San Pedro Mártir individuals at the incubation stage had significantly lower $\delta^{13}C$ values compared to the rest of the stages (Paired t -test, $t_{5,6} = 0.79$, $p < 0.001$, $t_{6,7} = 0.57$, $p = 0.017$ and $t_{6,8} = 0.60$, $p < 0.002$ for courtship, early and late chick-rearing stages, respectively), while late chick-rearing individuals at Peña Blanca had significantly higher $\delta^{15}N$ values than those from incubation stage ($t_{2,4} = 0.63$, $p = 0.005$; Fig. 5). The standard ellipse areas estimated for each breeding stage at the study sites exhibited dissimilarities, with the widest isotopic niches during incubation at both colonies (SEA_C: San Pedro Mártir = 0.55 and Peña Blanca = 0.31), whereas the narrowest isotopic niches were estimated during early chick-rearing from San Pedro Mártir and late chick-rearing from Peña Blanca (SEA_C: 0.29 and 0.15, respectively; Fig. 5, Table 4). Likewise, all pairwise comparisons of niche breadth between breeding stages showed significant differences in their ellipse areas ($p < 0.001$; Table S5). The isotopic niches of the breeding stages in San Pedro Mártir had an overlap ranging from 28% to 39%, while in Peña Blanca, they ranged from 22% to 34% (Table S6). Particularly, the highest overlap in San Pedro Mártir occurred between the courtship and late chick-rearing, while in Peña Blanca it was observed between the early and late chick-rearing stages (Fig. 5, Table S6).

4. Discussion

4.1. Literature review on the diet

The results of this study agreed with previously reported data on the prey composition of the species, that consumed fish, cephalopods, and crustaceans (Stonehouse, 1962; Nelson 2006; Castillo-Guerrero et al., 2011; Almaguer-Hernández, 2016; Diop et al., 2018; Madden et al., 2022, 2023; Table S1). Unlike previous studies that relied on regurgitates (Stonehouse, 1962; Castillo-Guerrero et al., 2011; Almaguer-Hernández, 2016; Diop et al., 2018; Madden et al., 2022, 2023; Table S1) or stomach contents (North, 1946), our study highlights the advantages of DNA metabarcoding on faeces over conventional methods (e.g., Guillerault et al., 2017; Snider et al., 2022; Allen et al., 2023). Specifically, we achieved higher taxonomic resolution, identifying to species level more accurately than most previous studies, and obtained high amplification success even with minimal sample amounts. In contrast, when collecting regurgitates from colonies, prey may be at a significant stage of digestion that renders them unidentifiable (Scribner and Bowman, 1998; Barrett et al., 2007). This constrain for identifying digested prey at the species level may result in the omission of key dietary information required to understand marine food webs (Barrett et al., 2007; Allen et al., 2023; Querejeta et al., 2023). In addition, faeces collection does not affect the breeding success of the species studied by not interfering with prey captured by individuals (e.g., Almaguer-Hernández, 2016), and the number of samples necessary for a complete dietary mapping is only a fraction compared to studies using regurgitates or stomach contents.

In this study, the availability of trophic resources for both colonies was inferred from bibliographic information, considering the location, environmental characteristics, and phenology of the prey. However, since the presence, distribution, and abundance of prey can change annually, direct sampling of the fish community or other potential prey would have provided a more accurate assessment of prey availability. Nonetheless, this approach presents significant logistical and financial challenges. In that sense, environmental DNA (eDNA) analysis presents an alternative approach, to assessing biodiversity and identifying organisms in different habitats and oceans (e.g., Lima-Mendez et al., 2015; Andruszkiewicz et al., 2017; Deiner et al., 2017; Djurhuus et al., 2018). It can provide a detailed view of species composition, facilitating the inference of co-occurrence patterns and the detection of organisms

Table 1
Frequency of occurrence (% of samples; FO) for main prey categories consumed by Red-billed Tropicbird (*Phaethon aethereus*) at the study sites (San Pedro Mártir and Peña Blanca islands), and during four defined breeding stages (COU – courtship, INC – incubation, ECR – early chick-rearing, LCR – late chick-rearing). Prey depth ranges (m) are shown in the table. Sample sizes correspond to the number of DNA extractions from faecal samples, and in brackets to the number of successfully sequenced samples.

