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Assessing the impacts of trace element contamination on the physiology and health of seabirds breeding along the western and southern coasts of Portugal

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Abstract: Coastal seabirds serve as sentinels of ecosystem health due to their vulnerability to contamination from human activities. However, our understanding on how contaminant burdens affect the physiological and health condition of seabirds is still scarce, raising the uncertainty on the species' vulnerability vs tolerance to environmental contamination. Here, we quantified 15 trace elements (TE) in the blood of gull (yellow-legged gull *Larus michahellis* and Audouin's gull *Ichthyaetus audouinii*) and shearwater (Cory's shearwater *Calonectris borealis*) adults, breeding in five colonies along the Portuguese coastline. Additionally, stable isotopes of carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) were quantified to elucidate foraging habitat and trophic ecology of adults, to identify potential patterns of TE contamination among colonies. We used immuno-haematological parameters as response variables to assess the influence of TE concentrations, stable isotope values, and breeding colony on adults' physiological and health condition. Remarkably, we found blood mercury (Hg) and lead (Pb) concentrations to exceed reported toxicity thresholds in 25% and 13% of individuals, respectively, raising ecotoxicological concerns for these populations. The breeding colony was the primary factor explaining variation in five out of six models, underlining the influence of inherent species needs on immuno-haematological parameters. Model selection indicated a negative relationship between erythrocyte sedimentation rate and both Hg and selenium (Se) concentrations, but a positive relationship with $\delta^{13}\text{C}$. The number of immature erythrocyte counts was positively related to Hg and Se, particularly in yellow-legged gulls from one colony, highlighting the colony-site context's influence on haematological parameters. Further research is needed to determine whether essential TE concentrations, particularly copper (Cu) and Se, are falling outside the normal range for seabirds or meet species-specific requirements. Continuous monitoring of non-essential TE concentrations like aluminium (Al), Hg, and Pb, is crucial due to their potential hazardous concentrations, as observed in our study colonies.

Keywords: Animal haematology, Heavy metals, Laridae, Procellariidae, Risk, Stable isotopes

1. Introduction

Over the past century, marine ecosystems have faced cumulative threats due to the worldwide intensification of anthropogenic disturbances. The overexploitation of metal ores for several human activities (mining, metallurgical industry, construction) has led to growing concentrations of trace elements (TEs) in the marine environment (Rauch and Pacyna, 2009; Sen and Peucker-Ehrenbrink, 2012). TEs can readily integrate marine food webs through different mechanisms: *via* adsorption onto suspended particles or dissolved organic matter, *via* their uptake by microorganisms and phytoplankton, or directly from the water through gills and skin (Budko and Martynova, 2019; Galindo et al., 2012). Once prey are ingested, TEs can be assimilated through the intestine in marine consumers and be transferred from lower trophic levels to higher trophic levels (Szykowska et al., 2018). Thus, TEs may undergo bioaccumulation within consumers over time, and some of these elements like mercury (Hg) can also undergo biomagnification throughout food webs (Seco et al., 2021). This process can lead to harmful TE concentrations in top predators, such as seabirds (Bearhop et al., 2000; Campbell et al., 2005).

Seabirds are likely to be contaminated by TEs mainly through their diet (Correia et al., 2023; Fromant et al., 2016). Coastal seabirds are especially vulnerable to contamination by TEs due to increased anthropogenic activities, such as the growing urbanisation and the increase in the number of fishing vessels, which have contributed to the increased release of elements into their foraging habitats (*e.g.*, cadmium (Cd), copper (Cu), nickel (Ni), lead (Pb), zinc (Zn); Richir and Gobert 2016; Richir et al., 2021; Ye et al., 2022). This makes coastal seabirds good for biomonitoring local-scale contamination (Correia et al., 2023; Jouanneau et al., 2022; Thorne et al., 2021). Notably, Hg concentrations have been linked to a higher reliance on marine prey by seabirds (Anderson et al., 2009; Bustamante et al., 2016; Jouanneau et al., 2022). Meanwhile, Pb concentrations have been positively correlated with a higher exposure

to urbanised environments (Bauerová et al., 2017; Callendar and Rice, 2000; Hitt et al., 2023). Blood is useful for assessing short-term exposure to contaminants, *e.g.*, during the breeding season (*i.e.*, when seabirds are accessible for sampling; Jouanneau et al., 2022; Laranjeiro et al., 2021), and may be a good proxy of contamination in other internal tissues (see Albert et al., 2019 for Hg). Also, the use of stable isotopes of carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) as proxies of foraging habitat and trophic ecology, respectively, has proven valuable in unravelling sources of contamination in seabirds, highlighting geographical and species-specific variations in the bioaccumulation of contaminants (Anderson et al., 2010; Carravieri et al., 2020, 2017; Laranjeiro et al., 2020).

While considerable attention has been directed towards blood Hg and its toxicity in seabirds (see the reviews of Ackerman et al., 2016 and Chastel et al., 2022), our understanding of the exposure of seabirds to essential TEs – Cu, iron (Fe), selenium (Se), and Zn – or non-essential elements such as Cd and Pb remains limited (but see Carravieri et al., 2020, 2017, 2014; Carvalho et al., 2013; Laranjeiro et al., 2021; Sebastiano et al., 2017, 2016; Voulgaris et al., 2019). Essential TEs play crucial roles in biological processes, within a specific range of concentrations, but can become double-edge swords when deviating from their optimal range. For instance, Se is required as a cofactor for enzymes that fight oxidants, *e.g.*, glutathione peroxidase, which protect organisms against oxidative stress (Gamble et al., 1997). However, excessive levels of Se can be toxic to birds, leading to adverse effects during embryonic development (Spallholz and Hoffman, 2002), impaired immunity, and teratogenesis (Franson et al., 2000; Janz et al., 2010). Additionally, it is recognized for its capacity to mitigate Hg toxicity by obstructing Hg binding sites, forming an insoluble and inorganic selenous form – mercury selenide – thereby hindering Hg interaction with organic molecules (Manceau et al., 2021; Nigro and Leonzio, 1996). Thus, it is recommended to consider Se alongside Hg,

employing the Se:Hg molar ratio, when assessing the impacts of Hg and its associated toxicity risks to wildlife (Carravieri et al., 2017; Cruz-Flores et al., 2024; Goutte et al., 2014a).

Conversely, non-essential TEs can be toxic at very low doses; for instance, seabirds with blood-equivalent Hg concentrations above $0.95 \mu\text{g g}^{-1}$ dry weight (dw) (*i.e.*, $0.2 \mu\text{g g}^{-1}$ wet weight, ww; Ackerman et al., 2016) showed higher rates of egg neglect (Tartu et al., 2015a), a 50% reduction in fledging success (Tartu et al., 2015b), and reduced breeding success in the subsequent year (Goutte et al., 2014a, b). Indeed, several adverse health impacts stemming from excess TE concentrations, beyond Hg, have been documented in terrestrial and aquatic birds (see Ancora et al., 2008; Pain et al., 2019, Vallverdú-Coll et al., 2019). Some frequently reported sub-lethal effects include immune system disruption (Ibañez et al., 2024; Vallverdú-Coll et al., 2019), physiological impairment (Bauerová et al., 2020, 2017), oxidative stress (Costantini et al., 2014; Espín et al., 2014a, 2014b), or mortality when reaching lethal concentrations (De Francisco et al., 2003; Pain et al., 2019). For instance, higher blood concentrations of arsenic (As), Cd, chromium (Cr), Cu, and Pb were linked to decreased absolute erythrocyte counts and increased haematopoiesis (evidenced by increased immature erythrocyte frequencies) in the great tit *Parus major* (Bauerová et al., 2017). This may reduce haemoglobin (Hb) concentrations, impairing the oxygen-carrying capacity of adults (Minias, 2015), which was also reported for urban and suburban bird populations (Goodchild et al., 2022; Herrera-Dueñas et al., 2014). In another study, urban-living great tits with increased concentrations of Cd, Pb, and Zn exhibited increased total leukocyte counts and heterophil/lymphocyte ratio (H/L) (Bauerová et al., 2020), a general indicator of stress (Norte et al., 2022) which also increases in response to infection (Davis et al., 2008). Yet, the effects may vary according to the contaminant, the level of exposure, and species-specific tolerance to contaminants (*e.g.*, Espín et al., 2016), underlining that the ecological context of the species/population must be considered.

In this study, we quantified TE concentrations in adults of three seabird species: yellow-legged gull (*Larus michahellis*), Audouin's gull (*Ichthyaetus audouinii*), and Cory's shearwater (*Calonectris borealis*) in five colonies located in the west and south coasts of Portugal, comprising four natural colonies and one urban colony. Yellow-legged gulls are generalist, highly opportunistic, and exhibits a mixed-foraging strategy taking advantage of both marine and terrestrial resources (Calado et al., 2021; Pais de Faria et al., 2021b). Within urban colonies, these gulls heavily rely on anthropogenic-derived resources such as urban waste or items sourced from landfills (Fernandes, 2022; Pais de Faria et al., 2021b). In natural colonies, while they may also forage in nearby landfills, their primary reliance is on marine prey, acquired either naturally or through interactions with fishing vessels, *i.e.*, fishery discards (Calado et al., 2021). In contrast, Audouin's gulls and Cory's shearwaters are strictly marine foragers, although they exploit different marine habitats: Audouin's gulls predominantly forage within coastal marine habitats (Matos et al., 2018), whereas Cory's shearwaters use both coastal marine (*i.e.*, neritic regions) and oceanic habitats (Paiva et al., 2010; Pereira et al., 2020). Consequently, their diets reflect these differences, with Audouin's gulls primarily consuming pelagic fish, occasionally supplemented by demersal fish sourced from fishery discards (Calado et al., 2021; Matos et al., 2018), while Cory's shearwaters target epipelagic fish, although cephalopods may also constitute a significant part of their diet (Alonso et al., 2014; Paiva et al., 2010).

This study capitalised on the diverse foraging strategies of these three species to investigate TE exposure across geographically proximate seabird colonies and evaluate potential impacts on adults' physiology. The two main objectives were: firstly, to determine the blood TE concentrations in adults across five colonies, compare them with closely related species occupying similar ecological niches, and highlight geographical exposure patterns. Secondly, to evaluate the potential impacts of TEs on adults' physiological condition using general

immuno-haematological parameters. Using yellow-legged gulls breeding in different colonies facilitated the comparison of TEs contamination between urban *vs* natural ecological contexts, while Audouin's gulls and Cory's shearwaters enabled comparisons among species, but most importantly, between coastal marine *vs* oceanic habitats. We expect species and colony to influence blood TE concentrations and immuno-haematological parameters. Specifically, yellow-legged gulls breeding in urban environments should have elevated concentrations of Cd, Ni, and Pb which are related to industrial processes, urban runoff, and historical use of leaded gasoline (Ye et al., 2022). These elevated TE concentrations may alter physiological and immune responses, evidenced by decreased Hb concentrations, increased immature erythrocyte counts, and higher H/L ratios (*e.g.*, Bauerová et al., 2020, 2017). Conversely, we expect higher concentrations of As, Hg, and Se in strictly marine foragers, especially those relying on fishery discards, such as gulls breeding in natural colonies (Calado et al., 2021). Additionally, Cory's shearwaters should exhibit higher Cd concentrations due to the inclusion of cephalopods in their diet (Bustamante et al., 1998). Elevated levels of As, Cd, and Hg concentrations may increase the incidence of genotoxic events, particularly on erythrocytes, leading to nuclear abnormalities. Overall, this study contributes to provide baseline values of haematological parameters in wild seabirds, as well as new information about the TEs toxicity thresholds in these species.

