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Revisiting the 2018 Corsica oil spill - a preliminary case study of ecosystem recovery from PAHs in Yelkouan Shearwater (Puffinus yelkouan)

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Revisiting the 2018 Corsica oil spill - a preliminary case study of ecosystem recovery from PAHs in Yelkouan Shearwater (*Puffinus yelkouan)*

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Table of Contents

Abstract

Polycyclic aromatic hydrocarbons (PAHs) are a specific class of persistent organic pollutants (POPs) heavily associated with crude oil, and pose genotoxic and physiological threats to marine life when released into the aquatic environment during a petroleum spill. This study seeks to understand the residual impacts of the 2018 Corsica oil spill on *Puffinus yelkouan* (Yelkouan Shearwater), a seabird endemic to the Mediterranean basin, using the species as a bioindicator for contamination in the region. Though classified by the IUCN Red List as 'vulnerable,' this species remains grossly understudied in present monitoring literature. This study made use of blood samples and excreta taken from live *P. yelkouan* specimens in three different Mediterranean sites (Tavolara, Montecristo, and Villasimius) six months following the original spill. HP-LC was performed to assess individual PAH levels in the blood, followed by an assay for erythrocyte nuclear abnormalities (ENA), white blood cell differential counts (H:L ratio), and a fluorometric reading of porphyrin levels. While carcinogenic PAH levels did not significantly differ between islands, collective values were notably much higher than reported values in existing literature. H:L ratios and total porphyrin levels were not indicative of long or short term stress levels. Lobed ENA values were notably higher than reported literature indices, particularly in the Tavolara region, suggesting some level of genotoxic stress may be present in the area. However, no significant relationship was observed between total carcinogenic PAHs and total ENAs, leading us to believe other contaminants are likely present. Evidence does not suggest lingering effects from the 2018 spill are still present in *P. yelkouan*, but contamination of the area from other sources remains likely and warrants further study. Collectively, these findings present a foundational understanding for the health of *P. yelkouan* in the region, in the hopes of contributing to further conservation efforts.

Riassunto

Gli idrocarburi policiclici aromatici (IPA), un sottoprodotto del petrolio greggio, sono un gruppo specifico di inquinanti organici persistenti (POP) che hanno effetti genotossici e fisiologici sulla fauna marina quando sono rilasciati nell'ambiente marina durante una perdita di petrolio. Usando *Puffinus yelkouan (*Berta Minore), un uccello marino endemico del del bacino del Mediterraneo, come un bioindicatore della contaminazione nella zona, questo studio cerca di capire gli impatti residui su questa specie dopo un sversamento di carburanti nel 2018 al largo delle coste della Corsica. Monitoraggio di questa specie rimane decisamente sottorappresentato nella letteratura, anche se la specie è stata classificata *vulnerable* dalla IUCN Red List. Campioni di sangue ed escrementi prelevati da esemplari vivi di *P. yelkouan* in tre diversi siti del Mediterraneo (Tavolara, Montecristo e Villasimius) sono stati presi sei mesi dopo lo sversamento originario. Per valutare i livelli individuali di IPA nel sangue è stata eseguita una HP-LC. I *biomarker* sono stati scoperti con un'analisi delle anomalie nucleari degli eritrociti (ENA), una conta differenziale dei globuli bianchi (rapporto H:L) e una lettura fluorimetrica dei livelli di porfirine. I livelli di

IPA cancerogeni non differivano significativamente tra le isole, comunque i valori collettivi erano notevolmente superiori a quelli riportati nella letteratura attuale. I rapporti H:L e i livelli totali di porfirine non sono stati indicativi dei livelli di stress a lunga o breve durata. I valori dell'ENA a lobi sono risultati notevolmente superiori agli indici riportati in letteratura, in particolare nella regione di Tavolara, suggerendo che potrebbe essere presente un certo livello di stress genotossico in quel luogo. La presenza di altri contaminanti è probabile siccome non è stata osservata una relazione significativa tra gli IPA cancerogeni totali e gli ENA totali, suggerendo che gli effetti su i *biomarker* non sono un prodotto di contaminanti dallo sversamento nel 2018 ma probabilmente dovuti ad altre fonti di contaminazione che dovrebbe essere studiato più approfondito. Nel complesso, questo studio presenta una comprensione fondamentale per la salute di *P. yelkouan* nella regione con lo scopo di sostenere più eventi di monitoraggio per la conservazione di questa specie.

Introduction

Anthropogenic contaminants are harmful factors released into the environment by human activity, the study of which is known as environmental toxicology or ecotoxicology (Zhang et al. 2017). Under this discipline falls the study of POPs or persistent organic pollutants; these chemicals were once considered useful for industrial production purposes, but were ultimately recognized as serious toxins that linger in the environment due to their chemical structures. According to the EPA, POPs can be transported as particulate or gaseous matter and travel across biotic and abiotic vectors alike (EPA). A particular class of concern is the polycyclic aromatic hydrocarbon (PAHs), denoting a group of organic compounds that contain between two and ten adjacent benzene rings (Hylland 2006), and may possess additional N, S, or O substituent groups or substitutions (Albers 2006). These compounds are hydrophobic in nature, and thus can accumulate in lipid rich tissues of living organisms (Pampanin 2017). PAHs themselves are known to form naturally during incomplete combustion or within high pressure systems (CDC), but modern research is concerned with the contributions of fossil fuels to environmental PAH release. In humans, benzene hydrocarbon exposure is known to cause genotoxic and oxidative DNA damage (Göethel 2014) and has been linked to tumor formation since 1775 (Luch 2005). Marine organisms feel these same effects, where neoplasia and tumor formation have been observed in bivalve mollusks, fish, pinnipeds, and cetaceans (Pampanin 2017). Marine bird species have also been used to assess PAH contamination levels, particularly following oil spill events. A large body of these works were published following the Deepwater Horizon oil spill in 2010 and cited notable physiological impacts including oxidative stress, hematologic injury, flight impairment, damage to organ and tissue masses, decreased reproductive success, and induced mortality (Pritsos et al. 2017, Fallon et al. 2017, Perez et al. 2017, Horak et al. 2017, Beyer et al. 2016). Notably, PAHs are only genotoxic once they have been activated via metabolism (Luch 2005), leading to the creation of volatile oxidation products *in vivo* (Pampanin 2017). Additionally, UV interactions with PAH compounds have also been found to amplify toxicity by three times that of their original levels (National Academies 2022).

