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Molecular diet analysis of the marine fish-eating bat (*Myotis vivesi*) and potential mercury exposure

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Abstract: Mercury is a toxic element acquired by animals through feeding which can accumulate within food chains through biomagnification. This possesses particular risks to higher trophic levels and may unduly impact marine foraging species or individuals. The fish-eating bat (*Myotis vivesi* Menegaux, 1901) inhabits islands in the Gulf of California and can act as a predator in the marine environment. A predominantly marine diet and a high trophic position increase the risk of mercury exposure owing to increased bioaccumulation. Using molecular techniques to reconstruct diet, we show that *M. vivesi* regularly feeds on small fishes and crustaceans, particularly on the Californian anchovy (*Engraulis mordax* Girard, 1854) and a krill species (*Nyctiphanes simplex* Hansen, 1911). Additionally, we identify significant interannual variation in diet composition within this population, but measured levels of total mercury in faecal samples were not related to dietary diversity or trophic level.

Key words: biomagnification, DNA barcoding, diet analysis, mercury exposure, trophic transfer, *Myotis vivesi*, fish-eating bat.

Résumé : Le mercure est un élément toxique acquis par les animaux par leur alimentation et il peut s'accumuler dans les réseaux trophiques par le biais de la biomagnification, ce qui pose différents risques pour les niveaux trophiques supérieurs et pourrait avoir des impacts indus sur les espèces ou individus s'alimentant en milieu marin. La chauve-souris piscivore (*Myotis vivesi* Menegaux, 1901) vit dans des îles du golfe de Californie et peut agir comme prédateur dans le milieu marin. Un régime alimentaire à prédominance marine et une position trophique élevée accroissent le risque d'exposition au mercure en raison d'une bioaccumulation accrue. En utilisant des techniques moléculaires permettant de reconstituer le régime alimentaire, nous démontrons que la chauve-souris piscivore se nourrit régulièrement de petits poissons et crustacés, en particulier l'anchois du Pacifique (*Engraulis mordax* Girard, 1854) et une espèce de krill (*Nyctiphanes simplex* Hansen, 1911). En outre, si nous relevons une variation interannuelle significative de la composition du régime alimentaire au sein de cette population, les teneurs de mercure total mesurées dans les échantillons fécaux ne sont pas reliées à la diversité de l'alimentation ni au niveau trophique. [Traduit par la Rédaction]

Mots-clés : biomagnification, codes-barres d'ADN, analyse du régime alimentaire, exposition au mercure, transfert trophique, *Myotis vivesi*, chauve-souris piscivore.

Introduction

The Gulf of California is an important area for biodiversity conservation in both the terrestrial and the marine environments (Enríquez-Andrade et al. 2005). High levels of species endemism, as well as small populations on the mosaic of islands in this region, leave many species vulnerable to anthropogenic pressures (Enríquez-Andrade et al. 2005). One species of special concern is the endemic Mexican fish-eating bat (*Myotis vivesi* Menegaux, 1901), which is classified as vulnerable by the International Union for the Conservation of Nature (IUCN) (Arroyo-Cabrales and Ospina-Garces 2016) and endangered by the Mexican government (SEMARNAT 2010). This bat is mainly restricted to islands in the Gulf of California, and its roosting behaviour makes it vulnerable to predation by introduced species (e.g., cats and rats) (Herrera et al. 2019). In contrast to most other bat species, *M. vivesi* feeds primarily on marine fish and crustaceans (Otálora-Ardila et al. 2013; Aizpurua and Alberdi 2018). Due to its marine-based diet, this species may be at particular risk from the harmful impacts of bioaccumulation of heavy metals. For example, elevated concentrations of several heavy metals (copper, zinc, iron, manganese, and lead) have been

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detected in the liver of *M. vivesi* (Méndez and Alvarez-Castañeda 2000).

Mercury (Hg) is a toxic element; while naturally released into the atmosphere during volcanic activity and erosion, most Hg in the atmosphere today is released by anthropogenic activities, such as mining, and is a major contaminant of terrestrial and aquatic ecosystems (Gochfeld 2003; Selin 2009). When deposited in aquatic systems, sulphate-reducing bacteria convert inorganic forms of Hg into the highly toxic methylmercury (MeHg), which can then become magnified through the trophic food web (Selin 2009). As a consequence, high concentrations of MeHg can be detected in the tissues and organs of top predators, such as northern elephant seals (*Mirounga angustirostris*) (Peterson et al. 2018), ringed seals (*Pusa hispida*), and polar bears (*Ursus maritimus*) (Brown et al. 2018), as well as other fish and marine mammals (see species within Boening 2000). The amount of MeHg that a terrestrial predator is exposed to will depend on the level of connectivity it has with aquatic ecosystems. For example, previous work has shown that the greater bulldog bat (*Noctilio leporinus*; a piscivorous bat) has higher MeHg concentrations in hair compared with bats in other dietary guilds (Becker et al. 2018). Additionally, it was found that insectivorous bats feeding on invertebrates which have early aquatic life stages show higher levels of MeHg compared with frugivores (Becker et al. 2018). Although *M. vivesi* feds primarily on marine fish and crustaceans (Reeder and Norris 1954; Blood and Clark 1998), the relative contribution of these prey items varies seasonally (Otálora-Ardila et al. 2013), exposing the bats to food items that may vary in levels of dietary MeHg.

Faecal samples are a well-known biomarker of inorganic mercury (IHg) exposure, but may also provide insight into MeHg exposure. MeHg consumed orally quickly leads to a rise in blood MeHg levels and is excreted via faeces (Komsta-Szumska et al. 1983). In a comparison of MeHg vs. IHg in various tissues and faeces following exposure, both were detected in faeces at 2 days post exposure (the earliest sample point), suggesting that excretion and thus detectability can be rapid (Komsta-Szumska et al. 1983), although approximately two-thirds of MeHg were converted to the inorganic form within hours (Komsta-Szumska et al. 1983). Reports suggest that up to 95% of consumed MeHg can be absorbed by the gastrointestinal tract in humans before excretion in the faeces (Ishihara 2000). In both cases, the evidence suggests that MeHg is rapidly detectable in faeces following consumption but may underestimate actual exposure. When Wistar rats (albino laboratory rats, *Rattus norvegicus domestica*) were fed fish containing methylmercury or the inorganic methylmercury chloride salt, faecal MeHg was higher and tissue accumulation lower in those with MeHg-contaminated food sources suggesting that the form of Hg used in many laboratory experiments influences estimates of absorption and excretion (Berntssen et al. 2004). In the same experiment, faeces were shown to be a better biomarker for MeHg than urine (Berntssen et al. 2004), with faecal MeHg thought to represent a combination of excreted and unabsorbed MeHg (Berntssen et al. 2004), suggesting that it may act as a biomarker for both short- and moderate-term exposures. As a consequence, faecal analysis may be a viable and a less invasive way to assess potential exposure risks, particularly in conjunction with diet analyses, but will likely underestimate actual Hg.