2. Methods

2.1. Study areas and study species

Fieldwork was carried out during the 2021 breeding season at three locations along the West and South coast of Portugal (Fig. 1): Porto (41°08'N, 8°36'W), a large urban and industrial area, that lies ~8 km from a major fishing harbour (Matosinhos); Berlenga Island (39°24'N, 9°30'W), a small neritic island that lies ~11 km from Peniche, a small city with a

medium-sized fishing harbour; Deserta Island (36°57'N, 7°53'W), an uninhabited sandy island sited within the lagoon system of Ria Formosa, Algarve, that lies ~7 km from a large fishing harbour (Olhão). Yellow-legged gull (YLG) breeds in all study sites, although population densities are largely different among breeding colonies: 593-813 breeding pairs in Porto, *ca.* 2,400 breeding pairs in Berlenga Island, and *ca.* 540 breeding pairs in Deserta Island (Oliveira et al., 2023). The colony of Porto represented an urban population (Fernandes, 2022), the colony of Deserta represented a coastal population with a pronounced mixed-foraging strategy (Matos et al., 2018), and the colony of Berlenga represented a coastal population but with a marine biased foraging strategy (Mendes et al., 2018). Audouin's gull (AG) only breeds in Deserta Island, with a current increase of its breeding population, *ca.* 5,340 breeding pairs (ICNF, unpublished data), while Cory's shearwater (CS) only breeds in Berlenga Island and has a current stable population of *ca.* 300 breeding pairs (Oliveira et al., 2020).

2.2. Fieldwork and sample collection

At each breeding colony, incubating gulls were caught using a walking trap placed over the nest (from late April in Deserta Island to early and late May in Berlenga Island and Porto, respectively), while shearwaters were caught by hand at night, during the chick-rearing period (in late August), when adults return to their nesting burrow to feed their chick. All birds handled were ringed ($N = 75$), weighted using a Pesola Spring© balance (± 5 g), measured with a calliper (tarsus, ± 1 mm), and a ruler (flattened wing, ± 1 mm). A blood sample (0.8 to 1.2 mL, $< 2\%$ of the body mass of AG, the smallest in this study) was collected from the tarsal vein using heparinized syringes coupled to a 25-G needle, transferred into a 1.5 mL microtube, and immediately stored in a cool box. At the end of the day, blood was centrifuged and stored at -20 °C. Additionally, two heparinized capillary tubes of whole blood (~ 150 μ L) were collected

for the analysis of immuno-haematological parameters (see below for a more detailed description).

2.3. Trace elements analysis

Blood cells (hereafter 'blood') were freeze-dried and used to measure total mercury (Hg) and 14 other elements at the laboratory Littoral, Environment and Societies (LIENSs, CNRS-La Rochelle Université). Total Hg was quantified with an Altec AMA 254 spectrophotometer (N = 75, aliquots mass: 0.86 ± 0.69 mg dw). Samples were analysed in duplicate-triplicate until reaching a relative standard deviation < 10% (Bustamante et al., 2006). TORT-3 lobster hepatopancreas (Hg certified concentration: 0.292 ± 0.022 $\mu\text{g g}^{-1}$ dw) was used as Certified Reference Material (CRM) to check the accuracy of the method at each 15 samples. The measured values were 0.287 ± 0.004 $\mu\text{g g}^{-1}$ dw (N = 8), thus corresponding to a recovery rate of $99.1 \pm 1.5\%$. Blanks were run at the beginning of each set of samples and the limit of detection (LoD) was 0.1 ng. Aluminium (Al), As, Cd, cobalt (Co), Cr, Cu, Fe, manganese (Mn), nickel (Ni), Pb, Se, tin (Sn), strontium (Sr), and Zn were analysed using a Thermo Fisher Scientific X Series 2 ICP-MS (aliquot mass: 80 – 200 mg, N = 75) following Kojadinovic et al. (2011). Briefly, prior to analysis, blood samples were microwave digested in a mixture of 3 mL of suprapure nitric acid (HNO₃) and 1 mL of suprapure chloridric acid (HCl), and then diluted to 25 mL with deionized water. Accuracy and reproducibility of the preparation was tested through analytical blanks and replicates of CRMs (TORT-3 Lobster Hepatopancreas and DOLT-5 Dogfish Liver, both NRC, Canada) along each set of samples. Recovery rates of CRMs ranged from 84 to 101% more specifically, equal to $96 \pm 11\%$ for Al, $95 \pm 6\%$ for As, $92 \pm 2\%$ for Cd, $88 \pm 4\%$ for Co, $97 \pm 5\%$ for Cr, $101 \pm 4\%$ for Cu, $94 \pm 4\%$ for Fe, $97 \pm 7\%$ for Mn, $92 \pm 3\%$ for Ni, $84 \pm 3\%$ for Pb, $100 \pm 8\%$ for Se, $88 \pm 7\%$ for Sn, $90 \pm 8\%$ for Sr, and $101 \pm 2\%$ for Zn. Results are given as mean \pm SD in $\mu\text{g g}^{-1}$ dw.

2.4. Stable isotopes analysis

Freeze-dried blood samples were weighted (between 0.2 – 0.4 mg dw, N = 71) with a microbalance and packed into tin capsules for combustion. Relative abundances of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ were measured using a continuous flow mass spectrometer (Delta V Plus with a ConFlo IV interface, Thermo Scientific, Bremen, Germany) coupled to an elemental analyser (Thermo Scientific EA 1112) at the laboratory LIENSs. Results were expressed using the delta (δ) notation and computed using the following equation: $\delta\text{X} = [(R_{\text{Sample}}/R_{\text{Standard}}) - 1] \times 1000$, where X stands by ^{13}C or ^{15}N , and R being the respective ratio, *i.e.*, $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$. Standard values correspond to Vienna PeeDee Belemnite and atmospheric N_2 for carbon and nitrogen, respectively. Replicate measurements of reference materials (USGS-61 and USGS-63, US Geological Survey) were used to check the accuracy of the isotopic results, and analytical precision was $< 0.10\%$ for both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values.

2.5. Immuno-haematological parameters analysis

Erythrocyte sedimentation rate (ESR) was measured by collecting one heparinized capillary tube filled with whole blood and stored vertically at 4 °C for 4 h (Heylen and Matthysen, 2008). ESR is the ratio of the volume of the capillary tube not occupied by erythrocytes and the total blood volume in the tube (Heylen and Matthysen, 2008).

Haemoglobin (Hb) concentration (g dL^{-1}) was measured in whole blood (20 μL) following Pais de Faria et al. (2022) using the Haemoglobin Assay commercial kit obtained from SPINREACT according to the manufacturer's instructions. Hb analyses were conducted at the Marine and Environmental Sciences Centre laboratory (MARE, University of Coimbra).

A small drop of whole blood was smeared on a slide in the field, fixed in methanol (99% vol) for 3 min within 4 h after collection, left to dry at room temperature (20 °C), and stained with the Giemsa procedure (Palhares and Grisolia, 2002). The analysis of each blood smear

was carried in a two-step's procedure: identify monolayer fields with similar density of well-stained erythrocytes under 400x magnification; proceed to the identification and counts of leukocytes (*i.e.*, WBC), immature erythrocytes (IE), and erythrocyte nuclear abnormalities (ENA) under 1000x magnification following Zuñiga-González et al. (2000). WBC were classified as basophiles, eosinophils, heterophils, lymphocytes, and monocytes, according to nuclear size, lobules, and colouration intensity and/or granularity, and overall cell dimension (Fokidis et al., 2008; Mallory et al., 2015; Norte et al., 2008). WBC was estimated by counting the number of leukocytes *per* 10,000 erythrocytes (Thrall et al., 2012), and the ratio between heterophil/lymphocyte (H/L) was calculated in a total of 100 WBC (Davis et al., 2008). IE were identified based on their less oval to round nuclei and polychromatic cytoplasm. ENA were classified as bilobated/binucleated, budding, kidney-shape, micronucleus, notched, nucleoplasmic bridge, tailed, vacuolated nucleus, and unknown nuclear malformation (Pacheco and Santos, 2006; Skarphedinsdottir et al., 2010). The sum of total ENA was calculated for each blood smear. IE and ENA were estimated *per* 10,000 erythrocytes. Blood smears were examined under a Leica DMLS microscope at the laboratory MARE by a single operator to mitigate bias linked with different operators. For more details about the aim of each parameter analysed consult Table S1 in Supplementary Material.

2.6. Data analysis

First, to explore contaminant co-variance and to identify the TEs reflecting most of the total variance of data, a linear discriminant analysis (LDA) was computed on normalised and scaled TEs with concentrations above the limit of quantification (LOQ) in at least 70% of the total number of individuals. Concentrations below this threshold were replaced with a half of the LOQ value (LOQ/2) and included in the analysis (Hites, 2019). Thus, Cd, Co, Cr, Ni, Sn, and Sr were excluded from the LDA. Replacements occurred for 15 individuals for Pb (20%) and

one individual for As (1.3%). $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were also added to LDA to help disentangling patterns of TE concentrations among species breeding on the different study sites. $\delta^{13}\text{C}$ was used to explore TEs relationships with the feeding habitat, as an indicator of the use of terrestrial or oceanic habitats and pelagic food webs (lower $\delta^{13}\text{C}$ values) vs. exploitation of marine or coastal habitats and benthic food webs (higher $\delta^{13}\text{C}$ values) (Newsome et al., 2007); while $\delta^{15}\text{N}$ was used to identify co-variance of TEs with trophic level (Carravieri et al., 2017). Since $\delta^{13}\text{C}$ has a trophic component, a linear regression was carried out between blood $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ with species/colony as a fixed categorical variable ($F_{5, 65} = 80.5, p < 0.001, r^2 = 0.85$; Fig. S1 in Supplementary Material), and the residuals were then used instead of raw $\delta^{13}\text{C}$ values (Ceia et al., 2012). All predictors (Al, As, Cu, Fe, Hg, Mn, Pb, Se, Zn, $\delta^{15}\text{N}$, and residual $\delta^{13}\text{C}$) were inspected for multicollinearity issues prior to LDA, through the calculation of the variation inflation factor ($\text{VIF} > 2$) and spearman correlation coefficients ($r_s > 0.50$) under the *usdm* R package (Naimi, 2017). This inspection resulted in the exclusion of Cu, Zn, and $\delta^{15}\text{N}$ from discriminant analysis (Table S2 and Table S3 in Supplementary Material). The Se:Hg molar ratio was calculated for all individuals following to Cruz-Flores et al. (2024), although not included in LDA since it is highly correlated to Hg and Se concentrations. Since only YLG breeds in all the three study species, the lack of overlapping breeding sites for AG and CS prevents simultaneous comparisons across species and colonies. Consequently, we opted to create five categories based on the species and study site (hereafter ‘colony-site’ factor). One-way ANOVAs or Kruskal-Wallis were computed to compare the concentrations of each TE, Se:Hg molar ratios, and stable isotopes among colony-sites followed by post-hoc Tukey tests or pairwise tests on significant results (significance level of $\alpha < 0.05$, Table 1). *P*-values were adjusted using the Bonferroni correction to counteract potential Type I errors.