Of the hundreds of PAH structures that exist, petrogenic PAHs are generally characterized by many alkyl constituents and lower molecular weight (2-3 rings), which lead to higher toxicity rates (Pampanin 2017). Though generally not considered soluble in water, PAHs are highly photoreactive and degrade into forms that are readily uptaken by marine environments, especially in the low molecular weight petrogenic forms (Neff et al. 2000, Albers 2006). Once dissolved, these compounds are subsequently uptaken by marine organisms through lungs, gills, or contaminated food sources (Lourenço et al., 2016). While POPs are known for their bioaccumulative effects, PAHs are more readily metabolized and excreted in vertebrates (Albers 2006). Indeed, biomagnification is generally not observed in metabolically advanced organisms (Hylland 2006), indicating that toxicity in *P. yelkouan* and other birds is a product of direct environmental exposure. Nonetheless, bioaccumulation of PAHs has been observed in zooplankton and fish species alike, providing increasingly contaminated food sources to these organisms (Almeda et al. 2013, Ziling et al. 2019). According to the CDC, carcinogenic PAHs include benzo[a]pyrene, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, chrysene, dibenz[a,h]anthracene, and indeno[1,2,3-cd]pyrene (USEPA 1993).

Oil spills have long been considered a significant source of environmental contamination (Kingston 2002). Upon release into a marine ecosystem, petroleum undergoes a number of distinct processes: spreading, evaporation, emulsification, biodegradation, sedimentation, photolysation, and dissolution (Kingston 2002). The last two processes are most relevant for our purposes, as soluble PAHs are formed during photolysation, allowing for dissolution and reuptake by marine organisms.

Preliminary studies regarding PAH levels took place in bivalve species where bioaccumulation rates could be easily observed due to their stationary nature, proximity to contaminated sediments, and high PAH compound retention rates that were confirmed in further research (Mix et al. 1977, Kasiotis & Emmanouil 2015). Later studies moved into marine mammals such as cetaceans and pinnipeds as indicators for broader PAH concentrations in marine ecosystems. Marine birds have likewise been used, particularly in the context of oil spills as was previously mentioned. This paper will focus specifically on *Puffinus yelkouan*, a seabird endemic to the Mediterranean basin commonly known as the Yelkouan Shearwater (Raine et al. 2013). The species was selected due to its classification as vulnerable by the IUCN Red List in 2020, and is notably absent from past ecotoxicological survey studies. To address this problem, recent studies have begun to categorize breeding colony locations in order to assess species behavior. Bourgeois & Vidal 2008 synthesized the withstanding literature to create a unified figure (Fig. 1A) of *Puffinus yelkouan* breeding site distribution. This study focuses on three breeding colonies pictured here, located on Montecristo Island, Tavolara Island, and the coast of Villasimius, respectively (Fig. 1B).

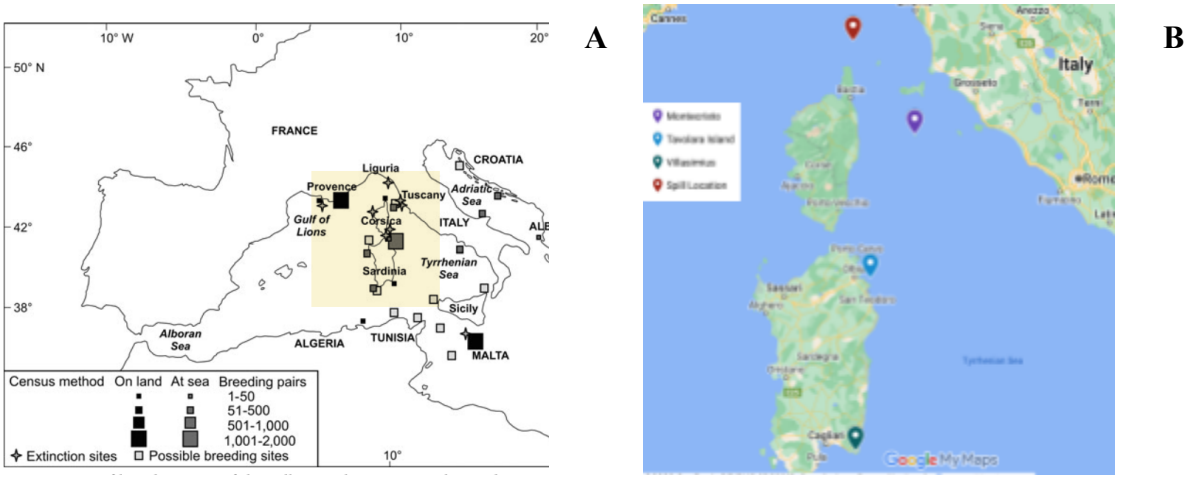

Figure 1. (A) Breeding site locations of *P. yelkouan* in the Mediterranean region, modified from Bourgeois & Vidal 2008. (B) Sampling sites from April 2019 around Corsica and Sardinia (Montecristo island, Tavolara & Molara islands , and the Capo Carbonara Marine Protected Area in Villasimius) in relation to the original 2018 spill site (see legend for color coding).

Indeed, the Tavolara archipelago located in northern Sardinia is considered to be the most important breeding site for the species globally (Pezzo et al. 2021). Between March and April, during the incubation period, *P. yelkouan* was observed to forage along the North and West coasts of Sardinia as well as the Southern coast of Corsica. The Southern end of Sardinia was noted to be a less preferable foraging site for the birds (Pezzo et al. 2021). Following the incubation period, *P. yelkouan* have been observed returning to their nesting sites beginning in mid-October (Borg et al. 2002).