Phylum	Class	Order	Family	Species	Common Name	Depth ^a	San Pedro Mártir				Peña Blanca					
							COU	INC	ECR	LCR	COU	INC	ECR	LCR		
							<i>n</i> = 25 (13)	<i>n</i> = 19 (5)	<i>n</i> = 10 (6)	<i>n</i> = 17 (7)	<i>n</i> = 19 (8)	<i>n</i> = 24 (6)	<i>n</i> = 12 (6)	<i>n</i> = 6 (3)		
Arthropoda	Malacostraca	Euphausiacea	Euphausiidae		Krill	0–600	–	–	–	–	12	–	–	–		
Mollusca	Cephalopoda	Myopsida	Loliginidae		Pencil squids	0–1000	7.7	–	16.7	–	–	16.7	16.7	–		
Chordata	Actinopterygii	Oegopsida	Ommastrephidae		Flying squid	–	–	–	–	–	–	–	16.7	33.3		
		Atheriniformes	Atherinopsidae	<i>Atherinops affinis</i>	Topsmelt silverside	0–26	–	60	50	–	–	–	–	–	–	
		Beloniformes	Exocoetidae		Flying fish	0–20 ^b	7.7	40	50	14.3	12.5	33.3	100	66.7	–	
				<i>Cheilopogon</i> sp.			0–5 ^b	–	–	–	–	–	50	–	–	
				<i>Cypselurus</i> sp.			0–20 ^b	–	20	33.3	–	12.5	16.7	83.3	33.3	
				Hemiramphidae		Halfbeak	–	–	–	–	28.6	25	–	50	–	–
					<i>Oxyporhamphus</i> sp.	Bigwing halfbeak	0–?	–	–	–	–	12.5	–	50	–	–
				Belonidae		Needlefish	–	–	–	–	–	–	16.7	–	–	–
				Perciformes	Carangidae	Jacks and pompanos	0–380	23	–	16.7	28.6	62.5	33.3	50	100	–
					<i>Caranx</i> sp.	Jacks	0–380	–	–	–	–	25	16.7	–	66.7	–
					<i>Decapterus macrosoma</i>	Shortfin scad	20–214	–	–	16.7	28.6	25	16.7	50	33.3	–
					<i>Selar</i>	Bigeye scad	0–170	–	–	–	–	25	–	–	–	–
					<i>crumenophthalmus</i>											
					Haemulidae	Grunts	–	7.7	–	–	–	–	–	–	–	–
					Mullidae	Goatfishes	2–113	–	20	33.3	–	25	16.7	33.3	33.3	–
					Serranidae	Sandperches	1–160	–	–	–	14.3	–	–	–	–	–
				Scombriformes	Trichiuridae	unid.	Cutlassfishes	2–500	7.7	–	–	–	–	–	–	–
					Scombridae			0–300	53.8	20	33.3	57.1	–	83.3	33.3	66.7
						<i>Auxis rochei</i>	Bullet tuna	0–200	–	–	–	–	–	33.3	16.7	33.3
						<i>Scomber japonicus</i>	Pacific chub mackerel	0–300	7.7	20	–	28.6	–	16.7	–	–
						<i>Scomber</i> sp.	Chub-Mackerels	–	30.8	20	33.3	57.1	–	33.3	16.7	33.3
						<i>Scomberomorus concolor</i>	Monterey Spanish mackerel	15–?	15.4	–	–	–	–	–	–	–
				Tetraodontiformes	Tetraodontidae		Puffer-Fishes	0–483	–	–	–	–	12.5	–	–	–
		Clupeiformes	Clupeidae		Herrings	–	38.5	20	–	42.8	–	16.7	–	–		
				<i>Sardinops sagax</i>	Californian pilchard	0–200	7.7	20	–	–	–	–	–	–		
				<i>Sardinops</i> sp.	Sardines	0–200	23.1	20	–	42.8	–	–	–	–		
			Engraulidae		Anchovies	–	46.1	40	33.3	57.1	37.5	16.7	–	66.7		
				<i>Engraulis mordax</i>	California anchovy	0–310	46.1	40	33.3	57.1	–	–	–	–		
		Batrachoidiformes	Batrachoididae		Toadfishes	1–225	7.7	20	16.7	–	–	–	–	–		
		Gadiformes	Bregmacerotidae	<i>Bregmaceros</i>	East Pacific codlet	0–1246	–	–	–	–	–	16.7	–	–		
				<i>bathymaster</i>												

? – Depth range unknown.

^a Depth range derived from Froese and Pauly, 2024; Robertson and Allen (2024).

^b Flying fish is encountered gliding over the sea level, indicated by the value 0 m here.