The objective of this study was to use novel genetic tools to establish the diet of *M. vivesi* from collected guano samples and then to test whether there is a relationship between diet and Hg concentrations in *M. vivesi* guano samples. We characterised the diet of *M. vivesi* using DNA metabarcoding of individual faecal samples, collected over 2 years, and analysed a subset of these faecal samples for total Hg (MeHg + IHg) concentration to assess potential exposure. We employed metabarcoding for faecal analysis, which can provide greater resolution of diet composition compared with traditional microscopy techniques, particularly when there are minimal visible remains (Symondson 2002) and for species where feeding behaviour is difficult to observe (Clare et al. 2009; Salinas-Ramos et al. 2015). To our knowledge, this is the first use of metabarcoding to establish the marine diet of terrestrial mammals and from this we investigated the diet of *M. vivesi* and address (*i*) the existence of interannual differences in their diet, (*ii*) whether individuals with a greater diversity of fish in their diets have higher total Hg concentrations in their faeces than those individuals feeding predominantly on one fish species, and (*iii*) how molecular identification of dietary items compare with previous morphological analysis of the diet of *M. vivesi*. We predicted that bats with a higher diversity of prey, as well as prey of a higher trophic position, in their diet will have a higher faecal Hg concentration.

Materials and methods

Sample collection and preparation

Myotis vivesi is the only species of bat roosting in Partida Norte Island, Mexico, where it uses rock crevices on talus slopes. Sampling took place from December 2012 to July 2013 (hereinafter referred to as the 2012 year) and from February to July 2014. Individuals were collected by hand from diurnal roosts at this location in the early morning (5–8 am). Between 2 and 4 bats per roost were placed in individual cloth bags for 30 min to 3 h and then returned to their roosts. Guano produced in the bags was stored in 75% ethanol at −20 °C before being transported to Queen Mary University of London (QMUL) for analysis. Total Hg analysis was conducted on guano from 2014 by dividing the pellet in half to generate matched samples. One half was analysed for Hg and the other half was analysed for diet.

Creation of a bespoke fish database

Metabarcoding relies on the comparison of unknown sequences to a DNA reference database; therefore, to aid in identifications, we created a bespoke reference dataset that consisted of DNA barcode sequences of fish species found within the study site and therefore are potential prey items. This is available as a public project "Fishes from Isla Partida (FIP)" on the BOLD database (Ratnasingham and Hebert 2007) and was transferred to the mBRAVE system for metabarcoding analysis (http://www.mbrave.net). In summary, this database contained 606 unique sequences, 185 BINs (Ratnasingham and Hebert 2013), 97 species comprising 70.1% Actinopterygii, 28.8% Malacostraca, 0.6% Elasmobranchii, and 0.6% Gastropoda.

The samples for this database were collected from five localities in the Gulf of California (Bahía de Kino and Bahía de los Angeles on the coast and close to the islands of Partida Norte, Angel de la Guarda, and Rasa) and one in the coast of Colima (Palo Verde estuary). The samples were obtained with a light trap deployed overnight (Elías-Gutiérrez et al. 2018) using a drag network (600 μm) and cast net. Individual larvae were fixed with 96% alcohol for transport to the laboratory. In case of juveniles and adult fish that could be taxonomically identified in the field, a sample of tissue was fixed for processing. DNA extraction, polymerase chain reaction (PCR), and sequencing of fish references were conducted at the laboratory in El Colegio de la Frontera Sur, Chetumal. All methods followed those described by Valdez-Moreno et al. (2010).

Molecular diet analysis

We extracted DNA from each guano sample using the QIAamp DNA Stool Mini Kit (Qiagen, UK), following the manufacturer's protocol and including modifications suggested by Zeale et al. (2011). Residual material was frozen for future analysis. PCR was conducted on all samples, targeting a 226 bp region of mitochondrial cytochrome *c* oxidase subunit I (COI) gene, using primers designed to amplify degraded fish material but with previously observed wider amplification success (CS1-Fish_miniE_F 5′-ACA

CTG ACG ACA TGG TTC TAC ACY AAI CAY AAA GAY ATI GGC AC-3′ and CS2-Fish_miniE_R 5′-TAC GGT AGC AGA GAC TTG GTC TCT TAT RTT RTT TAT 5CG 5GG RAA) adapted from Shokralla et al. (2015) for the Illumina MiSeq sequencing platform. PCR reactions were conducted in a total volume of 15 μL with 7.5 μL of Multiplex Master Mix (Qiagen, UK), 0.25 μL of both the forward and reverse primers (10 μmol/L), 5 μL of nuclease-free water and 2 μL of DNA template. The thermocycler conditions were 95 °C for 15 min, followed by 35 cycles of 94 °C for 40 s, 46 °C for 1 min, and 72 °C for 30 s, and a final extension of 72 °C for 5 min. PCR products were visualised on a 1% agarose gel. DNA quantification was conducted using QuBit dsDNA HS Assay Kit (Invitrogen, Life Technologies, UK) and further quality control was performed using the TapeStation D1000 (Agilent Technologies, UK). Sequencing was performed using 10 bp Fluidigm indexes and MiSeq2 Chemistry (Illumina, UK) for 2 × 150 bp target length at The Bart's and the London Genome Centre, London, UK.

We trimmed the primers and merged paired-end reads using AdapterRemoval (Schubert et al. 2016) before the merged reads were uploaded to mBRAVE (http://www.mbrave.net), which is a metabarcoding platform that is integrated with BOLD systems (Barcode of Life Data System; Ratnasingham and Hebert 2007). In mBRAVE, we set parameters to automatically trim, filter, and assign taxonomy to our data using the parameters: trim length = 600 bp; minimum QV = 0 qv; minimum length = 100 bp; maximum bases with low QV (<20) = 75%; maximum bases with ultra low QV (<10) = 75%; ID distance threshold = 1.5%; exclude from OTU threshold = 3%; minimum OTU size = 1; OTU threshold = 2%. Sequences were first compared with the FIP database generated for this project, in collaboration with the MEXBOL Network. Although the primary goal was to identify fish in the diet of this species, unassigned sequences following this initial screening were compared with three additional public reference databases available on mBRAVE: (1) chordates (SYS-CRLCHORDATA), (2) non-insect arthropods (SYS-NONINSECTARTH), and (3) non-arthropod invertebrates (SYS-CRLNONARTHINVERT). From the resulting taxonomic assignments, we removed any hits with less than 200 reads assigned (threshold set from blanks, controls, and control mixes "mock communities" (E.L. Clare, QMUL, London, UK, personal communication, January 2020) to reduce false positives. The data were then standardised to presence or absence because using read abundances is not advised due to differential digestion processes and PCR stochasticity in uncontrolled systems (Deagle et al. 2019). Any sequences matched to *M. vivesi* or human were removed. All taxa identified were assessed for known geographic location. A single species, the European seabass (*Dicentrarchus labrax* (Linnaeus, 1758)), was removed as a likely false positive. Many names have been applied to similarly appearing but often distantly related fishes in Mexico; for example, "bass" is sometimes used for representatives of several families of spiny-rayed fishes (Page et al. 2013), thus the common name "bass" can be used for fish in the area though there are no records of the family of the European seabass (Moronidae) from the Eastern Pacific. With >18 000 reads assigned to this species, it may represent a problem in the reference database reflecting common name confusion.