In order to assess the impacts of PAH levels on *P. yelkouan*, three biomarker indices were selected: erythrocyte nuclear abnormalities, heterophil-to-lymphocyte ratios, and porphyrin levels. On their most basic level, avian erythrocytes are nucleated red blood cells which have otherwise evolved to lose their nuclei in mammals including humans (Yap & Zhang 2021). Genotoxic stress is known to induce genome instability in these cells, and literature has reported nuclear change induced during cell division (Canedo 2021). The mechanism of damage is contingent on the class of contaminant as well as the cell cycle stage in which it is induced (Canedo 2021). Micronuclei (MN) and nuclear abnormality(NA) tests have been well documented as indices of genome instability caused by exposure to genotoxic compounds across nucleated species (Braham et al. 2017). The nuclear abnormalities test for this study, referred to here as an erythrocyte nuclear abnormality (ENA) assay, was conducted following the methods developed by Pacheco and Santos (1996). Exact details of this induction in bird species are not currently well understood, though in zebrafish and mammalian cells the mechanisms have been well documented (Kursa, Fenech 2011). These abnormalities, as well as micronuclei, turn out to be irreversible lesions and are considered indicators of genotoxic damage in the short term

(Marques et al., 2016; Casini et al., 2018). Targeted DNA repair mechanisms or apoptosis may remove some of this damage after the fact, also acknowledging that avian blood is completely recycled between 35 and 45 days *(*Rodnan et al. 1957). However, continued division of cells containing damaged nuclei may lead these genotoxic effects to accumulate over time (Brandts et al. 2022).

The second metric is heterophil-to-lymphocyte ratios, or H:L, an indicator of long term immune stress (Kursa & Bezrukov 2007, Lentfer er al 2015). Lymphocytes are white blood cells which induce the activity of T and B cells in the adaptive immune system response, while heterophils directly phagocytize foreign pathogens as a part of the innate immune system (Minias 2018). The relationship between the two in ratio form is a direct indicator of immune activity, wherein a higher value indicates a higher degree of biological distress (Minias 2018).

Finally, porphyrins are intermediates and byproducts of biological processes that accumulate in erythrocytic tissues, the liver, and the kidney in the presence of contaminants and are excreted in the form of urine or feces (Casini 2001). A relationship is thus thought to exist between PAH exposure and porphyrin production, which can in turn be measured indirectly from excreta (Casini 2003).

Zhang et al. 2017 delineated the steps required to study ecotoxicological impacts beginning with the selection of a model organism in assessing ecotoxicity, followed by the exploration of a contaminant scenario. Here, we explore the impacts of polycyclic aromatic hydrocarbons (PAHs) on *Puffinus yelkouan* specimens in the Mediterranean Sea following the 2018 oil spill located just north of the Corsican coastline. In this study, we first quantify measured PAH levels against toxicity values in previous literature (Pérez et al. 2008). We then assess biomarkers of genotoxic and physiological stress in relation to the derived PAH levels with regard to physical distance from the spill to quantify stress metrics and recovery of *P. yelkouan* in the Mediterranean basin.

Methods

1. Environmental Context

   The 2018 Corsica oil spill (Lat. 43.246167, Long. 9.4795) released 600 m^3 of fuel oil into the marine environment (Fig. 1). The slick expanded to around 20 km off of the coast of Cape Corso, and reached distances from the original site as far as 300 km (REMPEC).

2. Live specimen sampling

   In April of 2019, three Yelkouan Shearwater (*Puffinus yelkouan)* nesting sites were selected following the oil spill off the coast of Corsica in October of 2018. These sites,

respectively, were located along the coastlines of Montecristo island, Tavolara and Molara islands (constitute the same population), and the Capo Carbonara Marine Protected Area in Villasimius, located in southern Sardinia. Once nests were identified in each site, a blood sample of around 1mL was taken from each adult specimen via the brachial vein using a heparinized syringe and subsequently transferred to a "vacutainer", where it was frozen and stored in the dark. Samples were taken carefully to ensure good health and no residual bleeding, and animals were immediately returned to the capture site to minimize subsequent stress responses. When ready for analysis, a portion of each sample was set aside for use in a PAH analysis, while the rest was used to prepare glass slide blood smears for an ENA assay, white blood cell differential, and H:L ratio calculation. When possible, excreta was collected for porphyrin analysis.

3. Polycyclic aromatic hydrocarbon (PAHs) analysis

A portion of each frozen blood sample was conserved and stored at -20 °C prior to being dehydrated in an Edwards freeze-dryer. Each sample was then pulverized and run through a soxhlet extraction following the protocols established by Griest & Caton (1983) and Holoubek et al. (1990) and modified by Marsili et al. 1997. Aluminum foil was used to cover all instruments for the duration of this analysis to prevent photodegradation of the PAHs. Using this method, the samples were run through a soxhlet system for 4 hours with 100mL of a KOH-methanol mix (of a 1:4 ratio) and held at 70 °C, slightly higher than the boiling point of the solvent. After being allowed to cool at room temperature, the solution was transferred to a separatory funnel and shaken twice with 100 mL of cyclohexane for a total of 10 minutes. Shaken funnels were left to sit for 30 minutes until separation occurred, and the supernatant of cyclohexane and PAHs was carefully transferred to a new beaker using a 1 mL glass pipette. The cyclohexane was evaporated from the isolated supernatant using a Rotavapor device at 45 °C, and the remaining contaminants were resuspended in 10mL of an acetone-hexane mixture (1:1). This solution was then passed through a column packed with 3cm of activated Florisil, which had been previously heated to 120 °C for one hour. Subsequent elution through the Florisil was conducted using 90 mL of the acetone-hexane mixture for a total of 100 mL, and the resulting solution was reduced to a single drop using the Rotavapor and resuspended in 0.5 mL of acetonitrile.

The extracted solution was analyzed via high performance liquid chromatography with a fluorescence detector. A reverse phase column (Supelcosil LC-18.25 cm x 4.6 mm i.d, 0.5 μm particle diameter) with an acetonitrile/water gradient was used with a flow rate of 1.5 mL/min. The initial gradient concentration was 60% acetonitrile to 40% water for the first 20 minutes of the chromatography and was subsequently changed to 100% acetonitrile for the next 10 minutes, after which it was returned to the initial

concentration. Analysis was performed against a Supelco external 16-PHI standard (EPA 610).

4. Erythrocyte nuclear abnormality (ENA) assay

   The method developed by Pacheco and Santos (1996) was used to evaluate the presence and number of nuclear abnormalities in red blood cells induced by genotoxic agents. Slides were smeared and allowed to air dry. After staining with Diff-Quick dye, slides were rinsed with deionized water. For this test, only mature erythrocytes were quantified. 1000 cells were counted on each slide under an immersion light microscope (Olympus BX41), and results were expressed as the total frequency of anomalies (‰, parts per thousand).