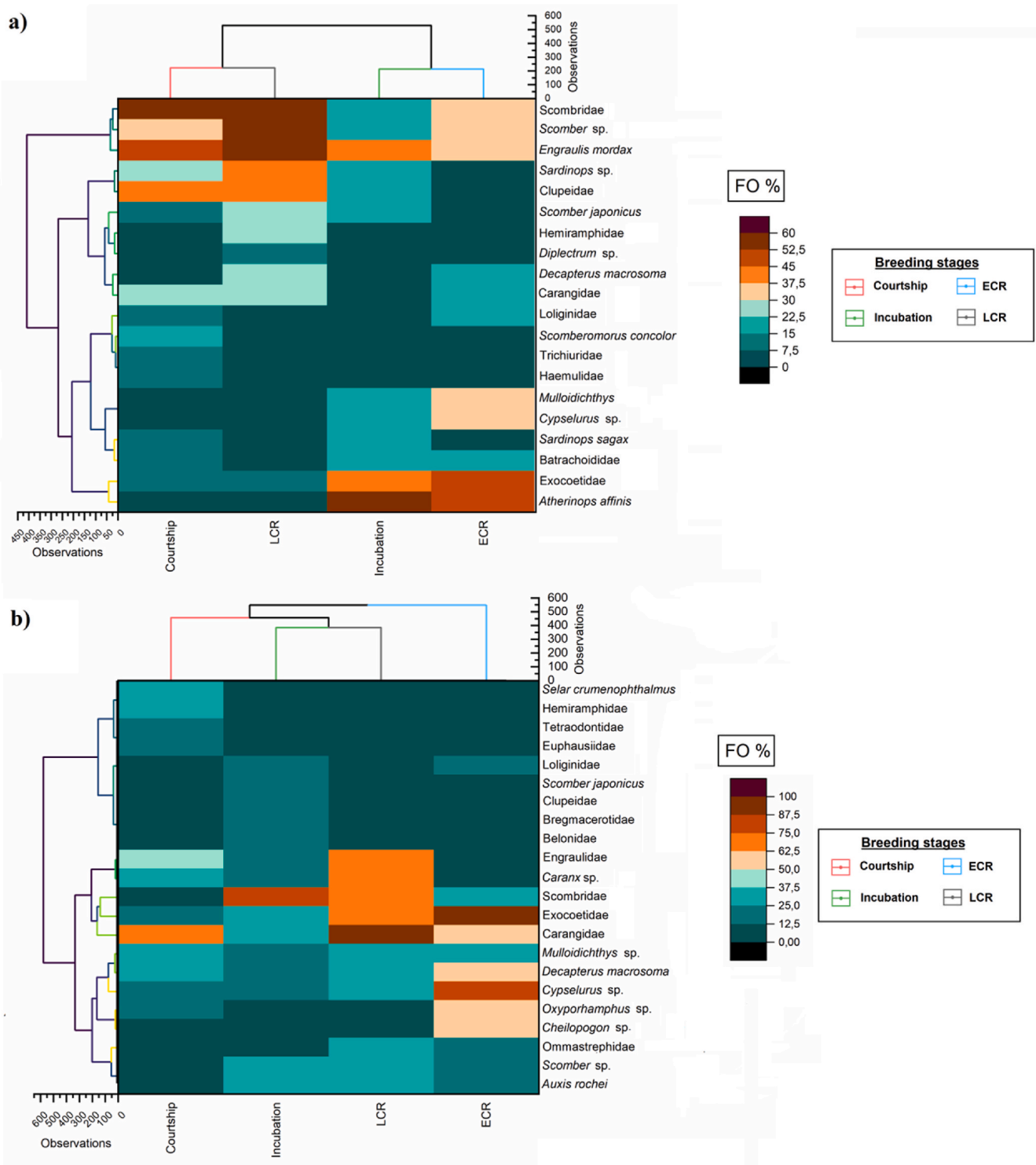


Fig. 3. Cluster maps highlighting the hierarchical cluster analysis performed for the MOTUs using the frequency of occurrence (FO) of the respective taxonomic class with the associated reads and the breeding stages (ECR = early chick-rearing, LCR = late chick-rearing, Ward’s cluster method CityBlock or Manhattan type) with the dendrograms of each study site [a) San Pedro Mártir Island, b) Peña Blanca Islet] are outlined below showing associated observation clusters. MOTUs were clustered based on the number of generated observations.

across wide marine areas (Djurhuus et al., 2020). The integration of metabarcoding and stable isotope data revealed that red-billed tropicbirds hold a top-predator role in marine pelagic systems, primarily preying on mesopelagic and epipelagic open-ocean fish species and to a lesser extent, benthic and reef-associated fish, cephalopods and crustaceans. The Californian anchovy, Pacific chub mackerel and South American pilchard (*Sardinops sagax*) were identified

as main prey for San Pedro Mártir, whereas flying fish were prominent in the diet from Peña Blanca individuals, consistent with prior studies (Stonehouse, 1962; Castillo-Guerrero et al., 2011; Almaguer-Hernández, 2016; Diop et al., 2018; Madden et al., 2022, 2023). Specifically, Spotfin flying fish (*Cheilopogon furcatus*), Stained flying fish (*C. spilopterus*), Whitetip flying fish (*C. xenopterus*), and *Cypselurus* sp. (likely Ornamented flying fish [*C. callopterus*]) were noted prey for Peña Blanca

Table 2

Results of Permutational analysis of variance (Permanova) test using the Adonis base model with added effects for each study location and different taxonomic resolution. ECR – early chick rearing, LCR – late chick rearing. Significant *p*-values: * = 0.05, ** = 0.01, *** = 0.001.

Model	AIC	R	F	<i>p</i>	Significance level
General data set (combined)					
<i>Sites</i>	-60.93	0.07	4.16	0.005	**
San Pedro Mártir					
<i>stages</i>	-33.05	0.16	1.62	0.095	
<i>collection date</i>	-44.74	0.77	2.35	0.002	**
<i>age</i>	-29.33	0.26	1.17	0.239	
<i>stages*age</i>	-30.44	0.33	1.37	0.094	
<i>stages*collection date</i>	-83.21	0.96	3.88	0.002	**
<i>chickstages ~ ECR*LCR</i>	-13.76	0.17	2.39	0.067	
Peña Blanca					
<i>stages</i>	-30.43	0.33	3.06	0.003	**
<i>collection date</i>	-19.80	0.65	0.71	0.873	
<i>age(metric)</i>	-20.56	0.20	0.68	0.897	
<i>stages*collection date</i>	-29.60	0.81	0.95	0.553	
<i>stages*age(metric)</i>	-24.05	0.37	1.27	0.182	
<i>chickstages ~ ECR*LCR</i>	-16.77	0.30	3.06	0.050	*

(Almaguer-Hernández, 2016). In addition, Carangidae dominated in Peña Blanca diet, with MOTUs like *Caranx* sp. and Shortfin scad (*D. macrossoma*), aligning with previous records (Almaguer-Hernández, 2016; Diop et al., 2018). Among the most frequent prey of the San Pedro Mártir individuals, the topsmelt silverside (FO = 19.3%) was identified, which simultaneously represent the first record for the diet of the species.

Clupeidae and Hemiramphidae were previously recorded in the prey of the focal species like the Pacific thread herring (*Opisthonema libertate*) and *Oxyporhamphus* sp. (i.e., Bigwing halfbeak [*O. micropterus*]; Castillo-Guerrero et al., 2011; Almaguer Hernández, 2016). Belontiidae was found in the diet of Peña Blanca individuals, recently recorded in the Caribbean and the Eastern Atlantic populations (Diop et al., 2018; Madden et al., 2022). Other fish taxa identified had not been previously recorded and represent probably opportunistic prey, evidenced by low read counts or single records (e.g., Trichiuridae, Batrachoididae, Tetraodontidae, *Diplectrum* sp. and *Bregmaceros bathymaster*). Although the possibility that these observations are the result of secondary predation (i.e., DNA carry-over from ingested prey) cannot be excluded either.