To facilitate comparisons with published data, we used a conversion factor of 4.76 by multiplying the wet mass concentrations to obtain the dry mass equivalents. This assumption

is based on an estimated humidity of approximately 79% in wet tissues such as blood, as indicated by Eagles-Smith et al. (2008). These results must be interpreted cautiously, considering that the conversion factor may vary among species and individuals.

Second, to explore the occurrence of patterns on immuno-haematological parameters among colony-sites, a second LDA was carried out using those quantified in at least 50% of the individuals. ESR was not included in the LDA because it was not quantified in YLG breeding in Deserta. One individual was excluded from this LDA because none of immuno-haematological parameters was quantified. Isolated missing values were replaced by the average value computed using the available values for each colony-site, corresponding to 16% of missing values for Hb, 10% for IE and ENA counts, and 4% for H/L ratio and WBC counts. Since the first two axes of the LDA explained more than 95% of total variance of immuno-physiological parameters among colony-sites (see Fig. S2 and Table S4 in Supplementary Material), this factor was added as a predictor in subsequent regression-type analyses (see below).

Third, univariate analyses (linear or generalized linear models, GLM) were used to test the effect of the colony-site (fixed categorical variable with five levels), LD scores of axes 1 and 2 extracted from LDA of TE and stable isotopes (fixed continuous variables), and their interactions on each immuno-haematological parameter – ESR, Hb, H/L, WBC, IE, and ENA (response variables) – in a total of six models (Table S5 in Supplementary Material). Full models were adjusted to the family data distribution (*e.g.*, Gaussian, Gamma (log link function), Poisson (log link function), Negative binomial) and the shearwater was used as the reference colony-site, as the representative for coastal and oceanic habitats. Models were checked for residuals normality, homoscedasticity, and outliers using ‘check_model’ function within the *performance* R package (Lüdecke et al., 2021), before going through model selection. Overdispersion was checked to ensure the appropriateness of the chosen modelling

approach in regression models of variables with Poisson distribution, *i.e.*, counting data variables (WBC, IE, and ENA). Whenever overdispersion was observed, the negative binomial family was chosen and adjusted for skewness with θ shape parameter (Zuur et al., 2009). Models were run using the ‘dredge’ function within the *MuMIn* R package (Barton, 2023), and model selection was carried out following the Akaike's Information Criterion corrected for small sample sizes (AICc) to identify the most parsimonious models ($\Delta\text{AIC} < 2$; Burnham and Anderson, 2002). AICc weights – representing the normalised weight of evidence in support of a particular model – along with the explained deviance (reflecting the proportion of variation explained by the model, Zuur et al., 2009) were employed for deducing the effect of explanatory variables on the response variables (Burnham and Anderson, 2002). When several models outperformed the null model and had $\Delta\text{AICc} < 2$, we employed model averaging for inference. This process generated averaged parameter estimates (β) for the predictor variables included in the models, weighted by their respective AICc weights. If the null model ranked as the optimal model, all explanatory variables were statistically deemed non-significant. Predicted values and confidence intervals (CI) were extracted from the most parsimonious model or average models using the ‘ggpredict’ function within the *ggeffects* R package (Lüdtke, 2018) and plotted using *ggplot2* R package (Wickham et al., 2023). Candidate models with both significant and non-significant effects are presented in Table 2.

Fourth, scaled and centred isotopic data were employed with the *SIBER* R package – Stable Isotope Bayesian Ellipses in R – to calculate Bayesian estimates of standard ellipse areas (SEA_B) and draw bivariate standard ellipses for each colony (Jackson et al. 2011). For visualisation purposes, standard ellipse areas corrected for small sample sizes (SEA_C) were computed using 40% of the data (Fig. S3 in Supplementary Material). Data exploration, visualisation, and model computations were carried out in R version 4.3.1 (R Core Team, 2023).

3. Results

3.1. TE concentrations and stable isotopes

Among the 15 targeted TEs, essential elements including Cu (overall range: 1.18 – 4.92 $\mu\text{g g}^{-1}$ dw), Fe (1608 – 2501 $\mu\text{g g}^{-1}$ dw), Mn (0.07 – 0.53 $\mu\text{g g}^{-1}$ dw), Se (2.2 – 89.5 $\mu\text{g g}^{-1}$ dw), Zn (18.8 – 57.0 $\mu\text{g g}^{-1}$ dw), and non-essential TEs like Al (2.40 – 18.27 $\mu\text{g g}^{-1}$ dw) and Hg (0.30-15.47 $\mu\text{g g}^{-1}$ dw) were detected in all individuals (Table 1). The highest concentrations of Cu were found in YLG populations and the lowest in CS, while Al concentrations were higher in YLG breeding in Berlenga rather than in CS (ANOVA, $F_{4, 70} = 5.29$, $p = 0.02$). Se concentrations were higher in AG and in CS compared with YLGs, while Hg concentrations were the highest in AG and YLG breeding in Deserta and the lowest in YLG breeding in Porto ($38.67 < F_{4, 70} < 40.95$, $p < 0.001$). Se:Hg molar ratio was the highest in CS ($F_{4, 70} = 15.83$, $p < 0.001$; Table 1) and not different among the other colonies ($p > 0.05$). As concentrations (0.13 – 18.45 $\mu\text{g g}^{-1}$ dw) were detected in all individuals, apart from one YLG breeding in Porto and were higher in AG and YLG breeding in Berlenga compared with CS and YLG breeding in Porto ($F_{4, 69} = 8.38$, $p < 0.001$). The detection frequencies of Cd (0.010 – 0.099 $\mu\text{g g}^{-1}$ dw), Pb (0.01 – 2.63 $\mu\text{g g}^{-1}$ dw), and Sr (0.04 – 0.46 $\mu\text{g g}^{-1}$ dw) varied widely among colonies (Table 1). Cd was higher in CS and not detected in YLG breeding in Deserta, while Pb concentrations were the highest in YLG breeding in Porto (Kruskal-Wallis, $\chi^2 = 47$, $\text{df} = 4$, $p < 0.001$). Co, Cr, and Ni were detected in low concentrations in a few individuals, while Sn was only detected in two AGs (Table 1).

Blood $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values varied widely among adults from different colonies (ANOVA, $F_{4, 66} = 18.1$ and 59.7, respectively for carbon and nitrogen, both $p < 0.001$). Notably, urban-dwelling YLGs and YLGs breeding in Berlenga occupied distinct isotopic niches compared to the other colonies, as evident from non-overlapping standard ellipses (Table 1, Fig. S3-A in Supplementary Material). Urban YLGs and those breeding in Deserta exhibited the broadest

isotopic niches, while the strictly marine AG and CS exhibited narrower niches (Fig. S3-B in Supplementary Material).

3.2. TE and isotopic patterns in the ordination space

Among the 7 TEs (Al, As, Fe, Hg, Mn, Pb, and Se) and the residual $\delta^{13}\text{C}$ values included in the LDA, Hg and Se contributed most to LD1, while the residual $\delta^{13}\text{C}$ values contributed most to LD2 (Table S6 in Supplementary Material). The LDA highlighted a clear segregation of urban YLGs along axis 1, strongly associated to the lower blood Hg and Se concentrations in this population. On the other hand, YLG breeding in Berlenga was clearly segregated along axis 2, strongly associated to the higher residual $\delta^{13}\text{C}$ values (Fig. 2). LD scores of the two main axes were retained for subsequent univariate analyses, and TEs here reported were used for further inferences (Table S6 in Supplementary Material).

3.3. Association of TEs and trophic ecology with immuno-haematological parameters

The best model explaining ESR (range: 0.01 – 0.29) included the colony-site, LD1, and LD2 as predictors, with no interaction (Table 2). ESR was higher in CS than in urban YLGs, and those breeding in Berlenga exhibited a moderate negative association with LD1, and a very strong positive association with LD2 (Fig. 3, Table 2). This suggests an overall increase of ESR with $\delta^{13}\text{C}$ values. Model averaging indicated that there was a positive and strong effect of the interaction between YLG breeding in Deserta and LD1 on the number of IE (Table 2, Fig. 4). This suggests an increase in the number of IE in YLGs breeding in Deserta with the increase on Hg and Se concentrations. Hb concentrations, WBC, and ENA counts only included the colony-site as significant predictor in the best model (WBC counts) or average models (Hb and ENA counts) (Table 2). More specifically, CS presented higher concentrations of Hb, higher numbers of WBC and ENAs than AG and YLGs breeding in natural colonies, while urban

YLGs presented higher numbers of ENAs than all other gull populations. Heterophil/lymphocyte ratio (H/L) exhibited no association with either the colony-site or any of the LD axes (Table 2).

4. Discussion

This study analysed blood concentrations of 15 trace elements (TEs) in three seabird species breeding along the western and southern coasts of Portugal. It indicates a diverse exposure to essential and non-essential TEs, possibly driven by colony-site and species-specific trophic ecology and foraging habitats, as evidenced by the distinct isotopic niches and their sizes. Immuno-haematological parameters were strongly influenced by the colony-site, suggesting that species-specific requirements together with the ecological context of the breeding colony could be the primary factors explaining these differences. Intriguingly, ESR and IE counts exhibited moderate and strong relationships with $\delta^{13}\text{C}$ values and Hg and Se concentrations, respectively, rather than solely the colony-site, which might warrant further investigation.

4.1. Comparison to previous studies on blood TE concentrations in seabirds

The majority of ecotoxicological studies on TEs have been carried out using feathers collected from adult seabirds, while only a few studies have used blood (but see Anderson et al., 2010; Carravieri et al., 2014; Fromant et al., 2016; Sebastiano et al., 2017, 2016). Different tissues have diverse turnover rates, preventing direct comparison between them, thus here we focus on blood studies' comparisons. In the present study, Se and Hg were the elements which contributed the most for separation among colony-sites in the ordination space along the LD1, evidencing a clear segregation of the urban YLGs from the natural colonies. In fact, the range of Se concentrations was very wide among colonies, being 3- to 10-fold lower in the urban-

dwellers than in other Larids (Correia et al., 2023; Sebastiano et al., 2017). Notably, most urban YLGs had Se concentrations in the range of seabird chicks reared in natural habitats ($< 10 \mu\text{g g}^{-1} \text{dw}$, Carravieri et al., 2020; Kim et al., 2013; Sebastiano et al., 2017, 2016). This underlines the lower bioavailability of Se in terrestrial and urban habitats, highly linked to the lower intake of marine prey by YLGs from Porto (Pais de Faria et al., 2021b), when compared to species strictly foraging on marine resources (range $30\text{--}200 \mu\text{g g}^{-1} \text{dw}$; Anderson et al., 2010; Carravieri et al., 2014; Correia et al., 2023; Fromant et al., 2016; Sebastiano et al., 2017).