5. White blood cell differentials & H:L ratio

   White blood cell counts were conducted using the same staining protocol as the ENA assay where 200 cells per slide were identified and recorded under an immersion light microscope (Olympus BX41). Leukocytes (monocytes, lymphocytes, heterophils, eosinophils and basophils) were identified and classified according to the method developed by Casal and Orós (2007). Thrombocyte counts were conducted using the methods of Davis et al. (2008). Results for each cell type were expressed as percentages of the total cell count. The H:L ratio was calculated following the quantification of white blood cell differentials by deriving the ratio between the total number of heterophilic cells to the total number of leukocytes. The resulting value was a numerical index representing the stress state of the specimen.

6. Porphyrin concentrations

   Porphyrins were extracted from specimen excreta using a modified methodology from Casini et al. (2003). Excreta was first dehydrated using an Edwards freeze-dryer, and 50 g aliquots were taken from each sample. 1 mL of 5N HCl was added to each sample, and each was vortexed four times. 3 mL of diethyl ether was then added and the sample was vortexed until an emulsion was reached, after which 3 mL of distilled water was added. The resulting solution was centrifuged at 580 x g to separate the diethyl ether supernatant containing chlorophyll derivatives and carotenoids from the central layer of insoluble materials and the aqueous subnatant of “free-acid” porphyrins. Using the methods of Grandchamp et al. (1980), a fluorimetric reading was performed on the aqueous subnatant. This procedure allowed percentages and concentrations, respectively, of

coproporphyrins, uroporphyrins, protoporphyrins and total porphyrins to be quantified. In this method, each porphyrin corresponds to a particular excitation-emission wavelength (coproporphyrins: 400/595 nm; uroporphyrins: 405/595 nm; protoporphyrins: 410/605 nm). Fluorescence emissions were recorded for each of these three value pairs to obtain three linear functions and compared to three corresponding standard curves that represented the relative concentrations of each porphyrin type present in solution. Porphyrin concentrations were expressed in pmol x g excreta in dry weight.

7. Data Analysis

A log transformation of total carcinogenic PAHs and total porphyrins was performed to correct right skew, and two outliers removed from the dataset before beginning analysis (one from MC and one from TAV, respectively). A Levene's test for variance was performed to determine if the assumptions of an ANOVA were met for each factor. Once confirmed, an ANOVA was run for each and followed by a post-hoc Tukey test if appropriate. Additionally, Pearson correlation tests were performed for each biomarker response as compared to the total carcinogenic PAH values. Significance was determined at 0.05 for all tests. All statistical tests and relevant plots were run in Rstudio, and baseline mean values with standard errors were calculated in excel, when appropriate.

Results

An initial assessment of the data's normality distribution identified two outliers in the Tavolara and Montecristo datasets, respectively, which were removed prior to beginning the analysis. Transformations were conducted to correct right skew in the total carcinogenic PAHs and total porphyrin levels, allowing the assumption of normality to be met (Fig. 2). A preliminary assessment of total carcinogenic PAH levels across islands was then conducted (Fig. 3), where MC was observed to have the highest average transformed value, but overlapping confidence intervals called into question the significance of this difference. Levene's test revealed unequal variances across islands, indicating a non-parametric test was required for further analysis. Welch's t-test was subsequently performed; $P>0.05$, so the null hypothesis was accepted and the three factors were not determined to be statistically distinct from one another.

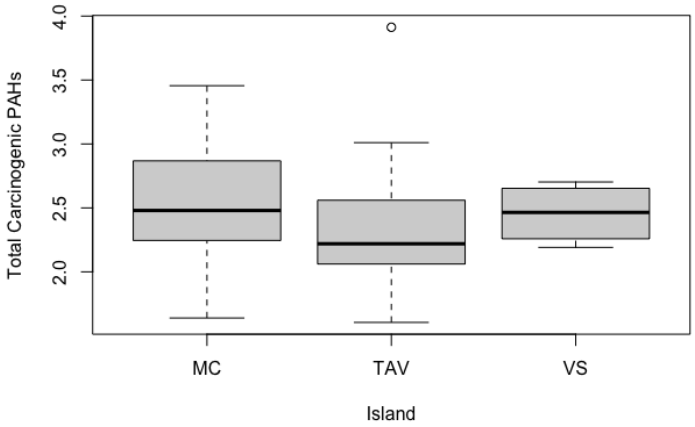

Figure 2. Box plot of log-transformed total carcinogenic PAH values by island with outliers (MC = Montecristo; TAV = Tavolara; VS = Villasimius), corrected for positive skew (n = 13, 4, 15, respectively).

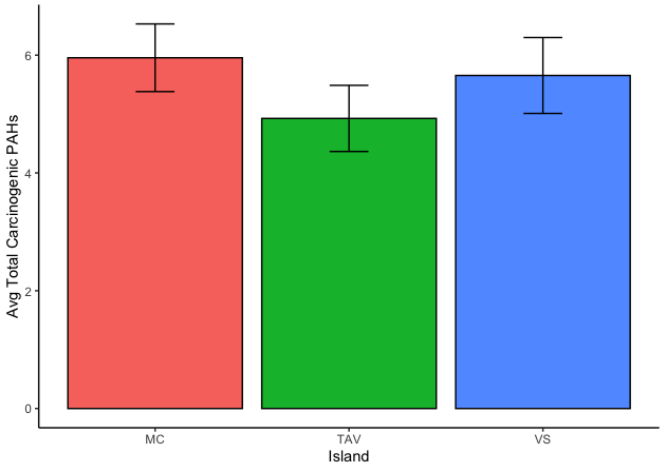

Figure 3. Transformed mean total carcinogenic PAH levels by island (MC = Montecristo; TAV = Tavolara; VS = Villasimius) with 95% confidence intervals.

An analysis of variance (ANOVA) was conducted for each biomarker response. Total ENAs ($F_{2,27} = 2.2963$, $p = 0.1199$), the H:L dataset ($F_{2,27} = 0.921$, $p = 0.4103$), and total porphyrin levels ($F_{2,39} = 2.6687$, $p = 0.08199$) possessed homoscedasticity, meeting the assumptions of a parametric ANOVA. A significant difference between islands was found across ENA totals ($F_{2,27} = 9.659$, $p = 0.000685$), and a post-hoc Tukey test revealed a significant difference between TAV and MC ($p = 0.0007175$), and TAV and VS ($p = 0.0436343$), respectively. No significant difference was observed between MC and VS for total ENAs ($p > 0.05$). Island did not significantly affect H:L ($F_{2,27} = 0.144$, $p = 0.867$). Island significantly affected total porphyrins ($F_{2,39} = 7.715$, $p = 0.0015$), and a post-hoc Tukey test revealed a significant difference between

TAV and MC (p = 0.0007175), and TAV and VS (0.0436343). No significant difference was observed between MC and VS (p > 0.05).