Further comparison of the prevalence of cephalopods and crustaceans in this study with previous research on the diet of tropicbirds reveals a minor role of both taxa in the colonies studied (Table S1). Therefore, the observed diet profiles align more closely with Caribbean and Atlantic Red-billed Tropicbird populations, where fish predominantly or entirely comprise the diet (Diop et al., 2018; Madden et al., 2022 et al., 2023). Despite previous studies indicating low crustacean frequencies, squid consistently appeared in regurgitates, ranging between 0 and 30% prevalence (Stonehouse 1962; Nelson 2006; Castillo-Guerrero et al., 2011; Almaguer-Hernández, 2016; Madden et al., 2023). Our findings suggest a lower importance of cephalopods as prey, nevertheless it is crucial to address the potential biases that influence low detection rates. While the cephalopod primers used here were confirmed to detect unspecific, non-target taxa with a generally low match rate possibly introducing a bias (non-target prey recorded in 96.2% of all reads; Young et al., 2020), we find this scenario highly unlikely for the following reasons. The COI and 16S regions have been widely employed for the amplification and identification of DNA from vertebrates and cephalopods in dietary studies (e.g., Carreiro et al., 2020; Young et al., 2020; Nimz et al., 2022); besides, the primers set was used for cephalopods already and was validated (see Peters et al., 2015; Berry et al., 2017; de Jonge et al., 2021). Also, we used two different primers to target cephalopods, leaving a low likelihood that cephalopods were possibly overlooked. Conversely, recent studies have pointed out the relatively lower success rates of 16S assays in dietary DNA

studies of pelagic seabirds (Doyle and Adams, 2018; Nimz et al., 2022). Therefore, it is advisable to perform an initial experiment with a subset of samples to identify the most effective primer sets for full analyses. This recommendation is based on the fact that new primer sets emerge rapidly, and the success of primer assays depends on factors such as primer binding efficiency and the availability of prey sequence data (Nimz et al., 2022). Additionally, it is worth mentioning that detection of uncommon or poorly studied taxa as cephalopods could be limited by the lack of complementary DNA sequences in genetic reference databases (i.e., Genbank), although a matter of time for sequence databases to improve the widely used metabarcoding targets to achieve more accurate taxonomic identifications (e.g., de Jonge et al., 2021).

4.2. Divergence in prey selection between breeding colonies

In accordance with our expectations, the diet of red-billed tropicbirds varied between breeding sites, reflecting differences in prey composition and abundance between regional marine systems (upwelling vs. oligotrophic). These differences could be linked to factors such as food availability, reflected in differences in foraging behaviour and competition for resources. The individuals from San Pedro Mártir exhibited lower prey diversity than those from Peña Blanca, with individuals from both sites primarily relying on fishes, comprising their diets mainly by 4–5 prey species (FO range: 19.5–45%, total RRA >80%; Tables 2 and S2, Fig. S3). Notably, individuals at each site prey on abundant species in their respective regions (e.g., *E. mordax*, *Sardinops* sp. [*sagax*] at SPM), showcasing trophic plasticity throughout their distribution range (Castillo-Guerrero et al., 2011; Diop et al., 2018; Madden et al., 2022, 2023). Trophic plasticity, a common strategy among widely distributed species, allows the use of fluctuating food resources driven by environmental variability in their home ranges (Carlign et al., 2019; Jafari et al., 2021; Song et al., 2021). Breeding seabirds can adjust their foraging behaviour based on prey accessibility near their colonies (McInnes et al., 2017; Jacoby et al., 2023; Querejeta et al., 2023). Indeed, variations in the availability and abundance of the main prey items could lead to differences in foraging behaviour and diet between breeding sites (Ainley et al., 1996; Mellink et al., 2001; Castillo-Guerrero et al., 2016). Foraging behaviour in red-billed tropicbirds varies according to the local marine environmental features (e.g., coastal upwelling vs. oceanic; Diop et al., 2018). The areas surrounding our study sites, used as tropicbird foraging grounds, exhibit contrasting local features (e.g., chlorophyll-a, air and sea surface temperature [SST]; Piña-Ortiz et al., 2023), as they are located in distinct marine ecoregions (see details Spalding et al., 2007). San Pedro Mártir, located in the Gulf of California, is an upwelling system, while Peña Blanca (Mexican Tropical Pacific) is primarily influenced by open, oligotrophic oceanic waters (Hernández-Vázquez et al., 2018). Preliminary data on foraging ecology indicate divergent foraging parameters between these colonies, with individuals from Peña Blanca undertaking longer and more distant trips compared to those from San Pedro Mártir (Figs. S4 and S5; Piña-Ortiz et al., unpubl. data). This suggests that differences in foraging behaviour are likely driven by variations in prey availability and resource distribution between the two locations.

Predators sharing common prey are thought to occupy similar ecological niches, potentially leading to competition for finite resources (Holt, 2009). During the breeding season, seabirds become central-place foragers, and those with overlapping breeding periods or confined foraging habitats often face heightened competition due to limited resource availability. Documented evidence indicates that intraspecific competition is more intense than interspecific competition among seabirds (Grémillet et al., 2004; Masello et al., 2010; Paredes et al., 2012; Rosciano et al., 2016; Lee et al., 2021). While San Pedro Mártir supports a greater diversity of breeding seabirds (8 species vs. 2 species), Peña Blanca has larger population sizes (~7500 vs. ~16,500 breeding seabird pairs; Hernández-Vázquez et al., 2017; Piña-Ortiz et al., 2018; Castillo-Guerrero et al., 2022). Therefore, we expect a scenario of increased