Total Hg analysis

Total Hg analysis was performed at Texas Christian University (USA). Prior to analysis, ethanol-preserved samples were dried for 48 h in a 60 °C oven. We determined total Hg concentrations in faecal samples in 35 of the 38 samples collected in 2014 using a Milestone Direct Hg Analyzer, which uses thermal decomposition, gold amalgamation, and atomic absorption spectroscopy (US EPA 1998). Mean (±SE) mass of faecal samples was 9.2 ± 1.2 mg. Quality assurance included reference (National Research Council of Canada Institute for National Measurement Standards) and duplicate faecal samples. The mean (±SE) percent recovery for reference samples (DORM-4) was 94.3% ± 0.96% (n = 17). Duplicate faecal samples were analyzed approximately every 20 samples and the mean (±SE) relative percent difference was 19.1% ± 8.14% (n = 2). The limit of detection was 2.84 ng/g based on a sample mass of 10 mg. All samples were above the limit of detection.

Statistical analysis

The diet types of the individual bats were classified based on the prey found in the DNA analyses, which was either (*i*) a fish-only diet, (*ii*) a crustacean-only diet, or (*iii*) a mixed diet, where DNA was from both fish and crustacean species in any proportion. In 10 samples, no DNA could be identified; these were assigned a fourth category of no-DNA diet. To investigate interannual differences in the diets of *M. vivesi*, we used Pearson's χ^2 to test the probability of observing differences in interannual dietary types. To compare within the dietary groups, post hoc pairwise tests were applied using the Bonferroni correction for multiple testing on the standardised residuals of the χ^2 test. We then used a general linear model (GLM) with a Gaussian distribution to test for differences in faecal Hg levels between individuals with different diet types. Log concentration of Hg was the response variable against the categorical variable of diet type. We did not include sex, age, or seasonal predictors given unbalanced and small sample sizes in subcategories (for example, only adults were caught in the 2012–2013 year and subadults were caught in only one sample trip of 2014). These analyses were conducted in R (R Core Team 2018) using packages "tidyverse" (Wickham et al. 2019) and "chisq.posthoc.test" (Ebbert 2021).

Ethics approval

The protocols for this study were approved by the Mexican Wildlife Service (Dirección General de Vida Silvestre) and were in accordance with the Canadian Council on Animal Care (CCAC) guidance and animal welfare procedures and guidelines from the American Society of Mammologists (Sikes and the Animal Care and Use Committee of the American Society of Mammalogists 2016). This process included ethical approval of sampling and field protocols. All procedures were in accordance with local laws (Dirección General de Vida Silvestre, Comisión Nacional de Áreas Naturales Protegidas, and Secretaría de Gobernación) under permit nos. 2082/12, 1947/13, and 4169/14.

Results

A total of 35 guano samples were sequenced from 2012 and 38 guano samples were sequenced from 2014, resulting in 2.13 million reads. After filtering out predator DNA, as well as other non-target DNA, 20 of the 35 samples from 2012 and 28 of the 38 samples from 2014 contained identifiable prey DNA meeting our quality filtering (above). In total, 11 prey taxa were detected across the 48 samples, representing four crustaceans and seven fish (Table 1). In 2012, the two most commonly identified prey items were a euphausiid krill (*Nyctiphanes simplex*) and the Californian anchovy (*Engraulis mordax*). *Engraulis mordax* was also the most commonly detected prey in 2014, along with the Panama lanternfish (*Benthosema panamense*) (Table 1). *Nyctiphanes simplex* was the most commonly detected crustacean in 2014. In 27 *M. vivesi* faecal samples, only fish items were detected; in 13 faecal samples, only crustaceans were detected; in 8 faecal samples, both fish and crustacean taxa were detected (Table 1). The mixed-diet samples contained only one crustacean taxon, which was most frequently *N. simplex* (7 out of 8 samples). Most mixed diet contained only one fish taxon too; however, one sample contained three different fish taxa. Mercury was detected in 35 samples with levels ranging from 53.3 to 757.4 ng/g and a mean concentration of 225.5 ng/g.

Interannual differences in *M. vivesi* diet composition

There was a significant difference between the observed number of samples of each diet type for each yearand the expected

Table 1. Identity of prey species recovered from COI DNA barcoding of fish-eating bat (*Myotis vivesi*) guano samples.

Prey type	Taxonomic name	Common name	2012	2014
Crustacean	Euphausiacea	Unidentified krill	1	2
	Farfantepenaeus californiensis (Holmes, 1900)	Yellow-leg shrimp	0	2
	Litopenaeus vannamei (Boone, 1931)	White-leg shrimp	1	0
	Nyctiphanes simplex Hansen, 1911	Euphausiid krill	13	6
Fish	*Benthosema panamense* (Tåning, 1932)	Panama lanternfish	4	10
	Coryphaena sp.	Dolphinfish	1	0
	Engraulis mordax Girard, 1854	Californian anchovy	8	10
	Mugil curema Valenciennes, 1836	White mullet	1	0
	Opisthonema libertate (Günther, 1867)	Pacific thread herring	1	0
	Prognichthys sp.	Sailor flyingfish	4	0
	Sphoeroides annulatus (Jenyns, 1842)	Bullseye pufferfish	1	0

Note: The number of detections for both years is given. There were 20/35 samples with amplifiable prey DNA in 2012 and 28/38 samples with amplifiable prey DNA in 2014.

Table 2. Contingency table and residuals of post hoc analysis for the χ^2 test between diet type and year of sampling.