Overall, no statistical difference for carcinogenic PAHs was detected between islands, but some biomarkers gave indication of varying levels of genome instability and immune function across locations. To assess whether any association between carcinogenic PAH levels and selected elements of *P. yelkouan* physiology existed, Pearson's product-moment correlations were then conducted for total ENAs, H:L ratios, and total porphyrins. Where MC and VS were not found to be statistically distinct from one another during the ANOVA portion of the analysis, they were considered collectively during the correlation calculations (ENAs and porphyrins) and compared to TAV. Likewise, no statistical difference was detected across H:L ratios in all three locations, so the distribution for this biomarker assessed all H:L measurements as a singular dataset.

A visual assessment of total carcinogenic PAHs to total ENAs reveals a random distribution, and a Pearson's correlation of the two datasets confirmed that no significant correlation exists between carcinogenic PAHs and total ENAs for either TAV (t_{12} = 0.60, p = 0.56, r = 0.17) or MC-VS (t_{14} = -0.46, p = 0.65, r = -0.12) (Fig. 4).

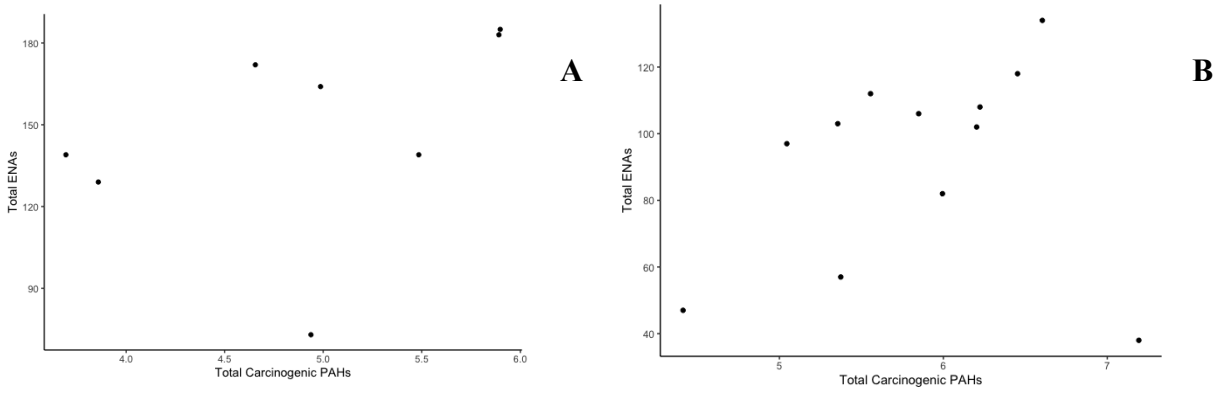

Figure 4. Scatter plots of transformed total carcinogenic PAHs to total ENAs where (A) TAV and (B) MC-VS (plotted together for clarity).

As carcinogenic PAH levels and H:Ls did not differ across islands, all data points were considered collectively. The scatter plot depicting the relationship between total carcinogenic PAHs and H:L ratios (Fig. 5) displays a random distribution with no discernible relationship between the two variables. A Pearson correlation test confirmed that there is no significant correlation between carcinogenic PAHs and H:L (t_{28} = 1.0054, p = 0.3233, r = 0.1866596). Likewise, there was no significant correlation between carcinogenic PAHs and total porphyrins for either TAV (t_{6} = 0.08, p = 0.94, r = 0.03) or MC-VS (t_{11} = 0.09, p = 0.93, r = 0.03) (Fig 6).

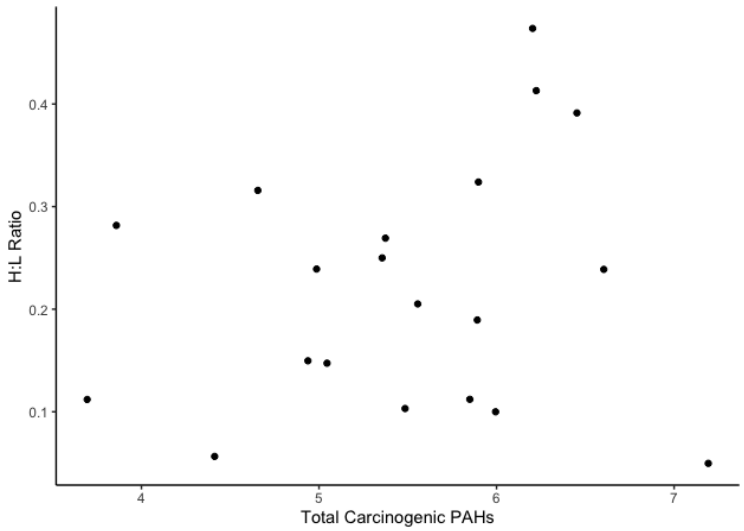

Figure 5. Combined scatter plot of total carcinogenic PAH values to H:L ratios across all three sites.