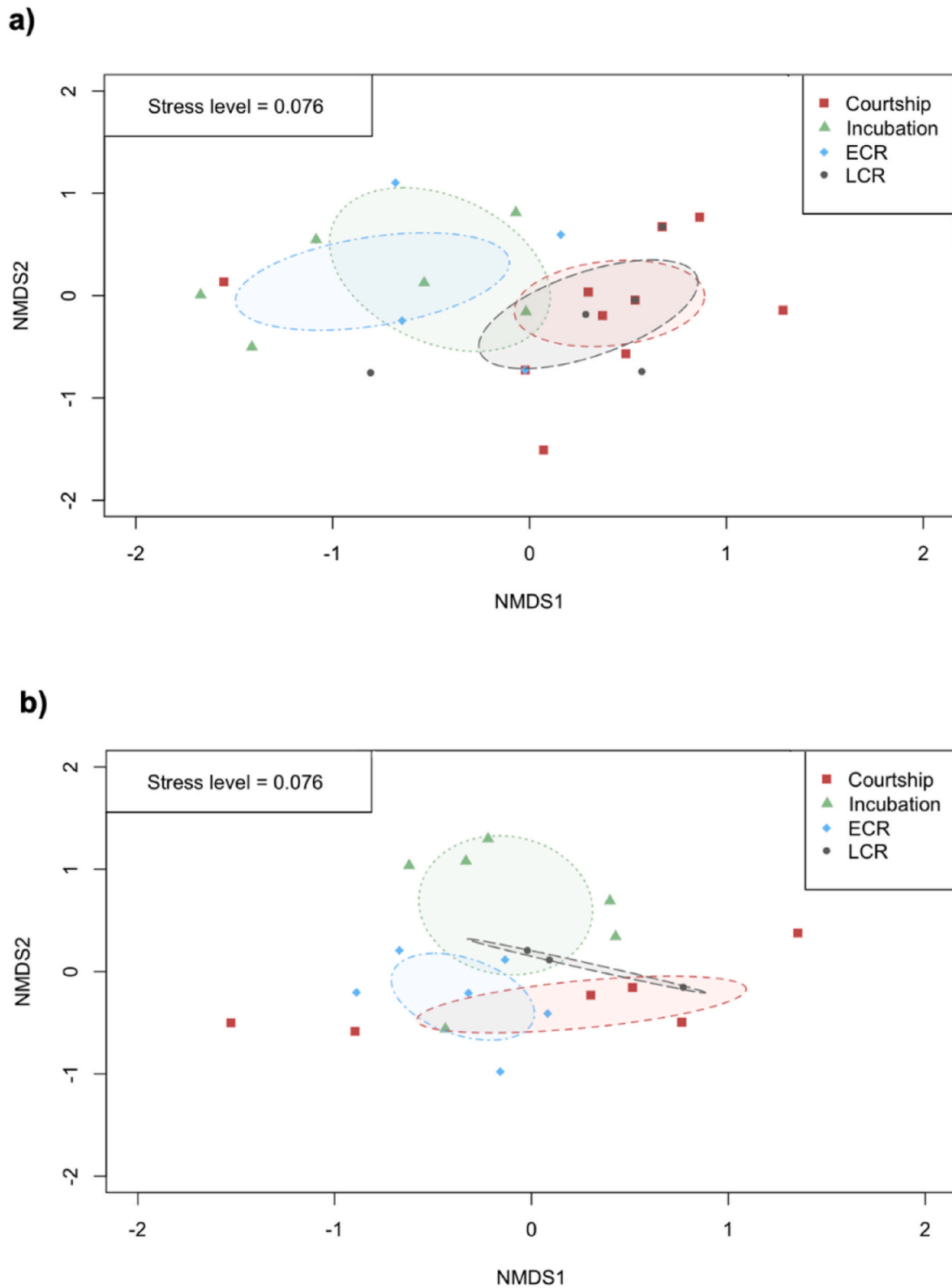


Fig. 4. Non-metric multidimensional scaling plots (Bray-Curtis $k = 2$) showing the dissimilarity patterns in prey compensation using the MOTUs between the four breeding stages (ECR = early chick-rearing, LCR = late chick-rearing) at **a)** San Pedro Mártir Island (stress level = 0.076) and, **b)** Peña Blanca Islet (stress level = 0.076).

competition for resources at the Peña Blanca colony, supported by differential foraging effort between study sites, which could result in a decrease in the abundance of preferred prey, and consequently, may result in a more diverse diet (Optimal foraging theory; MacArthur and

Pianka, 1966; Stephens and Krebs, 1986; Ratcliffe et al., 2018). Actually, studies have demonstrated that high intraspecific competition increases population niche width and individual specialization (Svanbäck and Bolnick, 2005, 2007; Ratcliffe et al., 2018). However, our results show a

Table 3

Summary statistics of the final selected general linear model (GLM) evaluating the effects of the site and breeding stage (courtship, incubation, early chick-rearing and late chick-rearing) on the nitrogen ($\delta^{15}\text{N}$) and carbon ($\delta^{13}\text{C}$) isotope ratios measured in whole blood of red-billed tropicbirds from the 2021 breeding season at San Pedro Mártir Island (temperate) and Peña Blanca Islet (tropical), Mexico. Sample sizes are as follows, courtship (San Pedro Mártir = 16, Peña Blanca = 11), incubation (San Pedro Mártir = 16, Peña Blanca = 15), early chick-rearing (San Pedro Mártir = 12, Peña Blanca = 18) and late chick-rearing (San Pedro Mártir = 18, Peña Blanca = 9). Significant terms are shown in bold.

Factors and interactions	$\delta^{15}\text{N}$			$\delta^{13}\text{C}$		
	F	df	p	F	df	p
site*stage	1.94	3, 106	0.13	2.70	3, 106	0.05
site	525.76	1, 106	< 0.001	24.0	1, 106	< 0.001
stage	6.50	3, 106	< 0.001	8.61	3, 106	< 0.001
date	1.83	1, 106	0.18	1.92	1, 106	0.17

surprisingly similar prey range and comparable niche breadth between sites (Layman's metric of convex hull area [TA]: San Pedro Mártir = 0.090 and Peña Blanca = 0.086; Fig. 2 and S6). Although resource availability and colony size generate a competition gradient (Ashmole, 1963; Gaston et al., 2007), this competition will not necessarily translate into an expansion of niche breadth (Correa and Winemiller, 2014). In fact, variability in niche breadth and individual specialization could arise from an "ecological opportunity" (e.g., spatio-temporal availability of resources) to exploit different prey rather than direct competition (see Araújo et al., 2012; Correa and Winemiller, 2014). Despite our findings agreeing on a relationship between resource availability and competition, disentangling the role of each in the diet of the Red-billed Tropicbird remains challenging to obtain. Future research could explore the diet of breeding seabirds from both sites and assess the interplay with the intra- and interspecific competition.