	2012–2013			2014		
Diet type	Observed	Expected	Residual	Observed	Expected	Residual
Crustacean-only	6	5.28	0.45 ($p = 1$)	8	8.72	−0.45 ($p = 1$)
Fish-only	9	10.93	−1.02 ($p = 1$)	20	18.07	1.02 ($p = 1$)
Mixed	7	3.02	3.12 ($p = 0.01$*)	1	4.98	−3.12 ($p = 0.01$*)
No DNA identified	1	3.77	−1.98 ($p = 0.38$)	9	6.23	1.98 ($p = 0.38$)

Note: Observed and expected values used in the Pearson's χ^2 test are given ($\chi^2_{[3]} = 12.42$, $p < 0.05$*). Post hoc analysis was done using standardised residuals to test the significance of each category with the Bonferroni correction used to account for multiple testing. An asterisk indicates where the p value is significant at $\alpha = 0.05$.

values ($\chi^2_{[3]} = 12.42$, $p < 0.05$; Table 2). The post hoc pairwise analysis showed significant differences between two of the groups. There were more individuals with a mixed diet in 2012–2013 compared with 2014, and a greater number of bats with a predominantly fish-based diet in 2014 compared with those with a mixed diet in 2012–2013 (Fig. 1; Table 2).

Effects of predator diet types on total Hg in guano

The GLM showed that Hg concentration does not vary between different diet types ($R^2 = 0.06$; df = 2, 31; $p = 0.38$) (Table 3). The bat with mixed diet was not included in the model because it was a single observation. There was no difference in the mean total Hg concentration of the individual guano samples where only one prey type was detected (Fig. 2). Samples where no prey DNA could be detected (only *M. vivesi* or contaminant DNA was detected) had the highest absolute total Hg concentration and also the greatest variability (Fig. 2); however, this was not significantly different (Table 3).

Discussion

Our molecular dietary reconstruction was able to provide detailed taxonomic information on the fish consumed by *M. vivesi* and we were also able to confirm a large number of crustaceans as a primary food sources as previously described (Walker 1950; Reeder and Norris 1954; Bloedel 1955; Maya 1968; Otálora-Ardila et al. 2013). We identified *N. simplex*, *E. mordax*, and *B. panamense* as the most frequently consumed species. Although we confirmed the presence of *B. panamense* and *E. mordax*, other well-known prey species such as the Mexican lampfish (*Triphoturus mexicanus*) were not present in the bespoke reference database, but specimens were included in the system chordate database though they produced no confirmed matches in our samples. Matches to *B. panamense* and *E. mordax* were found in most samples, suggesting a significant role in supporting *M. vivesi* diet. *Benthosema panamense* is very small even as an adult (e.g., <5 cm in length), but some of the other species identified as prey can be much larger. Molecular data does not inform the life stage (larva to adult) being consumed, but it is likely that potentially large prey are only eaten as larva or juveniles.

DNA barcoding of fish from environmental DNA (eDNA) has often focused on the use of the 12s ribosomal DNA region (Miya et al. 2015) and the same region has also been used for piscivorous diet analysis (e.g., Deagle and Tollit 2007; Deagle et al. 2009, 2013), but both applications have had limited taxonomic scope. Here we demonstrate that COI primers designed for degraded fish material (Shokralla et al. 2015) provide an excellent level of detection and identification for fish as dietary items. Although it was not our primary objective, these primers were also useful for the detection of crustaceans in their diet. It should be noted that the crustacean databases available are small and their taxonomy in need of curation, which may limit the certainty in some of our identifications. Our bespoke database was augmented by the extensive COI databases available in BOLD and mBRAVE to make these non-fish identifications. The COI primers used here have not previously been tested for their binding affinity to non-fish DNA, thus it is possible that some crustacean species are not amplified and could have been missed. We hesitate to advise the use of these primers as a tool for analysis of dietary arthropods without further robust testing; however, results of such tests may demonstrate an advantage in other DNA barcoding applications. Although 12s primers work exceptionally well for fish communities (Miya et al. 2015), COI primers are frequently designed with broad taxonomic ranges in mind. For example, common fish primers used for full length barcoding also amplify a wide range of vertebrate and non-vertebrate species including a large number of crustaceans (Valdez-Moreno et al. 2012). The fact that the COI primers used here amplify a wider range of taxa than the fish which they were designed to target makes them a potentially excellent choice for marine dietary analysis, particularly when some non-fish items are expected. Their testing for eDNA studies of aquatic communities and across other taxonomic groups should be prioritised where a mixed community is expected (for example see Hallam et al. 2021). A combined approach may be particularly advantageous for cross validation and to minimize

Fig. 1. The dietary composition of guano samples collected from the fish-eating bat (*Myotis vivesi*) in 2012–2013 and 2014. Individual samples were assigned a diet type based on DNA metabarcoding; these were samples where only fish was detected (fish-only diet), where only crustaceans where detected (crustacean-only diets), or where both fish and crustacean DNA were detected in any proportion (mixed diet). Additionally, there were also samples for which no DNA from dietary taxa could be identified (no-DNA diet).

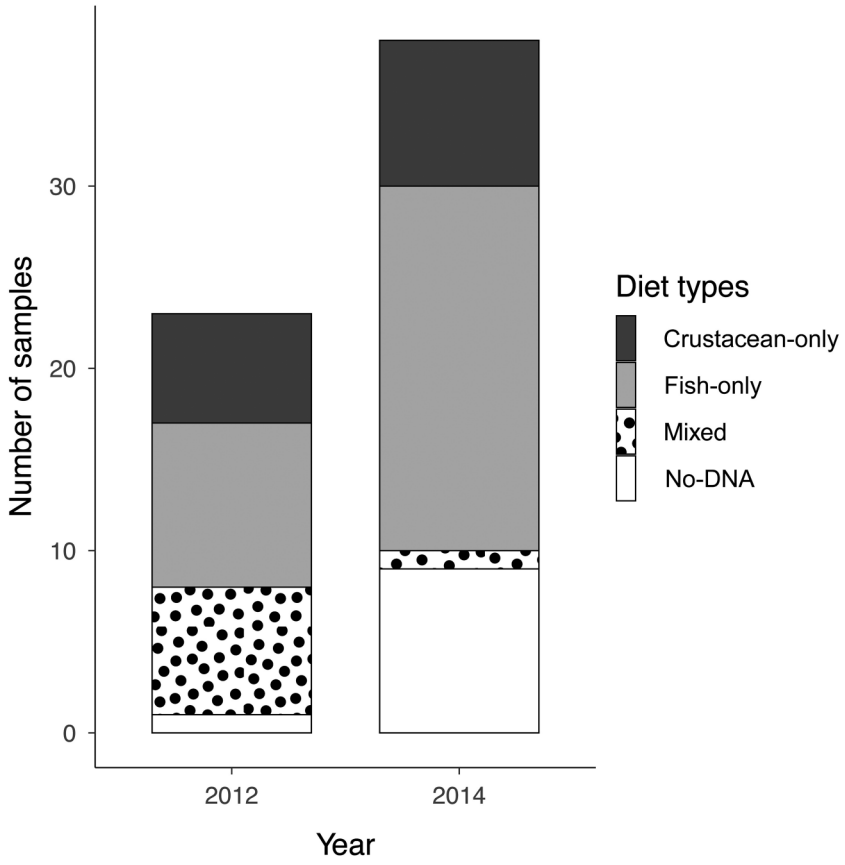


Table 3. Results of GLM showing the effect of the individual bat's predominant diet type on the log-transformed concentration of Hg detected from its guano sample.