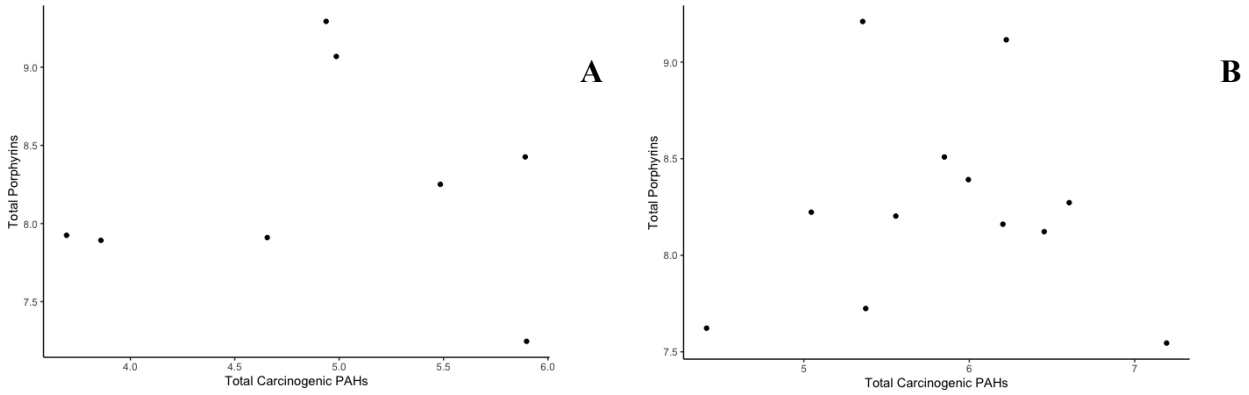

Figure 6. Scatter plots of total carcinogenic PAHs to total porphyrins where (A) TAV and (B) MC-VS.

Converted from ppm to % of 1,000 cells, the average lobed ENA values, the highest constituent nuclear abnormality, were 16.46±1.09, 9±1.31, and 9.58±0.55 for TAV, MC, and VS, respectively, with a collective mean of 12.31±1.17%. The collective mean H:L across islands was determined to be 0.24±0.02. Average total porphyrin levels were 4.62±1.04, 2.85±0.20, and 6.81±1.60 nmol/g for TAV, MC, and VS, respectively, with a collective mean of 3.59±0.34 nmol/g.

Discussion

Across the study, we found no compelling evidence of severe physiological or genotoxic damage to *P. yelkouan* associated with PAH levels in the sampling region. While a good indication for recovery from the original spill, recorded contaminant levels remained high and some genome instability in the form of lobed nuclei was nonetheless observed, suggesting the region may be experiencing other forms of toxicity from novel sources. The degree of marine ecosystem remediation following the incident and potential physiological distress in sampled birds across the region, respectively, are discussed in greater detail below.

2018 Spill Recovery - *P. yelkuan* as Bioindicators

The first point of inquiry addressed potential differences in PAH exposure by location and magnitude of recovery in the blood of *P. yelkouan* six months following the 2018 spill. From a baseline contaminant perspective, the locations are not significantly different from one another ($p > 0.05$). However, the measured contaminant levels are significantly higher than precedent values set by Pérez et al. (2008) following the Prestige oil spill off the Spanish coast in 2002. In fact, the values are between one and two orders of magnitude higher than those previously recorded for unoiled, oiled, and recovered locations, with the exception of benzo[a]anthracene levels that remained fairly consistent across both studies (Table 1). However, high variation and low sample sizes led to very high SE values across the present study, making it difficult to definitively conclude high rates of contamination were indeed present. Even so, the presence of such high numbers remains concerning from an environmental health standpoint and likely warrants further investigation.

	Present study			Pérez et al. 2008			
	Apr 2019, 6 mo. following 2018 Corsica spill			May-Jun 2004, 17 mo. following *Prestige* spill			
	TAV	MC	VS	LO	VI	ON	CI
benz[a]anthracene	11.51±2.01	20.96±6.19	39.79±14.54	29.03±15.84	11.71±3.61	12.47±4.48	9.54±2.94
crisene	76.33±19.27	131.14±55.09	69.84±18.11	4.73±3.24	1.25±0.59	0.86±0.24	2.03±0.48
benzo[b]fluorantene	148.77±85.04	176.88±41.65	119.24±36.74	22.23±17.96	1.88±0.60	2.69±1.14	1.69±0.36
benzo[k]fluorantene	11.48±3.37	29.34±8.04	13.00±3.37	9.86±7.79	1.88±0.38	2.32±0.66	1.68±0.41
benzo[a]pirene	8.78±2.99	27.69±8.82	10.60±4.49	0.73±0.21	0.58±0.34	0.35±0.16	1.58±0.55
dibenzo[a,h]antracene	12.96±2.80	36.62±12.22	44.91±34.00	0.52±0.28	0.05±0.00	0.06±0.01	0.21±0.11
indeno[1,2,3-cd]pirene	14.20±5.91	16.42±4.46	20.84±6.15	1.81±1.00	0.66±0.55	1.41±0.98	1.42±0.60

Table 1. Mean carcinogenic PAH values ± SE (in ng/g) in the present study and values collected by Pérez et al. (2008) 17 months following the *Prestige* spill off the coast of northwestern Spain in Nov. 2002. Site abbreviations are as follows: TAV = Tavolara; MC = Montecristo; VS = Villasimius; LO = Lobeiras;
VI = Vionta; ON = Ons; CI = Cíes.

As has been previously discussed, PAHs do not bioaccumulate in vertebrates due to efficient metabolism and excretion, though genotoxic effects are still observed following contamination. With this in mind, we know that levels of PAHs in the blood are a byproduct of environmental exposure from contaminated food, inhalation, or direct physical contact. The numbers above are notably high, but do not statistically differ across sample locations. Without the possibility of bioaccumulation, the values must necessarily be an indication of contamination during the specific time of sampling. This then offers two possibilities: either contaminants from the 2018 spill are lingering across the region in a uniform distribution, or the consistent PAH distribution is a product of some other source of contamination in the region. The first explanation is fairly unlikely, considering the natural influence of ocean diffusion and sequestration processes. Previous findings which looked to estimate the approximate time between exposure and final sequestration of petroleum contaminants found that, in a closed marine system, the PAH benz[a]anthracene took around 2 months to become fully sequestered in sediment form (Hinga & Pilson 1987). Additionally, following the Deepwater Horizon spill, researchers tracked the sequestration of petroleum through the sedimentation of marine snow and concluded that complete deposition of particles occurred anywhere from six weeks to 13 months after (Passow & Stout 2020). Based on these findings, we can assume that contaminants from the Corsica spill would have been partially-to-fully sequestered in the six months between the incident (October

2018) and the sampling date (April 2019), allowing us to conclude that the PAH contamination is coming from a different source.