Overall, CS had generally low Hg concentrations, comparable to those found in other small and medium-sized Procellariiformes (range $0.3 - 3.3 \mu\text{g g}^{-1} \text{dw}$; Anderson et al., 2009; Carravieri et al., 2021, 2018; Carvalho et al., 2013; Fromant et al., 2016). YLGs and AGs breeding in natural colonies had similar Hg concentrations to those of western gulls (*L. occidentalis*) breeding on the west coast of the USA (Clatterbuck et al., 2021) and to YLGs and lesser black-backed gulls (*L. fuscus*) breeding in Île de Ré on the west coast of France (Jouanneau et al., 2022), although not exceeding records reported for the great black-backed gull (*L. marinus*) breeding in Île de Ré (range $7.0 - 21.5 \mu\text{g g}^{-1} \text{dw}$; Jouanneau et al., 2022). Conversely, our urban-dwelling YLGs had low Hg concentrations, similarly to gulls breeding in the Baltic Sea (Szumilo-Pilarska et al., 2017). The variation in Hg concentrations detected in our study aligns with the prevalent diet at gull colonies: YLGs and AGs from Deserta feed on demersal and mesopelagic fish made available via trawler discards, highly abundant in Algarve (Bueno-Pardo et al., 2017), whereas YLGs from Berlenga and Porto typically rely on crustaceans and anthropogenic-derived resources, respectively (Calado et al., 2021; Pais de Faria et al., 2021b). This highlights the role of diet, trophic position, and foraging habitat in Hg exposure (Anderson et al., 2009; Jouanneau et al., 2022; Mills et al., 2022, 2020), evidencing Hg biomagnification (Seco et al., 2021). Demersal and mesopelagic fishes are known to be of higher trophic position ($\delta^{15}\text{N}$ -enriched; Navarro et al., 2010), and more enriched in Hg (Blum

et al., 2013; Chouvelon et al., 2012; Choy et al., 2009) compared to crustaceans and anthropogenic-derived resources (Chouvelon et al., 2012; Thorne et al., 2021). The higher rate of microbial-mediated methylation of Hg in low oxygen waters, such as the mesopelagic layer, prompts the formation and accumulation of methyl-Hg (MeHg) in the mesopelagic layer (Choy et al., 2009). This may help explaining the higher Hg concentrations found for AGs and YLGs breeding in Deserta when compared to CS, which rely on cephalopods and epipelagic fish, typically lower in Hg (Choy et al., 2009; Minet et al., 2021).

$\delta^{13}\text{C}$ contributed most to the separation of YLGs breeding in Berlenga from those breeding in the urban colony along LD2. As previously noted for $\delta^{15}\text{N}$, $\delta^{13}\text{C}$ is generally higher in marine than in terrestrial food webs, and in demersal than in pelagic food webs (Newsome et al., 2007). The lower $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values ($r_s = 0.623$), indicating a diet poorer in marine resources, together with higher Pb concentrations, distinguished urban YLGs from other populations in the ordination space. Pb is a non-essential element associated with higher exposure to urban and landfill environments (Kim and Williams, 2017; Meillère et al., 2016), explaining why YLGs showed the highest detection frequencies of Pb (85-100%). This is likely due to their use of both marine and terrestrial/anthropogenic resources (Pais de Faria et al., 2021b). Markedly, 13% of the individuals surpassed the lowest benchmarks of Pb reported to elicit inhibitory effects on delta-aminolaevulinic acid dehydratase (ALAD) in Baltic eiders ($0.24 \mu\text{g g}^{-1} \text{dw}$; Franson et al., 2000; Franson and Deborah, 2011). This enzyme is essential in the initial steps of heme synthesis – a precursor of Hb necessary to bind oxygen (Gibson et al., 1998) – and its inhibition occurs due to the replacement of Zn atoms by Pb atoms, required for ALAD activity (Astrin et al., 1987). All YLGs exceeding $0.24 \mu\text{g g}^{-1} \text{dw}$ of Pb in blood were urban dwellers, except one individual from Deserta Island. Thus, these results underscore the elevated Pb exposure in the urban centres, attributable to YLG's use of anthropogenic facilities such as

landfills (Fernandes, 2022; Lopes et al., 2021; Matos et al., 2018), but also suggest a high resilience of YLGs to contamination.

Other TEs, such as Co, Fe, Mn, Ni, and Zn were within the typical seabird concentration ranges (Anderson et al., 2010; Carravieri et al., 2014; Correia et al., 2023; Fromant et al., 2016; Sebastiano et al., 2017), denoting their role in homeostatic processes (Walsh, 1990). Despite including cephalopods in their diet, a major source of Cd for predators (Bustamante et al., 1998), CS had low concentrations of this metal in its blood, suggesting a low exposure risk (e.g., Carravieri et al., 2014; Sebastiano et al., 2017, 2016; but see Carvalho et al., 2013; Voulgaris et al., 2019). Al and Cu concentrations found in YLGs were higher compared to those from other seabirds (Al: range 0.7 – 6.9 $\mu\text{g g}^{-1}$ dw, Cu: range 0.5 – 1.3 $\mu\text{g g}^{-1}$ dw; Correia et al., 2023; Finger et al., 2016, 2015; Fromant et al., 2016; Kim et al., 2013; Sebastiano et al., 2017, 2016). YLGs are known to feed on considerable amounts of the Henslow's swimming crab (*Polybius henslowii*), especially in Berlenga Island (Alonso et al., 2015; Calado et al., 2021; Pais de Faria et al., 2021b). In fact, crustaceans usually contain high Cu and Zn enzymatic requirements (mean Cu: 26.3 $\mu\text{g g}^{-1}$ dw, mean Zn: 34.5 $\mu\text{g g}^{-1}$ dw; White and Rainbow, 1985); in crustaceans, haemocyanin is the respiratory pigment used to transport oxygen (Taylor and Anstiss, 1999), leading to an increased requirement of Cu (White and Rainbow, 1985). Nevertheless, Cu concentrations were similar across all YLG colony-sites, suggesting that these elevated levels of Cu may be related to the foraging ecology of YLG (i.e., species-specific factors) or potentially to a metabolic disturbance. Usually, Cu concentrations are expected to remain constant due to homeostatic processes (Walsh, 1990). Conversely, As concentrations varied widely among and within colony-sites (range 0.3 – 18.5 $\mu\text{g g}^{-1}$ dw), with higher concentrations in gulls than shearwaters, consistent with previous reports (Carravieri et al., 2014; Carvalho et al., 2013; Correia et al., 2023; Fromant et al., 2016; Sebastiano et al., 2017). Multiple sources of As challenge the tracking of its origin, especially for wide-ranging

foragers like shearwaters or flexible foragers like gulls. These species rely on diverse foraging habitats and prey, *e.g.*, marine *vs* terrestrial and pelagic *vs* demersal, which may ‘dilute’ As concentrations in blood, making it difficult to detect contamination patterns. Despite the uncertainty surrounding As toxicity thresholds, concentrations in our study did not exceed $50 \mu\text{g g}^{-1} \text{ dw}$ (Eisler, 1994; Neff, 1997; Sánchez-Virosta et al., 2015), suggesting minimal threat to these populations. Therefore, when assessing TE concentrations, we must acknowledge the multiple sources of each element (natural and anthropogenic), as well as the potential species-specific requirements for essential elements that may influence the overall concentrations.

4.2. Hg toxicity and Se:Hg molar ratio

Remarkably, 25% of the individuals, primarily AGs and YLGs from Deserta, exceeded the internationally defined toxicity threshold for Hg ($1 \mu\text{g g}^{-1} \text{ ww}$), which poses a moderate risk to Hg toxicity (range $4.8 - 14.3 \mu\text{g g}^{-1} \text{ dw}$; Ackerman et al., 2016). Such concentrations are known to impact physiology, behaviour, and reproduction in several seabird species (Ackerman et al., 2016; Chastel et al., 2022; Goutte et al., 2015, 2014a, b; Tartu et al., 2015a, b). However, it is worth noting that all individuals, with the exception of one urban-dwelling YLG (Se:Hg = 3.9), exhibited a Se:Hg molar ratio above 4, a recent threshold outlined as the Se-Hg relationship needed to effectively demethylate MeHg, the most toxic organic form of Hg (Manceau et al., 2021). This suggests an overall effective protection against Hg toxicity (Cuvin-Aralar and Furness, 1991; Nigro and Leonzio, 1996). In particular, AGs and YLGs had similar mean Se:Hg molar ratios, despite elevated Se concentrations found in AGs. If we consider a 1:1 stoichiometry for our study species, it suggests that AG and YLG populations may have lower protection against the toxic effects of Hg compared to CS. This contrasts with expectations based solely on the absence of differences in Hg concentrations observed between CS and YLG from Berlenga. These results underscore the importance to consider Hg and Se

levels at the same time in predators (Cruz-Flores et al., 2024) and prey to validate our findings, and assess potential Hg exposure risks for coastal breeding seabirds.

4.3. Impact of TEs on physiology and health of seabirds

Our study found that immuno-haematological parameters of gulls and shearwaters were not related to TEs exposure. The parameters used here did not show any evidence of negative health impacts on the immune response and physiological condition of breeding adults. Despite widely used in ecology, it should be noted that these parameters are relatively coarse, thus it is possible that they simply could not detect certain impacts due to the lack of specificity to TE contamination. Yet, it is worth noting the elevated levels of Hg and Pb in some individuals, which were correlated with demographic consequences in other species (Ackerman et al., 2016; Chastel et al., 2022; Franson and Deborah, 2011; Goutte et al., 2014a, b).

Of particular interest was the overall negative relationship between ESR and $\delta^{13}\text{C}$ values, as well as the relationship between IE with Hg and Se concentrations within YLG breeding in Deserta. A higher ESR often indicates systemic inflammation, due to infections, chronic diseases, or acute inflammatory responses (Heylen and Matthysen, 2008; Norte et al., 2022). However, it can also reflect physiological stress, including exposure to pollutants or other stressors (aggressions, competition, starvation, etc.) (Norte et al., 2022). Our results suggested that higher $\delta^{13}\text{C}$ values favoured lower ESR. In gulls, higher $\delta^{13}\text{C}$ values are often linked to a marine foraging strategy, while lower $\delta^{13}\text{C}$ values are usually linked to a more terrestrial foraging strategy (Garthe et al., 2016; Mendes et al., 2020; Pais de Faria et al., 2021b). Thus, it would be predicted that urban YLGs exhibit higher ESR than the other YLG populations. This prediction is contradictory with a previous study that assessed the several immuno-haematological parameters in the YLG adults and chicks in the same natural and urban colonies (Pais de Faria et al., 2022). Here, adults and chicks followed the same pattern: individuals from

urban colonies exhibited lower ESR than individuals from natural colonies. In fact, in natural colonies the nest density could be much higher than in urban environments, causing more intraspecific negative interactions. Higher amounts of IE indicate a necessity for replenishing erythrocytes due to haemorrhage or haemolysis, typical of regenerative anaemias caused by ectoparasites (Boyd, 1951; Leighton et al., 1983). Interestingly, our results revealed that within YLG breeding in Deserta, the number of IE would be higher in individuals with higher Hg and Se concentrations. This indicates that YLG with a more marine foraging strategy would have higher numbers of IE. A higher amount of IE reveals that the individual is replacing the erythrocytes that were depleted due to a haemorrhagic event (Minias, 2015). Haemorrhagic events can occur when competing for food resources while at sea, or during interactions with fishing vessels that prompt aggressions among individuals (Costa et al., 2020). Indeed, the amount of Hb synthesised in IE does not exceed 20% of the level found in mature erythrocytes, meaning that Hb concentrations would be lower and are expected to be replenished with a substantial delay (Minias, 2015). Moreover, Hg is known to induce oxidative stress by generating reactive oxygen species (ROS) (Henry et al., 2015), which damage cells including the erythrocytes, thereby lowering Hb levels. Despite the significance of these relationships, we recommend caution when interpreting these results, since the number of studies analysing these parameters in wild seabirds is still limited, which challenges the establishment of baseline values, even more with low sample sizes (but see Pais de Faria et al., 2022).