Diet type	Estimate (±SE)	*t*	*p*
Intercept	5.28 (0.22)	23.70	<0.05*
Fish-only	−0.15 (0.26)	−0.55	0.59
None	0.24 (0.33)	0.75	0.46

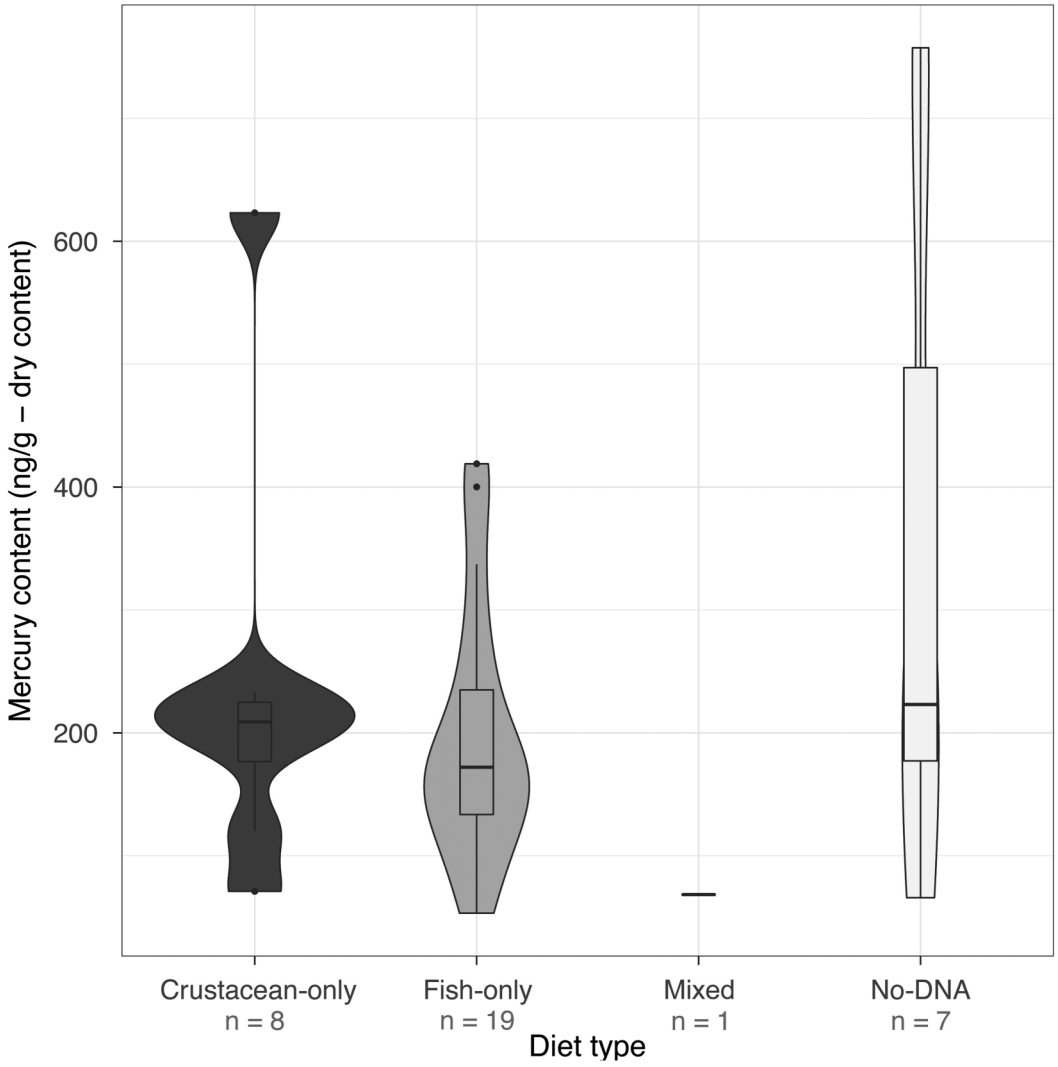
Note: Only relates to those 35 samples collected in 2014, for which Hg was analysed. Diet type is assigned based on the predominant taxa detected from DNA metabarcoding. An asterisk indicates where the *p* value is significant at $\alpha = 0.05$.

primer biases. Combinations of COI and 12s would be ideal for this work where a fish community is of particular interest, but a broader taxonomic perspective is useful.

We observed some differences in dietary composition between years with mixed fish–crustacean diets much more common in 2012–2013. Timing of sampling could have an effect on our measurement of overall diet composition. Past literature concluded that fish were more commonly consumed by *M. vivesi* in June, September, and October, whereas crustaceans were more dominantly consumed in February, April, and December (Otálora-Ardila et al. 2013). Sampling for both years covered the months in which the ratio of fish to crustaceans fluctuates, and while this could be driven by physiological demands, it may also reflect availability of the correct size class of prey species. Small sample sizes preclude further dividing these data to analyse seasonal variation. Interestingly, diel migration patterns may also play a part in predation by *M. vivesi*. For example, lanternfish species (Myctophidae) have different patterns of diel migration (Watanabe 1999), with some species coming to shallower areas at night in pursuit of prey. This vertical migration may bring them to the surface at the time that bat foraging peaks.