Moreover, the isotopic differences between sites closely mirrored those identified in the dietary analysis. Despite variations in consumed species, attributing all nitrogen value differences solely to the feeding

ecology of predominant prey proved challenging. Both sites featured predominantly planktivorous (e.g., Atherinopsidae, Exocoetidae, Engraulidae) and secondarily carnivorous fish species (e.g., Scombridae, Carangidae). However, observed isotopic variations may be significantly influenced by spatio-temporal fluctuations in isotopic baselines between sites, driven by environmental differences in the foraging grounds (Quillfeldt et al., 2005; Cherel and Hobson, 2007; Bond and Jones, 2009). The isotopic levels of red-billed tropicbirds from both study sites align with those of other seabirds in their respective regions. By comparing $\delta^{15}\text{N}$ values in their blood with those of other seabirds known to consume fish and cephalopods, consistent trophic positions were observed (Peña Blanca: ~17.7‰; San Pedro Mártir: 18.6–20.4‰; Auriolos-Gamboa et al., 2013; Castillo-Guerrero et al., unpubl. data). This consistency underscores the ecological connections within the seabird community in each marine region. Additionally, the observed $\delta^{15}\text{N}$ values in zooplankton, cephalopods, and flying fish across the study sites further support these ecological connections (Peña Blanca: ~10.7‰, 16.2‰, and 16.5‰; San Pedro Mártir: ~12.3‰, 16.3‰, and 16.1‰, respectively; Auriolos-Gamboa et al., 2013; López-Ibarra et al., 2018; Castillo-Guerrero et al., unpubl. data). Considering trophic

Table 4

Summary of the SIBER computational analysis evaluating the two-dimensional isotopic niche breadth based on Bayesian ellipses for red-billed tropicbirds from different breeding stages at San Pedro Mártir Island and Peña Blanca Islet during the 2021 breeding season. The Layman's metric of convex hull area (TA) and the area of the corrected standard ellipse (SEA_C). Sample sizes are as follows, courtship (San Pedro Mártir = 16, Peña Blanca = 11), incubation (San Pedro Mártir = 16, Peña Blanca = 15), ECR (San Pedro Mártir = 12, Peña Blanca = 18) and LCR (San Pedro Mártir = 18, Peña Blanca = 9).

Breeding stage	TA		SEA_C	
	San Pedro Mártir	Peña Blanca	San Pedro Mártir	Peña Blanca
Courtship	1.19	0.34	0.42	0.18
Incubation	1.36	0.71	0.55	0.31
Early chick-rearing	0.55	0.70	0.29	0.26
Late chick-rearing	0.88	0.24	0.32	0.15

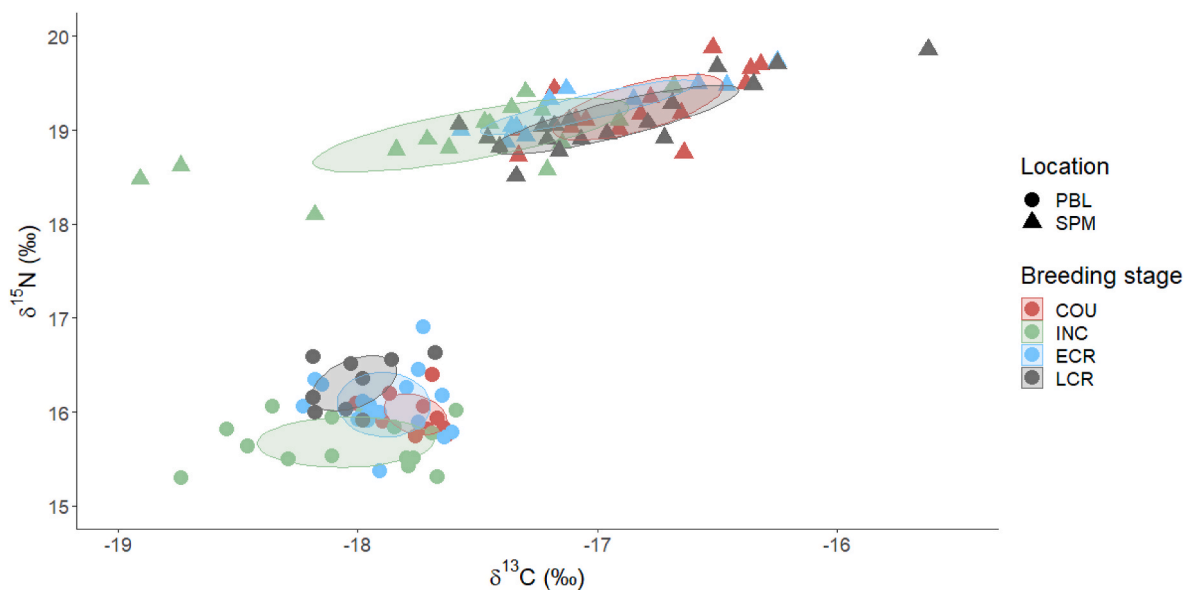


Fig. 5. Bayesian standard ellipse areas (SEA_B) estimated from stable isotope ratios ($\delta^{15}\text{N}$ and $\delta^{13}\text{C}$) of whole blood in red-billed tropicbirds during courtship [COU], incubation [INC], early chick-rearing [ECR] and late chick-rearing [LCR] at San Pedro Mártir [circle] and Peña Blanca [triangle] during the 2021 breeding season. Sample sizes are as follows, courtship (San Pedro Mártir = 16, Peña Blanca = 11), incubation (San Pedro Mártir = 16, Peña Blanca = 15), ECR (San Pedro Mártir = 12, Peña Blanca = 18) and LCR (San Pedro Mártir = 18, Peña Blanca = 9).

enrichment factors (2–3.5‰ blood tissue; Bond and Jones, 2009), the $\delta^{15}\text{N}$ values observed in red-billed tropicbirds are within the expected range for piscivorous seabirds in both study locations.