WBC and ENA counts were notably higher in CS than in the other two species examined here, suggesting increased immune responses and greater genotoxicity (*i.e.*, DNA damage), respectively. However, these values were comparable to those reported for other seabirds (Colominas-Ciuró et al., 2022; D'Amico et al., 2016; Olmastroni et al., 2024, 2019). Among gulls, urban-dwelling YLGs exhibited higher ENA counts than AGs and YLGs breeding in natural colonies. This is in line with previous reports on the higher prevalence of genotoxicity,

e.g., DNA adducts, micronucleus and other ENAs, in urban and suburban birds (Baesse et al., 2019, 2015; Skarphedinsdottir et al., 2010) or in more polluted areas (Quirós et al., 2008), when compared to more ‘natural’ environments. We should not overlook the potential genotoxic effect of the high Pb concentrations on urban-dwelling YLGs, despite the low number of ENAs found in our study (range 0-27), when compared with previous studies (D’Amico et al., 2016; Olmastroni et al., 2024, 2019). A higher frequency of ENAs is often indicative of sensitivity to contaminants such as essential and non-essential TEs (Christopher et al., 2004; Shah et al., 2020), however it might not be directly related to overall exposure. Instead, ENAs have been linked to increased oxidative stress, infectious diseases, nutritional deficiencies, and physiological stress (Bourgeon et al., 2012; Keilen et al., 2022).

Other factors such as age, sex, body condition, breeding stage, and the presence and loads of other stressors, like organic pollutants or parasites, may influence individual physiological and immunological responses (Bustnes et al., 2004; Davey et al., 2000; D’Amico et al., 2016; Wells et al., 2024). This should be particularly critical during the breeding period when adults are more vulnerable. For instance, Hb concentrations tend to increase from the egg-laying to the chick-rearing period due to the high demands of oxygen-carrying capacity while feeding the chicks (Davey et al., 2000), which may partly explain the increasing pattern found for Hb between gulls (incubation) and shearwaters (chick-rearing). On the one hand, we advise caution when interpreting immuno-haematological parameters alone as indicators of individual or populational vulnerability to contaminants. We advocate for a critical look when interpreting these relationships in the context of environmental contamination. On the other hand, the lack of values outside the normal ranges and the lack of species-specific toxicity benchmarks for most of TEs do not preclude potential physiological effects, as the species may play a pivotal role in such cause-effect relationships.

5. Conclusion

This study represents a significant contribution in our understanding of blood TE contamination in coastal and oceanic seabirds. A key finding was the large variation in TE concentrations among colony-sites, including some essential elements which may reflect species-specific requirements. Notably, differences found in Hg, Pb, and Se concentrations among colony-sites underscore the influential role of diet and trophic ecology in shaping TE accumulation patterns in adult breeding seabirds. The marked impact of the colony-site effect highlights the necessity for additional research on the toxicity thresholds of TEs, within the specific ecological context of each population, *i.e.*, urban *vs* natural, coastal marine *vs* inshore. Our findings raise ecotoxicological concerns, particularly regarding Pb concentrations in urban-dwelling YLGs and Hg concentrations in AGs and YLGs which rely on fishery discards (Calado et al., 2021). Continuous monitoring during both breeding and non-breeding periods is essential, especially for AGs, which are classified as Vulnerable according to the IUCN Red List (BirdLife International, 2024). Finally, the limited associations observed between TEs concentrations and immuno-haematological parameters emphasises the need for more comprehensive investigations and larger sample sizes. Such studies could shed light on physiological and immunological responses to environmental contamination. This appears to be particularly relevant in light of the expanding presence of gulls in urban environments (Pais de Faria et al., 2021a), raising questions into the physiological adaptations of generalist animals inhabiting urbanised areas (García et al., 2023; Nos et al., 2024).

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Table 1. Trace elements (TEs) concentrations ($\mu\text{g g}^{-1}$ dw) measured in adults' blood, Se:Hg molar ratio, stable isotope values ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, ‰) and Bayesian estimation of standard ellipses (SEA_B ; 95% confidence intervals, CI), and immuno-haematological parameters calculated for each colony. Values are mean \pm SD and quantifiable sample sizes (N). Differences in TEs concentrations and stable isotopes were evaluated using one-way ANOVA or Kruskal-Wallis followed by post-hoc Tukey or pairwise tests are indicated by different letters ($\alpha < 0.05$). Cr and Sn were not tested for differences due to low detection frequency (< 10 adults in total).

	Cory's shearwater (CS)		Audouin's gull (AG)		Yellow-legged gull (YLG)					
					Porto		Berlenga		Deserta	
	N	Mean \pm SD	N	Mean \pm SD	N	Mean \pm SD	N	Mean \pm SD	N	Mean \pm SD
<i>Trace elements ($\mu\text{g g}^{-1}$ dw)</i>										
Al	15	6.60 \pm 3.25 ^a	15	7.62 \pm 3.34 ^{ab}	15	10.28 \pm 2.79 ^{bc}	15	11.36 \pm 4.11 ^c	15	9.12 \pm 2.50 ^{abc}
As	15	1.32 \pm 0.68 ^a	15	6.40 \pm 4.84 ^c	14	1.69 \pm 2.52 ^{ab}	15	7.48 \pm 2.25 ^c	15	5.27 \pm 5.69 ^{bc}
Cd	14	0.047 \pm 0.024 ^b	9	0.013 \pm 0.003 ^a	2	0.022 \pm 0.008 ^{ab}	12	0.029 \pm 0.016 ^{ab}	All $<$ 0.01	
Co	4	0.23 \pm 0.15 ^a	3	0.23 \pm 0.02 ^a	2	0.01 \pm 0.01 ^a	2	0.02 \pm 0.01 ^a	7	0.02 \pm 0.01 ^a
Cr	5	0.52 \pm 0.45	All $<$ 0.10		All $<$ 0.20		1	0.79	1	0.12
Cu	15	1.69 \pm 0.61 ^a	15	2.44 \pm 0.37 ^b	15	3.27 \pm 0.69 ^c	15	3.24 \pm 0.80 ^c	15	3.17 \pm 0.53 ^c
Fe	15	2264 \pm 76 ^b	15	2328 \pm 99 ^b	15	2097 \pm 213 ^a	15	2300 \pm 117 ^b	15	2048 \pm 149 ^a
Hg	15	2.08 \pm 0.52 ^b	15	6.58 \pm 2.71 ^c	15	1.08 \pm 0.75 ^a	15	2.69 \pm 1.12 ^b	15	5.63 \pm 2.73 ^c
Mn	15	0.15 \pm 0.11 ^a	15	0.10 \pm 0.04 ^a	15	0.15 \pm 0.06 ^a	15	0.11 \pm 0.03 ^a	15	0.11 \pm 0.03 ^a
Ni	6	0.34 \pm 0.25 ^a	2	0.08 \pm 0.05 ^a	1	0.09 ^a	3	0.42 \pm 0.59 ^a	3	0.20 \pm 0.10 ^a
Pb	9	0.03 \pm 0.02 ^a	8	0.03 \pm 0.02 ^a	15	0.83 \pm 0.82 ^b	13	0.04 \pm 0.04 ^a	15	0.07 \pm 0.06 ^a
Se	15	56.2 \pm 17.9 ^d	15	56.8 \pm 12.7 ^d	15	6.6 \pm 3.9 ^a	15	20.1 \pm 9.0 ^b	15	31.3 \pm 12.9 ^c
Sn	All $<$ 0.03		2	0.10 \pm 0.06	All $<$ 0.04		All $<$ 0.04		All $<$ 0.02	
Sr	5	0.08 \pm 0.03 ^a	7	0.09 \pm 0.05 ^a	6	0.11 \pm 0.04 ^a	13	0.15 \pm 0.10 ^a	12	0.11 \pm 0.05 ^a
Zn	15	23.5 \pm 4.5 ^a	15	25.2 \pm 3.1 ^a	15	25.5 \pm 3.0 ^a	15	30.2 \pm 8.4 ^a	15	25.0 \pm 2.2 ^a
Se:Hg	15	73.6 \pm 31.9 ^b	15	27.1 \pm 14.9 ^a	15	19.4 \pm 11.9 ^a	15	20.2 \pm 8.8 ^a	15	18.4 \pm 13.1 ^a
<i>Stable isotopes</i>										
$\delta^{13}\text{C}$ (‰)	13	-19.2 \pm 0.4 ^b	15	-19.4 \pm 0.4 ^b	15	-20.9 \pm 1.1 ^a	13	-19.4 \pm 0.5 ^b	15	-18.8 \pm 1.0 ^b
$\delta^{15}\text{N}$ (‰)	13	12.1 \pm 0.5 ^b	15	11.6 \pm 0.4 ^b	15	9.3 \pm 1.1 ^a	13	13.5 \pm 0.5 ^c	15	12.2 \pm 1.0 ^b
SEA_B [95% CI]	13	0.57 \pm 0.17 [0.27-0.90]	15	0.26 \pm 0.07 [0.14-0.40]	15	3.20 \pm 0.89 [1.71-4.92]	13	1.08 \pm 0.33 [0.53-1.68]	15	2.20 \pm 0.61 [1.16-3.38]
<i>Immuno-haematological parameters</i>										
ESR (Pi)	13	0.30 \pm 0.14	15	0.14 \pm 0.05	15	0.05 \pm 0.03	13	0.08 \pm 0.05	-	
Hb (g dL ⁻¹)	13	12.16 \pm 3.34	12	10.47 \pm 1.41	15	11.91 \pm 2.11	12	9.96 \pm 1.53	7	9.37 \pm 3.52
H/L ratio	13	1.27 \pm 0.49	14	1.02 \pm 0.72	13	1.17 \pm 0.47	13	1.76 \pm 0.71	15	1.61 \pm 0.75
WBC (counts)	13	79 \pm 10	14	61 \pm 15	13	71 \pm 12	13	69 \pm 14	15	65 \pm 13
IE (counts)	13	215 \pm 68	13	133 \pm 38	11	166 \pm 73	13	170 \pm 75	15	154 \pm 51
ENA (counts)	13	12.3 \pm 6.5	13	0.9 \pm 1.1	11	5.7 \pm 3.3	12	0.9 \pm 0.9	15	1.9 \pm 2.2

Table 2. Summary outputs including model specification, sample size, and Akaike's Information Criteria (AIC). This table includes all the six response variables tested showing both significant (in bold) and non-significant effects. Only up to the best ranked models and the null model are shown. The best model outputs are specified when only one model was selected, while model average (full average) outputs are specified ($\beta \pm SE$ [95% CI]) when two or more models were selected ($\Delta AICc < 2$). Abbreviations: LD1 and LD2, scores on axis 1 and 2 of a LDA of TEs (Al, As, Fe, Hg, Mn, Pb, and Se) and residual carbon isotopes (residual $\delta^{13}C$); k , number of parameters; AICc, Akaike's information criterion corrected for small sample sizes; $\Delta AICc$, difference between AICc of the specific model and the best model; w_i , AICc weights; Exp. Dev., explained deviance; AG, Audouin's gull; YLG, yellow-legged gull; BER, Berlenga Island; DES, Deserta Island; POR, Porto.