We detected total Hg in bat guano samples, confirming that individuals were being exposed to Hg. Edwards et al. (2019) found that mean total Hg concentration of guano from insectivorous bats in the southern US was 550 ng/g, which is similar to the total Hg levels that we report here. Interestingly, we observed no relationship between the level of total Hg in faeces and the dietary trophic ranking. In contrast, a comparison of bats across trophic levels in Belize (Becker et al. 2018) found that the aquatic foraging *N. leporinus* had much higher MeHg concentrations in hair than terrestrial insectivorous species such as the elegant myotis (*Myotis elegans*). Assuming that *M. vivesi* is feeding on the younger life stages of the larger predatory fish species due to their smaller size, this size constraint may actually act to minimize exposure to mercury. The younger an organism is, the less time it has had to be exposed to MeHg and therefore less MeHg bioaccumulates within tissues (Sanz-Landaluze et al. 2015). Additionally, the composition of a fish species diet can change with each life stage. For example, fish at younger life stages will likely eat invertebrates, resulting in low levels of dietary uptake of MeHg. In contrast, adult fish may eat other fish, resulting in a higher intake of MeHg due to the increased trophic levels of their prey items (MacCrimmon et al. 1983; Mathers and Johansen 1985). The intake of MeHg by *M. vivesi* feeding on species at earlier life stages would be lower than expected compared

Fig. 2. Violin plot showing the differences in mercury concentration (ng/g dry mass) depending on one of four main diet types assigned to fish-eating bat (*Myotis vivesi*) guano samples collected in 2014. The four diet types were assigned based on the results of the DNA metabarcoding and the same as the previous analysis: (1) DNA detected from fish (fish-only), (2) DNA detected from crustaceans (crustacean-only), (3) both fish and crustacean DNA detected (mixed), or (4) no dietary taxa DNA detected (no-DNA).



with a predator consuming the same species at an adult stage. *Nyctiphanes simplex* and *E. mordax* are small prey species and a large quantity may be eaten at one time, especially in a taxon like *E. mordax* which performs shoaling behaviour (Pitcher 1986; Robinson 2004) and thus becomes an excellent target for foraging *Myotis*. It is interesting to note that bats with mixed diets appeared to have guano with lower total Hg content. It is important to consider the temporal aspect of this because we expect our analysis to reflect only recent consumption for any one individual (e.g., within 24 h given fast gut passage times), but the consistency across individuals suggest a seasonal change in diet. As it is not advisable to quantify abundance or biomass using molecular approaches in most cases (Deagle et al. 2019), it is quite possible that some species were detected from very small or very large DNA traces unrelated to their actual biomass, making mixed diets hard to quantity for trophic position. Another potential explanation is that the time scales of detection of MeHg and DNA may differ. While both markers are thought to appear in faeces rapidly after ingestion, the duration of their detection is not known. It is possible that an individual with a mixed diet would have DNA from multiple sources, but would have MeHg from the previous day diluting any signal, or the opposite may also be true, that is, an individual who appears to be a fish specialist by DNA may carry the MeHg signal of a mixed diet the day before. It is also important to consider that while Hg is known to accumulate with age, it is not clear if this Hg accumulation would be reflected in faecal Hg, which likely reflects recent consumption. Further analyses should consider the influence of sex and age classes (juvenile vs. adult), which have both been shown to influence dietary choices that may alter exposure to Hg and thus its presence in faeces, though the effect may be subtle.

MeHg concentrations are considered toxic at a threshold of 10 mg/kg in hair reported for a number of mammals (Nam et al. 2012). However, faecal analysis does not assess toxicity because it measures excretion and not bioaccumulation of MeHg (Petersson et al. 1991). Yet this method provides a relatively non-invasive way to measure potential exposure to Hg (Edwards et al. 2019). More work is needed to determine the relationship between Hg in faeces and other tissue matrices like hair and blood. Although our analysis of faeces will likely underestimate the level of Hg eliminated from the body (which primarily occurs via the urinary system) or stored in tissues, it is a simple non-invasive method

to compare relative risk of exposure. The next step will be to directly sample bat hair to assess bioaccumulation, which may be particularly important when placed in the context of a decreasing population size and vulnerable status (IUCN Red List of Threatened Species).

A number of interesting future directions are suggested by our observations. First, the variation in diet between years needs more consideration. Whether this represents stochasticity or tracking of seasonally abundant prey, it is important for conservation planning. Second, it is particularly interesting that samples with the highest total Hg readings failed to produce sequences that could be assigned to prey (only some predator DNA was recovered). It is unclear if high levels of Hg may interfere with laboratory biochemical processes (Dalecka and Mezule 2018) or cause higher degradation rates making DNA less accessible, but the content of these samples is highly suspicious. One possibility is that they represent primarily endogenous losses of Hg in individuals who had not recently eaten.

Our data represent the first molecular analysis of a marine foraging bat and provide an efficient methodology for future marine dietary analyses. We confirm previous morphological work and provide additional resolution on species of fish and crustacean supporting this rare and vulnerable species of bat. In particular, our data suggest potential Hg exposure and interesting potential patterns of interannual variation in diet.

Competing interests

The authors declare that there are no competing interests.

Contributor statement

R.D., A.G., and J.C. performed molecular analysis. J.M.K. and M.M.C. performed mercury analysis. L.G.H.M., M.V., and E.L.C. secured funding and designed the experiment. A.O.-A. and J.J.F.-M. provided support in Mexico. All authors contributed to the writing of the manuscript.

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Data availability

All molecular and mercury data are available on Figshare digital repository (https://dx.doi.org/10.6084/m9.figshare.14519463).

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References

Aizpurua, O., and Alberdi, A. 2018. Ecology and evolutionary biology of fishing bats. Mamm. Rev. **48**(4): 284–297. doi:10.1111/mam.12136.

Arroyo-Cabrales, J., and Ospina-Garces, S. 2016. Fish-eating bat, *Myotis vivesi*. *In* The IUCN Red List of Threatened Species. Version e.T14209A22069146. Available at https://dx.doi.org/10.2305/IUCN.UK.2016-1.RLTS.T14209A22069146.en [accessed 21 March 2020].

Becker, D.J., Chumchal, M.M., Broders, H.G., Korstian, J.M., Clare, E.L., Rainwater, T.R., et al. 2018. Mercury bioaccumulation in bats reflects dietary connectivity to aquatic food webs. Environ. Pollut. **233**: 1076–1085. doi:10.1016/j.envpol.2017.10.010. PMID:29042136.

Berntssen, M.H.G., Hylland, K., Lundebye, A.-K., and Julshamn, K. 2004. Higher faecal excretion and lower tissue accumulation of mercury in Wistar rats from contaminated fish than from methylmercury chloride added to fish. Food Chem. Toxicol. **42**(8): 1359–1366. doi:10.1016/j.fct.2004.03.013.

Bloedel, P. 1955. Hunting methods of the fish-eating bats, particularly *Noctilio leporinus*. J. Mammal. **36**(3): 390–399. doi:10.2307/1375681.

Blood, B.R., and Clark, M.K. 1998. *Myotis vivesi*. Mamm. Species, **588**: 1–5. doi:10.2307/3504455.

Boening, D.W. 2000. Ecological effects, transport, and fate of mercury: a general review. Chemosphere, **40**(12): 1335–1351. doi:10.1016/S0045-6535(99)00283-0. PMID:10789973.