4.3. Temporal variation in prey composition

Our findings highlight the trophic plasticity of red-billed tropicbirds across the breeding season. Resource partitioning and adaptive foraging behaviour in response to changing requirements during different breeding stages has been documented in several seabird species (Navarro et al., 2014; Dehnhard et al., 2016; Booth et al., 2018; Gaglio et al., 2018; Soanes et al., 2021). In the case of San Pedro Mártir red-billed tropicbirds, diet has been found to be influenced by breeding stage and collection date, suggesting that variations may be attributed, at least in part, to differential prey selection between stages (see below), and changes in prey availability related to local oceanographic conditions, such as SST, primary productivity and seasonal hydrographic circulation (Sánchez-Velasco et al., 2009; Garcés-Rodríguez et al., 2021). For Peña Blanca, variations in diet were only influenced by breeding stage. Simultaneous sampling of all breeding stages at San Pedro Mártir allowed us to identify changes in diet associated with both stage and date. However, at Peña Blanca, sampling at different breeding stages followed a more chronological pattern, which limited our ability to discern the role of temporal food availability in dietary variation related to breeding stage. In addition, important inter-annual climatic variations are observed in the region, mainly linked to the ENSO cycle. Our findings highlight the relevance of local conditions on food availability, highlighting the importance of conducting research under variable conditions to understand the constraints and trophic dynamics of each colony.

In the Gulf of California, small pelagic fish availability is impacted by thermal fronts and mesoscale eddies, influencing the abundance of key prey species like the Californian anchovy, Pacific chub mackerel and South American pilchard (Garcés-Rodríguez et al., 2021). The absence of tropical fish, as flyingfish, during low SST in winter-spring contrasts with their increased presence during spring-summer, affecting prey availability for red-billed tropicbirds (Froese and Pauly, 2024). During courtship, individuals make various adjustments, including changes in behaviour, nest selection, defence, mating and egg production, aiming for higher energetic and physiological compensation compared to non-breeders. Previous studies suggest that a higher food quality and lipid metabolic profile during the pre-laying period are linked to increased breeding success and earlier reproduction in the season (González-Medina et al., 2018). In the case of red-billed tropicbirds, timely breeding is crucial, as both colonies are synchronised with peaks in food availability and breeding of other seabirds (Tershy and Breese, 1997; Hernández-Vázquez et al., 2017). A delay in the onset of breeding could increase competition for limited resources and reduce breeding success. As courting individuals exhibited higher $\delta^{15}\text{N}$ values compared to incubating individuals, indicating distinct foraging patterns. Unlike individuals in advanced breeding stages (e.g., incubation or chick-rearing), courting birds are not bound by temporal constraints or the physiological demand to provision for a chick. Their foraging trips, although the range is largely unknown, is comparable to adults during the late chick-rearing stage, where adults can spend more time foraging for oceanic prey offshore that is contemporary available. In Peña Blanca, Carangidae, particularly bigeye scads with high lipid content ($3.31 \pm 0.25\%$; Chedoloh et al., 2011), dominated the diet during courtship (FO ~40%). Conversely, courting individuals in San Pedro Mártir showed a diet reflecting the occurrence of profitable prey, including the Californian anchovy and *Scomber* sp.

Red-billed tropicbirds exhibit biparental care, with parental shifts during incubation lasting up to 12 days, significantly more to the <1–3 days recorded for the early chick-rearing stage (Fig. S5; Piña-Ortiz et al., 2024). Prolonged shifts during incubation require parents to endure prolonged fasting periods, emphasising the importance of diet

adjustments to maintain their metabolic needs. This probably involves the choice of larger prey or prey with higher lipid content, providing sustained energy release (Hilton et al., 2000; Jacobs et al., 2011). At Peña Blanca, the predilection of *Scomber* sp. (likely *japonicus*) for incubating individuals aligns with this strategy, given their higher muscle lipid content in larger individuals (size >20 cm: $23.2 \pm 1.2\%$, vs. <20 cm: $8.5 \pm 0.5\%$; Shulgina et al., 2019). A similar pattern was observed at San Pedro Mártir, where topsmelt silversides and Californian anchovies contributed significantly to the diet.

The dietary challenges faced by red-billed tropicbirds during chick-rearing are notable. For instance, adults exhibit a bimodal foraging strategy during this stage to meet the needs for chicks and themselves. Parents adjust their foraging behaviour, opting for shorter trips to coastal areas to enhance chick feeding frequency. Short foraging trips limit the effective time available for foraging, ensuring that adults can return faster to the nest and feed the chick more frequently (Piña-Ortiz et al., 2024). Although coastal areas offer cost-effectiveness, competition (particularly intraspecific, see above) there is markedly higher due to the predictability of food resources (see Weimerskirch, 2007), which poses a trade-off for red-billed tropicbirds, as foraging entirely near coastal areas could inflict finite resource depletion and increase competition (Weber et al., 2021). During chick-rearing, red-billed tropicbirds seem to adjust their prey choice, feeding selectively on higher caloric prey to meet the nutritional needs of the chick's growth. In fact, the stable isotope values varied between adults and chicks for both rearing stages in Peña Blanca, indicating that the diet selection for the adults is likely more selective, choosing prey that is abundant around pelagic waters, whereas prey provided to chicks are obtained closer to the coast and with higher $\delta^{15}\text{N}$ enrichment, as they require to fulfil their energetic demands (Piña-Ortiz et al., 2024). Previous research in seabirds linked higher-quality food, characterized by elevated caloric, protein, and lipid levels, with better weight gain, body condition, and higher breeding success (Albano et al., 2011; van Donk et al., 2017; González-Medina et al., 2017). Our study was limited to the analysis of adult faecal samples, but further studies should consider incorporating faecal samples of chicks, to confirm dietary divergence between age groups. Due to the differences in isotopic values between adults and chicks, we can assume that our data set reflects the diet of the adults rather than the prey selected for the chicks. For instance, early chick-rearing adults in Peña Blanca primarily consumed flying fish, representing a consistent and abundant prey resource in oligotrophic waters. In San Pedro Mártir, cold SST during winter-spring may limit flying fish availability, leading adults to shift preference towards other abundant epipelagic fish like topsmelt silversides and Californian anchovies.