Immuno-haematological parameter	k	AICc	$\Delta AICc$	w_i	Exp. Dev.
Erythrocyte sedimentation rate (ESR) (N = 53)					
<i>GLM Gamma family ('log' link)</i>					
Colony + LD1 + LD2	6	-176.3	0	0.55	0.66
Null model	2	-128.9	47.42	0	0
	$\beta \pm SE$ [95% CI]	z value	p (> z)		
Intercept	-1.21 \pm 0.19 [-1.60 – -0.78]	-6.45	$p < 0.001$	***	
ColonyAG	-0.29 \pm 0.20 [-0.68 – 0.10]	-1.42	0.16		
ColonyYLG BER	-0.92 \pm 0.37 [-1.72 – -0.15]	2.50	0.02	*	
ColonyYLG POR	-2.93 \pm 0.40 [-3.81 – -2.06]	-7.33	$p < 0.001$	***	
LD1	-0.18 \pm 0.07 [-0.32 – -0.03]	-2.62	0.02	*	
LD2	0.23 \pm 0.07 [0.10 – 0.36]	3.53	$p < 0.001$	***	
Haemoglobin concentration (Hb) (N = 58)					
<i>Linear model (Gaussian family)</i>					
Colony	5	648.4	0	0.38	0.21
Colony + LD2	6	648.8	0.41	0.31	0.23
Null model	2	655.8	7.46	0.01	0
	$\beta \pm SE$ [95% CI]	z value	p (> z)		
Intercept	122.30 \pm 6.29 [109.98 – 134.61]	19.46	$p < 0.001$	***	
ColonyAG	-17.54 \pm 8.52 [-34.25 – -0.84]	2.06	0.04	*	
ColonyYLG BER	-28.03 \pm 12.83 [-53.18 – -2.88]	2.18	0.03	*	
ColonyYLG DES	-27.55 \pm 8.48 [-44.17 – -10.93]	3.25	0.001	**	
ColonyYLG POR	-0.36 \pm 9.12 [-18.23 – 17.52]	0.04	0.97		
LD2	-1.66 \pm 2.60 [-9.08 – 1.66]	0.64	0.52		
Heterophil/lymphocyte ratio (H/L) (N = 68)					
<i>GLM Gaussian ('log' link)</i>					
Colony ¹	5	95.8	0	0.38	0.20
Colony + LD1 ¹	6	97.2	1.87	0.19	0.21
Colony + LD2 ¹	6	97.2	1.38	0.19	0.21
Null model	2	102.3	6.51	0.01	0
White Blood Cells counts (WBC) (N = 68)					
<i>GLM negative binomial</i>					
Colony	5	572.4	0	0.51	0.20
Null model	2	578.5	6.17	0.02	0
	$\beta \pm SE$ [95% CI]	z value	p (> z)		
Intercept	4.38 \pm 0.05 [4.28 – 4.47]	89.95	$p < 0.001$	***	
ColonyAG	-0.27 \pm 0.07 [-0.40 – -0.13]	-3.93	$p < 0.001$	***	
ColonyYLG BER	-0.14 \pm 0.07 [-0.28 – 0.01]	-2.05	0.04	*	
ColonyYLG DES	-0.20 \pm 0.07 [-0.33 – -0.06]	2.90	0.004	**	
ColonyYLG POR	-0.12 \pm 0.07 [-0.25 – 0.02]	-1.73	0.08		
Immature erythrocytes (IE) (N = 61)					
<i>GLM negative binomial</i>					
Colony + LD1 + LD2 + Colony:LD1	11	704.5	0	0.25	0.41
Colony + LD1 + Colony:LD1	10	705.2	0.40	0.17	0.38
Colony + LD1 + LD2 + Colony:LD1 + Colony:LD2 + LD1:LD2	16	705.6	1.85	0.14	0.54
Null model	2	709.0	4.58	0.02	0

<i>Model average (full average)²</i>	$\beta \pm \text{SE [95\% CI]}$	<i>z value</i>	<i>p (> z)</i>		
Intercept	5.29 ± 0.10 [5.09 – 5.49]	51.79	<i>p</i> < 0.001	***	
ColonyAG	-0.05 ± 0.34 [-0.67 – 0.61]	0.15	0.88		
ColonyYLG BER	-0.23 ± 0.22 [-0.71 – 0.21]	1.01	0.31		
ColonyYLG DES	-0.47 ± 0.13 [-0.73 – 0.21]	3.56	<i>p</i> < 0.001	***	
ColonyYLG POR	-0.33 ± 0.31 [-0.93 – 0.28]	1.06	0.29		
ColonyAG:LD1	-0.13 ± 0.13 [-0.39 – 0.13]	0.98	0.33		
ColonyYLG BER:LD1	-0.10 ± 0.16 [-0.41 – 0.21]	0.62	0.53		
ColonyYLG DES:LD1	0.31 ± 0.09 [0.14 – 0.48]	3.60	<i>p</i> < 0.001	***	
ColonyYLG POR:LD1	0.01 ± 0.10 [-0.20 – 0.21]	0.07	0.95		
Erythrocyte nuclear abnormalities (ENAs) (N = 59)					
<i>GLM negative binomial</i>					
Colony	5	269.8	0	0.41	0.63
Colony + LD1	6	270.4	0.68	0.29	0.64
Null model	2	323.2	53.47	0	0
<i>Model average (full average)</i>	$\beta \pm \text{SE [95\% CI]}$	<i>z value</i>	<i>p (> z)</i>		
Intercept	2.29 ± 0.15 [2.00 – 2.57]	15.73	<i>p</i> < 0.001	***	
ColonyAG	-1.61 ± 0.24 [-2.08 – -1.13]	6.67	<i>p</i> < 0.001	***	
ColonyYLG BER	-1.70 ± 0.30 [-2.27 – -1.11]	5.72	<i>p</i> < 0.001	***	
ColonyYLG DES	-1.20 ± 0.20 [-1.60 – -0.80]	5.85	<i>p</i> < 0.001	***	
ColonyYLG POR	-0.52 ± 0.30 [-1.11 – 0.08]	1.71	0.09		
LD1	-0.03 ± 0.05 [-0.19 – 0.04]	0.60	0.55		

¹ there were no significant predictors in model averaging (full average).

² LD1, LD2, their interaction, and Colony:LD2 were not significant (*p* > 0.05).

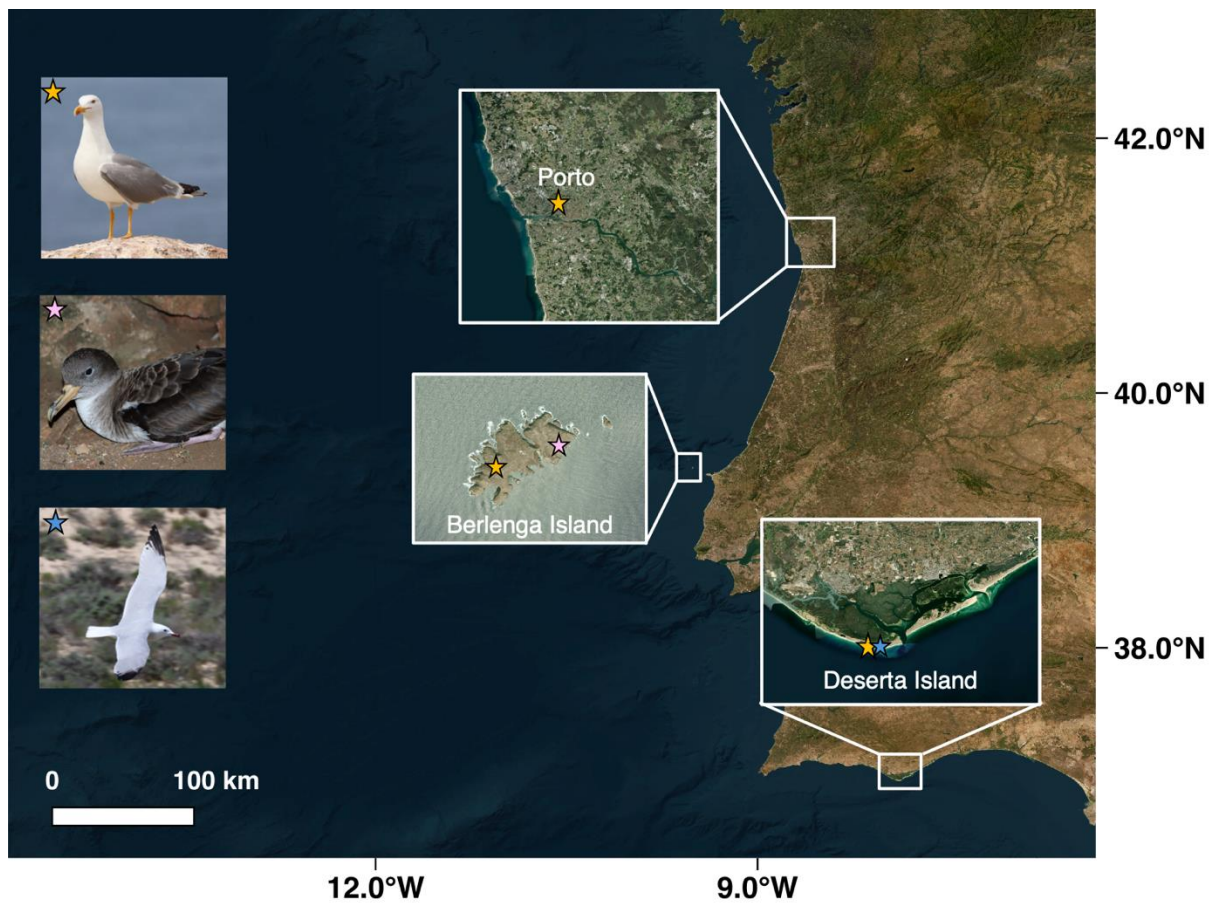


Fig. 1. Locations of the 5 breeding colonies (coloured stars) along the western and southern coasts of Portugal. Symbols: orange star, yellow-legged gull; pink star, Cory's shearwater; blue star, Audouin's gull. Photo credits M.I. Laranjeiro