Brown, T.M., Macdonald, R.W., Muir, D.C.G., and Letcher, R.J. 2018. The distribution and trends of persistent organic pollutants and mercury in marine mammals from Canada's eastern Arctic. Sci. Total Environ. **618**: 500–517. doi:10.1016/j.scitotenv.2017.11.052. PMID:29145101.

Clare, E.L., Fraser, E.E., Braid, H.E., Fenton, M.B., and Hebert, P.D.N. 2009. Species on the menu of a generalist predator, the eastern red bat (*Lasiurus borealis*): using a molecular approach to detect arthropod prey. Mol. Ecol. **18** (11): 2532–2542. doi:10.1111/j.1365-294X.2009.04184.x. PMID:19457192.

Dalecka, B., and Mezule, L. 2018. Study of potential PCR inhibitors in drinking water for *Escherichia coli* identification. Agron. Res. **16**(S2): 1351–1359. doi:10.1007/s42452-019-0533-1.

Deagle, B.E., and Tollit, D.J. 2007. Quantitative analysis of prey DNA in pinniped faeces: Potential to estimate diet composition? Conserv. Genet. **8**: 743–747. doi:10.1007/s10592-006-9197-7.

Deagle, B.E., Kirkwood, R., and Jarman, S.N. 2009. Analysis of Australian fur seal diet by pyrosequencing prey DNA in faeces. Mol. Ecol. **18**(9): 2022–2038. doi:10.1111/j.1365-294X.2009.04158.x. PMID:19317847.

Deagle, B.E., Thomas, A.C., Shaffer, A.K., Trites, A.W., and Jarman, A.N. 2013. Quantifying sequence proportions in a DNA-based diet study using ion torrent amplicon sequencing: which counts count? Mol. Ecol. Resour. **13**(4): 620–633. doi:10.1111/1755-0998.12103. PMID:23590207.

Deagle, B.E., Thomas, A.C., McInnes, J.C., Clark, L.J., Vesterinen, E.J., Clare, E.L., et al. 2019. Counting with DNA in metabarcoding studies: how should we convert sequence reads to dietary data? Mol. Ecol. **28**(2): 391–406. doi:10.1111/mec.14734. PMID:29858539.

Ebbert, D. 2021. chisq.posthoc.test: a post hoc analysis for Pearson's chi-squared test for count data. R package version 0.1.3. Available from http://chisq-posthoc-test.ebbert.nrw/.

Edwards, A.E., Swall, J.L., and Jagoe, C.H. 2019. Mercury concentrations in bat guano from caves and bat houses in Florida and Georgia. Virginia J. Sci. **70**(3). Available from https://digitalcommons.odu.edu/vjs/vol70/iss3/4/.

Elías-Gutiérrez, M., Valdez-Moreno, M., Topan, J., Young, M.R., and Cohuo-Colli, J.A. 2018. Improved protocols to accelerate the assembly of DNA barcode reference libraries for freshwater zooplankton. Ecol. Evol. **8**(5): 3002–3018. doi:10.1002/ece3.3742. PMID:29531713.

Enríquez-Andrade, R., Anaya-Reyna, G., Barrera-Guevara, J.C., Carvajal-Moreno, M.de los A., Martínez-Delgado, M.E., Vaca-Rodríguez, J., and Valdés-Casillas, C. 2005. An analysis of critical areas for biodiversity conservation in the Gulf of California region. Ocean Coast Manag. **48**(1): 31–50. doi:10.1016/j.ocecoaman.2004.11.002.

Gochfeld, M. 2003. Cases of mercury exposure, bioavailability and absorption. Ecotoxicol. Environ. Saf. **56**(1): 174–179. doi:10.1016/S0147-6513(03)00060-5. PMID:12915150.

Hallam, J., Clare, E.L., Jones, J.I., and Day, J.J. 2021. Biodiversity assessment across a dynamic riverine system: A comparison of eDNA metabarcoding versus traditional fish surveying methods. Environ. DNA. [Early view.]. doi:10.1002/edn3.241.

Herrera, M.L.G., Flores-Martínez, J.J., and Sánchez-Cordero, V. 2019. Geographical distribution and conservation status of an island endemic mammal: the vulnerable *Myotis* fishing bat (*Myotis vivesi*). Oryx, **53**(2): 388–393. doi:10.1017/S0030605317000874.

Ishihara, N. 2000. Excretion of methyl mercury in human faeces. Arch. Environ. Health, **55**(1): 44–47. doi:10.1080/00039890009603384. PMID:10735519.

Komsta-Szumska, E., Czuba, M., Reuhl, K.R., and Miller, D.R. 1983. Demethylation and excretion of methyl mercury by the guinea pig. Environ. Res. **32**(2): 247–257. doi:10.1016/0013-9351(83)90109-3. PMID:6641664.

MacCrimmon, H.R., Wren, C.D., and Gots, B.L. 1983. Mercury uptake by lake trout, *Salvelinus namaycush*, relative to age, growth, and diet in Tadenac Lake with comparative data from other Precambrian Shield lakes. Can. J. Fish. Aquat. Sci. **40**: 113–120. doi:10.1139/f83-020.

Mathers, R.A., and Johansen, P.H. 1985. The effects of feeding ecology on mercury accumulation in walleye (*Stizostedion vitreum*) and pike (*Esox lucius*) in Lake Simcoe. Can. J. Zool. **63**(9): 2006–2012. doi:10.1139/z85-295.

Maya, J.A. 1968. The natural history of the fish-eating bat, *Pizonyx vivesi*. Ph.D. dissertation, University of Arizona, Tucson.

Méndez, L., and Alvarez-Castañeda, S.T. 2000. Comparative analysis of heavy metals in two species of ichthyophagous bats *Myotis vivesi* and *Noctilio leporinus*. Bull. Environ. Contam. Toxicol. **65**: 51–54. doi:10.1007/s0012800093.

Miya, M., Sato, Y., Fukunaga, T., Sado, T., Poulsen, J.Y., Sato, K., et al. 2015. MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. R Soc. Open Sci. **2**(7): 150088. doi:10.1098/rsos.150088. PMID:26587265.

Nam, D.-H., Yates, D., Ardapple, P., Evers, D.C., Schmerfeld, J., and Basu, N. 2012. Elevated mercury exposure and neurochemical alterations in little brown bats (*Myotis lucifugus*) from a site with historical mercury contamination. Ecotoxicology, **21**: 1094–1101. doi:10.1007/s10646-012-0864-9. PMID: 22331394.