After about the fifth week of age, chicks experience longer periods of parental absence in the nest, corresponding to a reduction in the food supply. This prompts adults to modify their foraging behaviour, spending less time to the nest but compensating with more time foraging (Stonehouse, 1962; Piña-Ortiz et al., 2024). Likely, adults select prey with elevated lipid content or greater body mass to try the enhanced fasting endurance of older chicks, potentially favouring larger or slower-digesting prey with a higher lipid profile to provide offspring. During this stage, San Pedro Mártir adults consumed *Scomber* sp., *Sardinops* sp., and *E. mordax*, representing prey with high internal fat content (e.g., *S. sagax* 14.5% lipid content; Clark et al., 2010) or abundant during that period. In Peña Blanca, late chick-rearing adults mainly consumed Carangidae and Engraulidae, likely the most profitable prey in the area considering spawning events. *Caranx* sp. (likely *C. caballus*) could be encountered more often once the adults start foraging offshore on more pelagic sites as indicated by the lower $\delta^{13}\text{C}$ levels, despite that the species in question could represent in general a profitable prey due to the moderate lipid levels (3.74 ± 0.41 g/100 g body mass, Murillo et al., 2014).

In addition, fisheries in the Midriff Islands region, where San Pedro Mártir is located, have a significant impact on the small pelagic stocks,

which could affect the availability of prey for red-billed tropicbirds (Cisneros et al., 1990; Cisneros-Mata et al., 1995; Morales-Bojórquez et al., 2021). A decline in South American pilchards has shown a direct correlation between catch-per-unit rate and the proportion in the diet of pelagic seabirds in the Midriff region, suggesting targeted prey capture by industrial fisheries could induce temporal shifts and depletion in prey availability, prompting birds to adjust their diet or explore less affected foraging grounds (Velarde et al., 2013). For red-billed tropicbirds, most of the main prey items identified in this study coincide with the target species of small pelagic fishing fleets in the Gulf of California (Martínez-Zavala et al., 2010), which does not rule out the possibility that dietary variation could be influenced by the regional fisheries. However, future research must be conducted in the long term to elucidate the precise impact of fisheries on the diet and foraging patterns of the species.

5. Conclusion

Our study provides a comprehensive account on the diet of the Red-billed Tropicbird (*P. aethereus*) for two ecologically contrasting study sites (upwelling vs. oligotrophic) along the Mexican Pacific coast using DNA metabarcoding and stable isotope values. Our data highlights that breeding adults exhibit a divergent dietary profile between sites, regardless of epipelagic fish being the predominant prey for both locations. Spatial divergences in the diet were linked predominantly to prey availability and abundance determined by environmental parameters. Both colonies showed further trophic plasticity between the breeding stages, which seems to be related to changing physiological requirements (e.g., metabolic changes during different breeding stages), and environmental (SST and chlorophyll-a fluctuation) and biotic patterns (prey availability and competition). Due to the prolonged breeding cycle, red-billed tropicbirds adjust their diet continuously in response to the nutritional requirements associated with the respective breeding stage and the environmental changes taking place, efficiently utilizing profitable prey that is available through the breeding season. Although our study only covered one season of the breeding ecology of this species, our data provide an insight into the dietary plasticity of this species. Further research incorporating more breeding colonies, additional samples and multiple study years would be highly desirable to facilitate our understanding of the foraging ecology of red-billed tropicbirds.

Funding

This work was supported by the Sonoran Joint Venture [MBJV/F20AP11531-00]; Deutsche Ornithologische Gesellschaft [DO-G, Forschungsförderung to VM]; Universidad de Guadalajara; Justus Liebig University [Erasmus+ Traineeship Programme to APO]; CONAHCYT [scholarship for doctoral studies abroad No. 795355 to APO]; and the Pacific Seabird Group [student research grant - PSG SRGA 2021 to APO]. We further acknowledge CPER (Contrat de Projet Etat-Région) and the FEDER (Fonds Européen de Développement Régional) for funding the IRMS of LIENSs laboratory.

CRediT authorship contribution statement

Vladislav Marcuk: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Alberto Piña-Ortiz:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **José Alfredo Castillo-Guerrero:** Writing – review & editing, Visualization, Validation, Resources, Methodology, Investigation, Funding acquisition, Formal analysis. **Juan F. Masello:** Writing – review & editing, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis. **Paco Bustamante:** Writing – review & editing,

Validation, Resources, Methodology. **Sven Griep:** Writing – review & editing, Validation, Software. **Petra Quillfeldt:** Writing – review & editing, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

We thank Departamento de Estudios para el Desarrollo Sustentable de Zonas Costeras CUCSur – Universidad de Guadalajara and Comisión Nacional de Areas Naturales Protegidas (CONANP – Islas del Golfo de California – Reserva de la Biosfera Isla San Pedro Mártir) for logistical support in the field, specially J. Ventura-Trejo. We also thank Captain A. Alvariz-Martínez and Captain E. Ramírez-León for helping us access the islands and the fieldwork assistants for their hard work. Furthermore, we thank A. Espino-Herrera, S. Gómez-Hernández, L. Aguilar-Nuño, and L. Estrada-Osuna for their help with sample processing in the laboratory; S. Wagner, M. Austad, M. Brault-Favrou, and C. Churlaud for training and sample preparation; and G. Guillou from the ‘Plateforme analyses isotopiques’ of LIENSs for running stable isotope analyses. PB is an honorary member of the IUF (Institut Universitaire de France).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.marenvres.2024.106627>.

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