Furthermore, the Hinga and Pilson study also illustrated that even though pollutants had been sequestered, they continued to persist indefinitely within their sedimentary resting site. Given this, a study by Allan et al. (2012) detailing recovery a year after the Deepwater Horizon spill and subsequent re-elevation of levels in the summer months following becomes particularly pertinent. Their findings suggest delayed resuspension and renewed bioavailability of PAHs into the water column roughly equalling original contaminant levels is possible and should be considered following any petroleum spill, particularly along coastlines where wind and wave activity can induce mobility directly offshore of coastal breeding sites as modeled off of the Gulf Coast (Plant et al. 2013). This process may potentially explain some of the lingering contamination recorded in the region.

There is also evidence of secondary down-slope transport mechanisms of oil residues following initial deposition (Diercks et al. 2021), suggesting a high likelihood of resuspension of contaminants into the water column for a period of time, potentially allowing for upwelling or other upward directed currents to pull these contaminants back into surface waters.
The mechanism of resuspension from depth in the Mediterranean basin is unclear at this time. Ultimately, however, the literature supports a partial-to-full sequestration of the PAHs released during the 2018 spill leading up to the sampling time. There is also some evidence of PAH degradation by microbial communities in sediments along the Gulf Coast following the Deepwater Horizon spill, which may offer another route through which petroleum-derived contaminants are removed from marine environments (Kappell et al. 2014). This natural technology may have important health implications for coastal dwelling organisms, but it remains to be studied further.

Besides standard sequestration processes of contaminants, we must also consider lateral transfer of PAHs by ocean currents. The region in question in this study is characterized by both Atlantic [Deep] Water (AW) and Levantine Intermediate Water (LIW) (Fig. 7). AW is not directly pictured but mirrors LIW movement around point 15 in Figure 7. Given that the spill directly contaminated surface waters in its advent, movement of resulting PAHs would likely be characterized by the north-western directionality of the LIW deeper into the Corsica Channel and across the southern coast of France (Buffett et al. 2017, Cattaneo et al. 2017). Likewise, petroleum contaminants in the Gulf of Mexico have shown the potential to travel upwards of 517 km from the original release site before complete sequestration or degradation occurs (Romero et al. 2017). Given this, PAH exposure to *P. yelkouan* subjects may have been lower than originally expected, as significant amounts of contaminant may likely have been exported instead to the north-eastern Italian mainland and the large nesting populations along the coast of Provence,

France cited by Bourgeois & Vidal (2008) or more generally dispersed via diffusion effects (National Academies 2022).

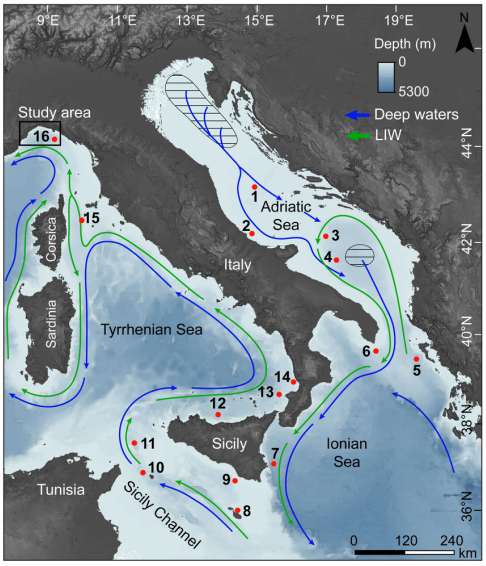

Figure 7. Ocean currents influencing water movement in the Mediterranean Basin, via Cattaneo et al. 2017.

Biomarkers & Bird Health

The second part of the study question addressed the relationship between carcinogenic PAH levels and three bird health biomarkers related to genotoxicity and physiological distress, for which we saw no statistically significant evidence to support. There were no significant correlations between contaminants and biomarker responses (Fig. 4-6). Individual responses are as follows.

H:L Ratios

H:L values did not vary significantly across islands. Collectively, *Puffinus yelkouan* specimens sampled displayed an average H:L ratio of 0.25. A research study exploring the evolution of H:L ratios in birds as a marker for physiological stress identified average H:L between passerine and non-passerine birds, further broken down by order and super-family (clade), (Fig. 8). *Puffinus yelkouan* is a part of the order Procellariiformes and is therefore classified as non-passerine. It then follows that the observed H to L ratio is significantly lower than the average value reported by Minias (2018). The left hand panel in Figure 8 also suggests that baseline H:L is highly species-specific, which may account for the large difference in values. Regardless, H:L ratios have been demonstrated as good indicators of long term avian stress (Kursa & Bezrukov 2007, Lentfer et al. 2015), and the low character of the observed value then suggests that the sampled individuals have not experienced severe chronic physiological distress.

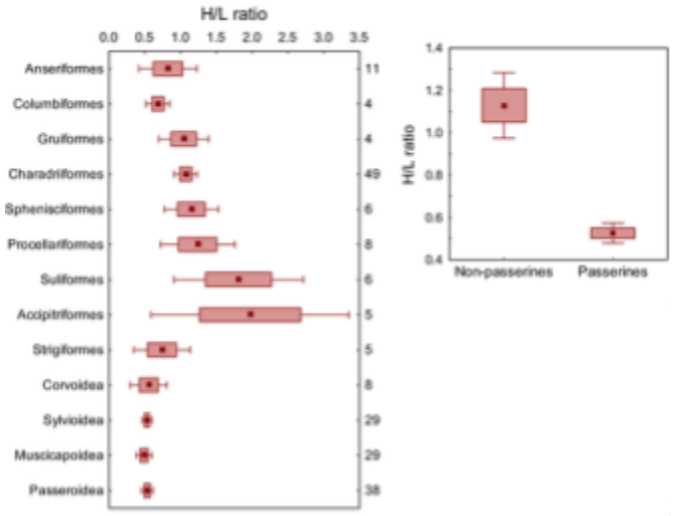

Figure 8. *Left* - H:L ratios in birds across order and superfamily (each corresponding n value is depicted to the right of the panel). *Right* - H:L ratios in non-passerine vs passerine birds. Dark red = mean; box = SE; whiskers = 95% CI. Modified from Minias (2018).