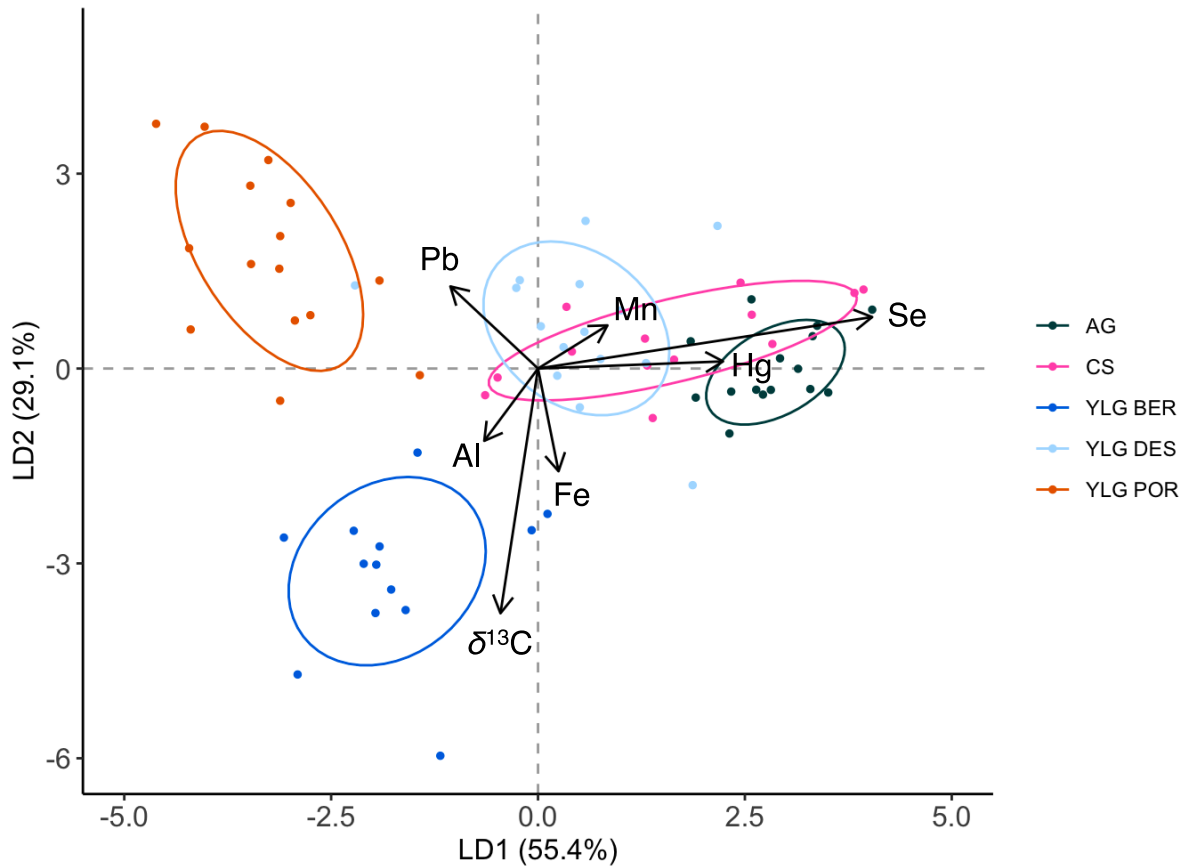


Fig. 2. Biplot of adult scores extracted by LDA on the two principal axes (LD1 and LD2) with log-transformed and scaled trace elements (TEs) and stable isotopes (residual $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) measured in adults' blood of gulls and shearwaters breeding along the western and southern coasts of Portugal. Ellipses include 70% of data. $\delta^{15}\text{N}$, Cu, and Zn were excluded due to multicollinearity issues ($\text{VIF} > 2$, $r_s > 0.50$) and As was removed from the plot for a clearer representation. Dashed lines indicate the axes where LD1 and LD2 are equal to zero. Abbreviations: AG, Audouin's gull; CS, Cory's shearwater; YLG: yellow-legged gull; BER, Berlenga Island; DES, Deserta Island; POR: Porto

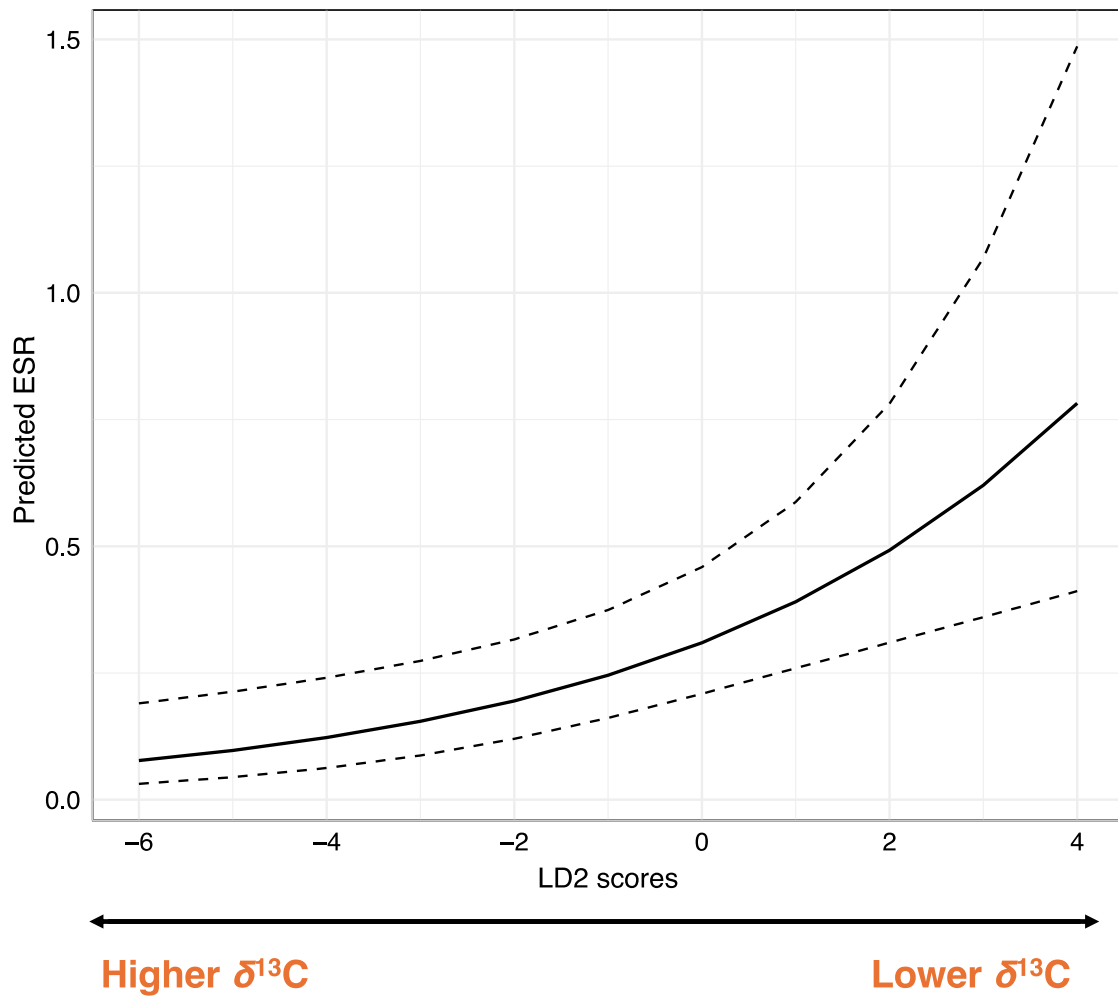


Fig. 3. Predicted erythrocyte sedimentation rate (ESR) increased with increasing LD2 scores ($\beta \pm \text{SE}$: slope 0.23 ± 0.07 , intercept -1.21 ± 0.19 , $N = 53$, $\Delta\text{AIC}_{\text{CNull model}} = 47.42$, see Table 2) expressive of a negative relationship with $\delta^{13}\text{C}$ values. Solid lines represent the predicted lines; dashed lines represent the 95% confidence intervals, CI

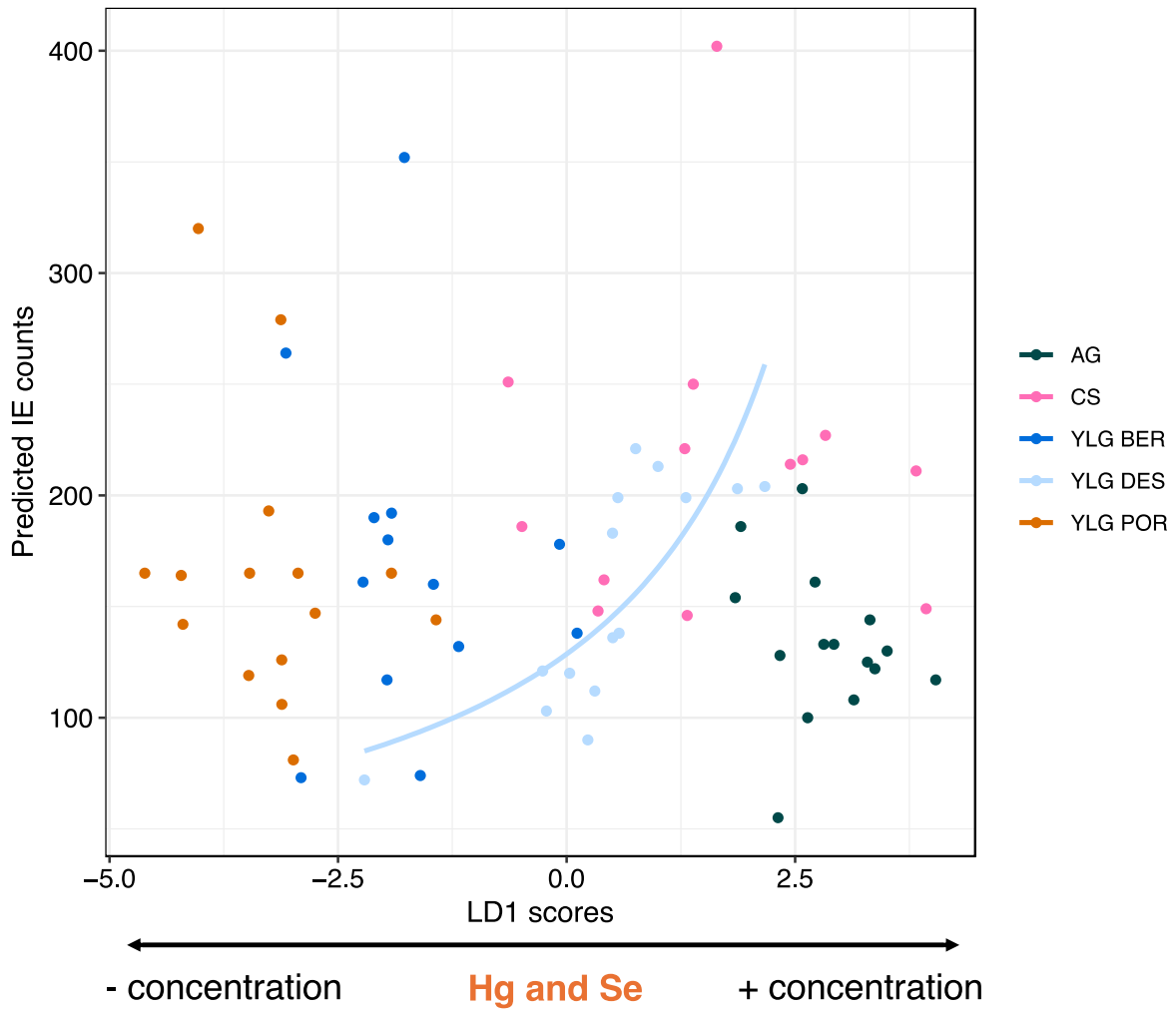


Fig 4. Predicted immature erythrocyte counts (IE) increased with increasing LD1 scores within the population of YLG breeding in Deserta (model average, $\beta \pm SE$: slope 0.31 ± 0.09 , intercept 5.29 ± 0.10 , $N = 71$, $\Delta AIC_{C_{Null\ model}} = 4.58$, see Table 2) expressive of a positive relationship with Hg and Se concentrations. Solid coloured points represent data from the model for each species breeding at each colony. Solid line represents the predicted line of the significant interaction.