Otálora-Ardila, A., Herrera M, L.G., Flores-Martínez, J.J., and Voigt, C.C. 2013. Marine and terrestrial food sources in the diet of fish-eating myotis (*Myotis vivesi*). J. Mammal. **94**(5): 1102–1110. doi:10.1644/12-MAMM-A-281.1.

Page, L.M., Espinosa-Perez, H., Findley, P.T., Gilbert, C.R., Lea, R.N., Mandrak, N.E., and Mayden, R.L. 2013. New seventh edition of common and scientific names of fishes: changes include capitalization of common names. Fisheries, **38**(4): 188–189. doi:10.1080/03632415.2013.767244.

Peterson, S.H., Ackerman, J.T., Crocker, D.E., and Costa, D.P. 2018. Foraging and fasting can influence contaminant concentration in animals: an example with mercury contamination in a free-ranging marine mammal. Proc. R. Soc. B Biol. Sci. **285**(1872): 20172782. doi:10.1098/rsp b.2017.2782. PMID:29436501.

Petersson, K., Dock, L., Söderling, K., and Vahter, M. 1991. Distribution of mercury in rabbits subchronically exposed to low levels of radiolabeled methyl mercury. Pharmacol. Toxicol. **68**(6): 464–468. doi:10.1111/j.1600-0773.1991.tb01271.x. PMID:1891441.

Pitcher, T.J. (*Editor*). 1986. Functions of shoaling behaviour in teleosts. In Behaviour of teleost fishes. Johns Hopkins University Press, Baltimore, Md. pp. 294–337.

Ratnasingham, S., and Hebert, P.D.N. 2007. BOLD: The barcode of life data system (www.barcodinglife.org). Mol. Ecol. Notes, **7**(3): 355–364. doi:10.1111/j.1471-8286.2007.01678.x. PMID:18784790.

Ratnasingham, S., and Hebert, P.D.N. 2013. A DNA-based registry for all animal species: the barcode index number (BIN) system. PLoS ONE, **8**(7): e66213. doi:10.1371/journal.pone.0066213. PMID:23861743.

R Core Team. 2018. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available from https://www.r-project.org.

Reeder, W.G., and Norris, K.S. 1954. Distribution, type, locality, and habits of fish-eating bat, *Pizonyx vivesi*. J. Mammal. **35**(1): 81–87. doi:10.2307/1376075.

Robinson, C.J. 2004. Responses of the northern anchovy to the dynamics of the pelagic environment: identification of fish behaviours that may leave the population under risk of overexploitation. J. Fish Biol. **64**(4): 1072–1087. doi:10.1111/j.1095-8649.2004.00372.x.

Salinas-Ramos, V.B., Herrera Montalvo, L.G., Leon-Regagnon, V., Arrizabalaga-Escudero, A., and Clare, E.L. 2015. Dietary overlap and seasonality in three species of mormoopid bats from a tropical dry forest. Mol. Ecol. **24**(20): 5296–5307. doi:10.1111/mec.13386. PMID:26369564.

Sanz-Landaluze, J., Pena-Abaurrea, M., Muñoz_Olivas, R., Cámara, C., and Ramos, L. 2015. Zebrafish (*Danio rerio*) eleutheroembryo-based procedure for assessing bioaccumulation. Environ. Sci. Technol. **49**(3): 1860–1869. doi:10.1021/es504685c. PMID:25590991.

Schubert, M., Lindgreen, S., and Orlando, L. 2016. AdapterRemoval v2: rapid adapter trimming, identification, and read merging. BMC Res. Notes, **9**: 88. doi:10.1186/s13104-016-1900-2.

Selin, N.E. 2009. Global biogeochemical cycling of mercury: a review. Annu. Rev. Environ. Resour. **34**: 43–63. doi:10.1146/annurev.environ.051308.084314.

SEMARNAT. 2010. Norma Oficial Mexicana Nom-059-SEMARNAT-2010. Protección ambiental — Especies nativas de México de flora y fauna silvestres — Categorías de riesgo y especificaciones para su inclusión, exclusión o cambio — Lista de especies en riesgo. Diario Oficial de la Federación, Segunda Sección, México (2010) 30 de diciembre de 2010. Mexico city, Mexico.

Shokralla, S., Hellberg, R.S., Handy, S.M., King, I., and Hajibabaei, M. 2015. A DNA mini-barcoding system for authentication of processed fish products. Sci. Rep. **5**: 15894. doi:10.1038/srep15894.

Sikes, R.S. the Animal Care and Use Committee of the American Society of Mammalogists. 2016. 2016 Guidelines of the American Society of Mammalogists for the use of wild mammals in research and education. J. Mammal. **97**(3): 663–688. doi:10.1093/jmammal/gyw078.

Symondson, W.O.C. 2002. Molecular identification of prey in predator diets. Mol. Ecol. **11**(4): 627–641. doi:10.1046/j.1365-294X.2002.01471.x. PMID:11972753.

US EPA. 1998. Mercury in solids and solutions by thermal decomposition, amalgamation, and atomic absorption spectrophotometry. US Environmental Protection Agency (EPA), Washington, D.C.

Valdez-Moreno, M., Vásquez-Yeomans, L., Elías-Gutiérrez, M., Ivanova, N.V., and Hebert, P.D.N. 2010. Using DNA barcodes to connect adults and early life stages of marine fishes from the Yucatan Peninsula, Mexico: potential in fisheries management. Mar. Freshw. Res. **61**(6): 655–671. doi:10.1071/MF09222.

Valdez-Moreno, M., Quintal-Lizama, C., Gómez-Lozano, R., and García-Rivas, M.D.C. 2012. Monitoring an alien invasion: DNA barcoding and the identification of lionfish and their prey on coral reefs of the Mexican Caribbean. PLoS ONE, **7**(6): e36636. doi:10.1371/journal.pone.0036636. PMID:22675470.

Walker, L.W. 1950. The fish bats of Pescadora. Audubon, **52**: 294–299.

Watanabe, H., Moku, M., Kawaguchi, K., Ishimaru, K., and Ohno, A. 1999. Diel vertical migration of myctophid fishes (family Myctophidae) in the transitional waters of the western North Pacific. Fish. Oceanogr. **8**(2): 115–127. doi:10.1046/j.1365-2419.1999.00103.x.

Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L., François, R., et al. 2019. Welcome to the tidyverse. J. Open Source Softw. **4**(43): 1686. doi:10.21105/joss.01686.

Zeale, M.R.K., Butlin, R.K., Barker, G.L.A., Lees, D.C., and Jones, G. 2011. Taxon-specific PCR for DNA barcoding arthropod prey in bat faeces. Mol. Ecol. Resour. **11**(2): 236–244. doi:10.1111/j.1755-0998.2010.02920.x. PMID:21429129.