Total ENAs

While H:L values did not differ between locations, a significant difference between the TAV site and the MC-VS sites for total ENA levels suggests that some ecological differences must exist in the upper region to induce physiological responses in the birds. The highest number of ENAs observed were classified as "lobed," a loosely defined deformity in the nuclear shape pictured in a schematic from Canedo et al. (Fig. 9). Across locations, the percentages of lobed nuclei present were significantly higher than the baseline literature value of 4.79 ± 1.64 % for Adelie penguins reported by Olmastroni et al. (2019). MC and VS presented very similarly for *P. yelkouan* with ENA percentage values of 9 and 9.575, respectively. TV was even 5 points higher than these values at 16.6%. Collectively, these values suggest the disruption of normal nuclear development during cellular division by an external genotoxic stressor. TAV was found to be statistically distinct from both MC and VS, suggesting that individuals are undergoing more extreme levels of physiological stress. No correlation was found between carcinogenic PAHs and the development of ENAs in any location, calling into question the cause of these nuclear abnormalities. Literature has recognized ENAs are caused by a loss of genome integrity during one or more periods of the cell division process, as has been previously mentioned (Fenech et al. 2011). Besides PAHs, notable pollutants also capable of inducing nuclear abnormalities include nanomaterials, metals, effluents, pesticides and pharmaceuticals, alkylating agents, steroids and hormones, industrial chemicals, minerals, and radiation (Canedo 2021). Therefore, other contaminants in the region may be responsible for the observed ENAs even if PAHs are not directly involved. Additionally, sample size may admittedly account for part of this difference as the literature tested from 19,000 cells and this study worked rather with a subsection of 1,000 cells, and ENA frequency may likewise be species specific. Further research on baseline ENA

levels in *Puffinus yelkouan* as well as other possible contaminant types in the area should be assessed in further studies.

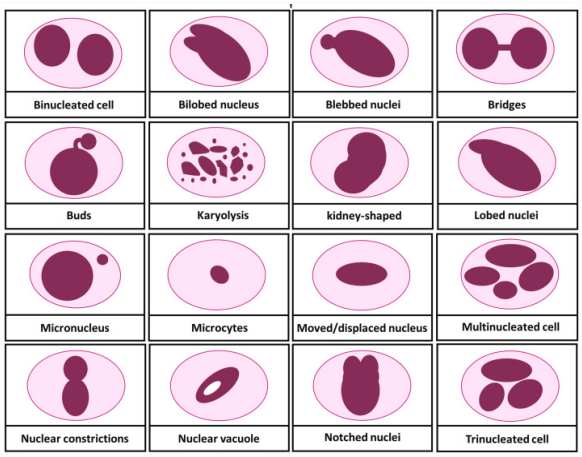

Figure 9. Possible classifications of erythrocytic nuclear abnormalities (ENAs). Modified from Canedo et al. (2021).

Total Porphyrins

A significant difference between locations was also observed for total porphyrins between TAV and the MC-VS sites. Surprisingly, VS individuals possessed significantly higher porphyrin levels than either other location. A small sample size (n = 4) may explain the high margin of error for the region, but more strikingly there is a significant difference between TAV and MC when variance is corrected by a transformation, as assessed by an ANOVA. These differences may be indicative of higher stress levels in Tavolara region individuals, but the high levels of porphyrins observed in Villasimius somewhat confounds these findings. A study looking into excreta porphyrins in Chilean sea birds (Casini et al. 2001) determined that in a coastal region influenced by high agricultural activity, total porphyrin levels were between 8-10 nmols/g dw at what seems to be the threshold of significance. This study recorded total porphyrin levels more closely aligned with the industrial and control sampling sites, between which there was no significant difference. Recognizing the contamination present in this study's own sampling sites and the lower porphyrin levels compared to the Casini study, we know that high agricultural activity leads to high levels of pesticide contaminant release, and therefore we can propose that high porphyrin levels in birds may be related to other kinds of POPs beyond PAHs.

Conclusions

This preliminary assessment exploring the health of *Puffinus yelkouan* subjects around the Italian island of Sardinia six months following an oil spill in the region found that carcinogenic PAH levels were not significantly higher along a distance gradient from the original site, suggesting that levels recovered from presumably high concentrations immediately following the spill. This

finding is a positive indication for long term environmental recovery, coupled with the low H:L ratios and porphyrin levels across study sites that show no evidence of long term physiological stress. However, elevated values in the magnitude of PAH compounds themselves as well as lobed ENAs observed in this work may be indicative of other forms of pollution or environmental stressors, where the sampled birds are bioindicators for environmental contamination levels.

The magnitude of average lobed ENAs across islands were notably elevated from baseline literature levels reported by Olmastroni et al. (2019). Additionally, there were significantly higher ENAs in the lobed form in the Tavolara site than in the Montecristo and Villasimius sites, respectively, potentially indicating an unspecified genotoxic agent is particularly concentrated in that region.

Total porphyrin levels were surprisingly highest in the Villasimius site, though a significant difference was observed between Tavolara-Villasimius and Tavolara-Montecristo, respectively. Tavolara displayed the second highest magnitude of porphyrins. However, the collective total porphyrin values are below the suggested threshold of significance indicated by Casini et al. (2001), again negating evidence of contaminant exposure stress.

Independent of the biomarker stability in these animals, PAH contamination in the blood - and the external environment by extension - is nonetheless evident. If PAH compounds from the original spill have since been sequestered, it prompts the question of where these lingering contaminants were sourced from. A dispersion effect is possible but unlikely, considering the direction of currents in the region (Buffett et al. 2017, Cattaneo et al. 2017). Potentially more likely is the local emissions impact of shipping vessels, considering the region experiences high levels of marine traffic. Future studies might look to address if a difference exists in reproductive success, which is known to be directly impacted by PAH exposure, as well as direct aqueous PAH levels in the waters surrounding the sample locations. Tavolara, as a significant breeding site, may be selected specifically to determine whether these contaminant values are coming from distant feeding grounds or exposure directly at the nesting site. Furthermore, previous literature offers the potential of synergism between PAH contaminants to amplify their genotoxic and negative physiological effects, and future studies would benefit from considering the magnitude of these interaction effects when multiple PAH compounds are present (Staal et al. 2007, Wassenberg & Di Giulio 2004).

We classify this work as a case study given the relatively low sample sizes analyzed, and further certainty of these results might require a more comprehensive study. Specifically, we urge the further assessment of basal contaminant and biomarker response levels in *P. yelkouan*, given the utility of many baseline metrics is species-specific. Given the vulnerable classification of this species in the Mediterranean, there is a demonstrated need to better understand the threats

environmental pollutants pose in this region, and this study provides a baseline understanding for further research to come.

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