Supplementary Material

Table S1. Immuno-haematological parameters measured in the current study. ESR, erythrocyte sedimentation rate; Hb, haemoglobin concentration; H/L, heterophil/lymphocyte ratio; WBC, IE, and ENA counts, number of white blood cell, immature erythrocytes, and erythrocyte nuclear abnormalities, respectively.

Immuno-haematological parameter	Definition and use	Reference
ESR	Rate at which red blood cells settle in a tube over a specific period of time. Non-specific measure of inflammation. When inflammation is present in the system, certain proteins, such as fibrinogen, increase in the bloodstream. These proteins cause red blood cells to clump together and fall more quickly than normal. Thus, an elevated ESR can be a sign of inflammation, infection, or other inflammatory conditions.	Heylen and Matthysen, 2008
Hb	Expressed as the mass of Hb <i>per</i> unit of blood. Indicator of blood oxygen-carrying capacity: high Hb concentrations improve aerobic capacity, low concentrations are linked with anaemia. Anaemias can be regenerative or non-regenerative. Regenerative anaemias are attributed to haemorrhage and haemolysis, caused by parasitism, injuries, and intoxication. Non-regenerative are attributed to chronic diseases, nutritional deficiencies, or starvation, since there is no response of red bone marrow to the need of erythrocytes. Thus, Hb concentration has been pointed out as a strong indicator of birds' physiological condition.	Minias 2015 and therein
H/L	Balance between two types of WBC: heterophils (involved in the immune response to bacterial infections) and lymphocytes (involved in the immune response to viral infections and other immune functions). Indicator of stress and overall health of an animal: under stressful conditions, such as environmental changes, parasite infection, among others, the balance between heterophils and lymphocytes can shift, leading to an increase in H/L ratio. Higher H/L ratios are often linked with stress, inflammation, or other challenges to the immune system.	Davis et al., 2008
WBC counts	Total number of WBC <i>per</i> 10,000 erythrocytes. WBC are a crucial component of the immune system and are involved in defending the organism against infections and foreign agents. High numbers of WBC (leucocytosis) may suggest infection, inflammation, stress, tissue damage, or other immune system responses, while low numbers (leukopenia) may suggest bone marrow problems, autoimmune disorders, or certain viral infections.	Thrall et al., 2012
IE counts	Total number of IE <i>per</i> 10,000 erythrocytes. Insights into the rate of erythropoiesis in response to factors such as anaemia, blood loss, or other conditions affecting erythrocyte turnover. Elevated count of IE may indicate increased erythropoiesis, which can be normal response to anaemia or environmental hypoxia, while a decreased IE count may suggest issues with erythropoiesis (red bone marrow disorders).	Campbell 2004
ENA counts	Total number of ENA <i>per</i> 10,000 erythrocytes. Abnormalities in the nuclei of erythrocytes can be indicative of diseases, nutritional deficiencies, or genotoxic damage. Such abnormalities may include changes in nuclear size, shape, or presence of multiple nuclei.	Pacheco and Santos, 1996

Table S2. Variation inflation factor (VIF) of trace elements and stable isotopes used for further computation of LDA. Bold values indicate variables which were excluded based on VIF value (VIF > 2).

Predictor	VIF
Al	1.243638
As	1.277711
Cu	6.092349
Fe	1.222651
Hg	1.299843
Mn	1.194276
Pb	1.473003
Se	1.582869
Zn	2.012410
Residual $\delta^{13}\text{C}$	1.177049
$\delta^{15}\text{N}$	3.677507

Table S3. Spearman's rank correlation between trace elements and stable isotopes. Bold values indicate correlations > 0.50.

	Al	As	Cu	Fe	Hg	Mn	Pb	Se	Zn	Residual $\delta^{13}\text{C}$
As	0.077									
Cu	0.815	0.198								
Fe	-0.117	0.179	-0.355							
Hg	-0.118	0.417	-0.019	0.142						
Mn	0.194	-0.231	0.186	-0.107	-0.430					
Pb	0.236	-0.307	0.438	-0.414	-0.491	0.545				
Se	-0.385	0.194	-0.507	0.341	0.513	-0.362	-0.647			
Zn	0.650	0.253	0.585	0.147	-0.036	0.246	0.150	-0.154		
Residual $\delta^{13}\text{C}$	0.005	0.370	0.024	0.190	0.132	-0.116	-0.295	0.164	0.323	
$\delta^{15}\text{N}$	0.060	0.387	0.088	0.086	0.429	-0.191	-0.340	0.144	0.115	0.623

Table S4. Blood immuno-haematological parameters on the two axes selected from the linear discriminant analysis (LDA) in adults of Audouin’s gull, Cory’s shearwater, and yellow-legged gull breeding in the West and South coast of Portugal. Abbreviations: Hb, haemoglobin concentration; H/L, heterophil/lymphocyte ratio; WBC, IE, and ENA counts, number of white blood cell, immature erythrocytes, and erythrocyte nuclear abnormalities, respectively.

Axis	Hb	H/L	WBC	IE	ENA
LD1	0.86	-1.90	2.94	0.83	7.02
LD2	-1.60	2.79	-0.99	1.28	1.02

Table S5. Model specification and significant predictors on explaining the variation of immuno-haematological parameters. Abbreviations: Group, species, and colony grouping factor (Audouin’s gull breeding in Deserta, Cory’s shearwater breeding in Berlenga, yellow-legged gull breeding in Berlenga, Deserta, or Porto); LD1 and LD2, scores on axis 1 and 2 of a LDA of trace elements and stable isotopes; ESR, erythrocyte sedimentation rate; Hb, haemoglobin concentration; H/L, heterophil/lymphocyte ratio; WBC, IE, and ENA counts, number of white blood cell, immature erythrocytes, and erythrocyte nuclear abnormalities, respectively.

Response	Initial predictors				N	Model specification	Significant predictors
ESR	Colony + LD1 + LD2 +	Colony :LD1 +	Colony :LD2 + LD1:LD2 +	Colony :LD1:LD2	53	GLM Gamma (link = ‘log’)	Colony, LD1, LD2
Hb	Colony + LD1 + LD2 +	Colony :LD1 +	Colony :LD2 + LD1:LD2 +	Colony :LD1:LD2	58	Linear model	Colony
H/L	Colony + LD1 + LD2 +	Colony :LD1 +	Colony :LD2 + LD1:LD2 +	Colony :LD1:LD2	68	GLM Gaussian (link = ‘log’)	None
WBC	Colony + LD1 + LD2 +	Colony :LD1 +	Colony :LD2 + LD1:LD2 +	Colony :LD1:LD2	68	GLM negative binomial	Colony
IE	Colony + LD1 + LD2 +	Colony :LD1 +	Colony :LD2 + LD1:LD2 +	Colony :LD1:LD2	61	GLM negative binomial	Colony, Colony:LD1
ENA	Colony + LD1 + LD2 +	Colony :LD1 +	Colony :LD2 + LD1:LD2 +	Colony :LD1:LD2	59	GLM negative binomial	Colony

Table S6. Blood cells trace elements and residual $\delta^{13}\text{C}$ loadings on the two axes selected from the LDA in adults of Audouin's gull, Cory's shearwater, and yellow-legged gull breeding in the West and South coast of Portugal. $\delta^{15}\text{N}$, Cu, and Zn were excluded due to multicollinearity issues ($\text{VIF} > 2$, $r_s > 0.50$).

Axis	Al	As	Fe	Hg	Mn	Pb	Se	Residual $\delta^{13}\text{C}$
LD1	-0.65	0.01	-0.25	2.24	0.84	-1.06	4.04	-0.45
LD2	-1.11	-0.70	-1.58	0.11	0.66	1.26	0.79	-3.77

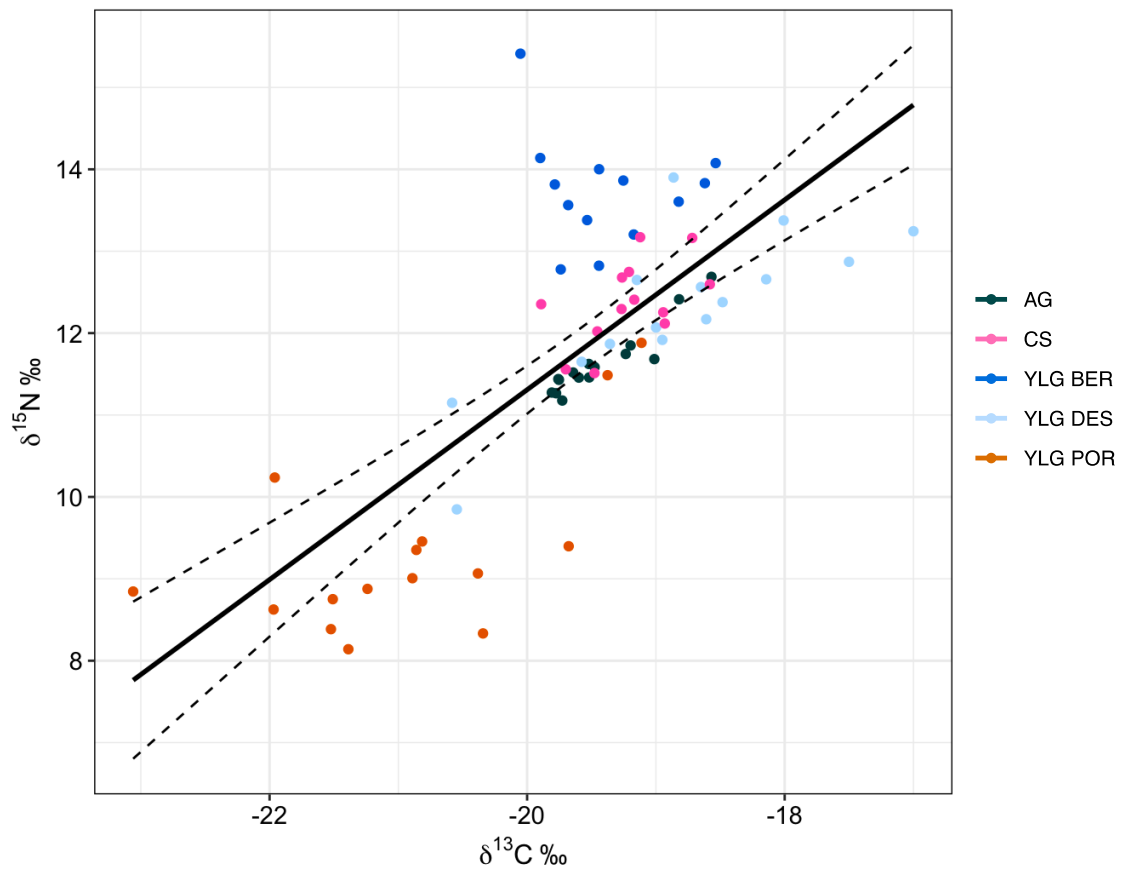


Fig. S1. Linear fit between blood carbon ($\delta^{13}\text{C}$) and predicted nitrogen ($\delta^{15}\text{N}$) values of adult shearwaters and gulls breeding along the West and South coast of Portugal. Solid coloured points represent data from the model for each species breeding at each colony. Solid black line represents the linear fit. Dashed lines represent the 95% confidence intervals (CI). Abbreviations: AG, Audouin's gull; CS, Cory's shearwater; YLG: yellow-legged gull; BER, Berlenga Island; DES, Deserta Island; POR: Porto.

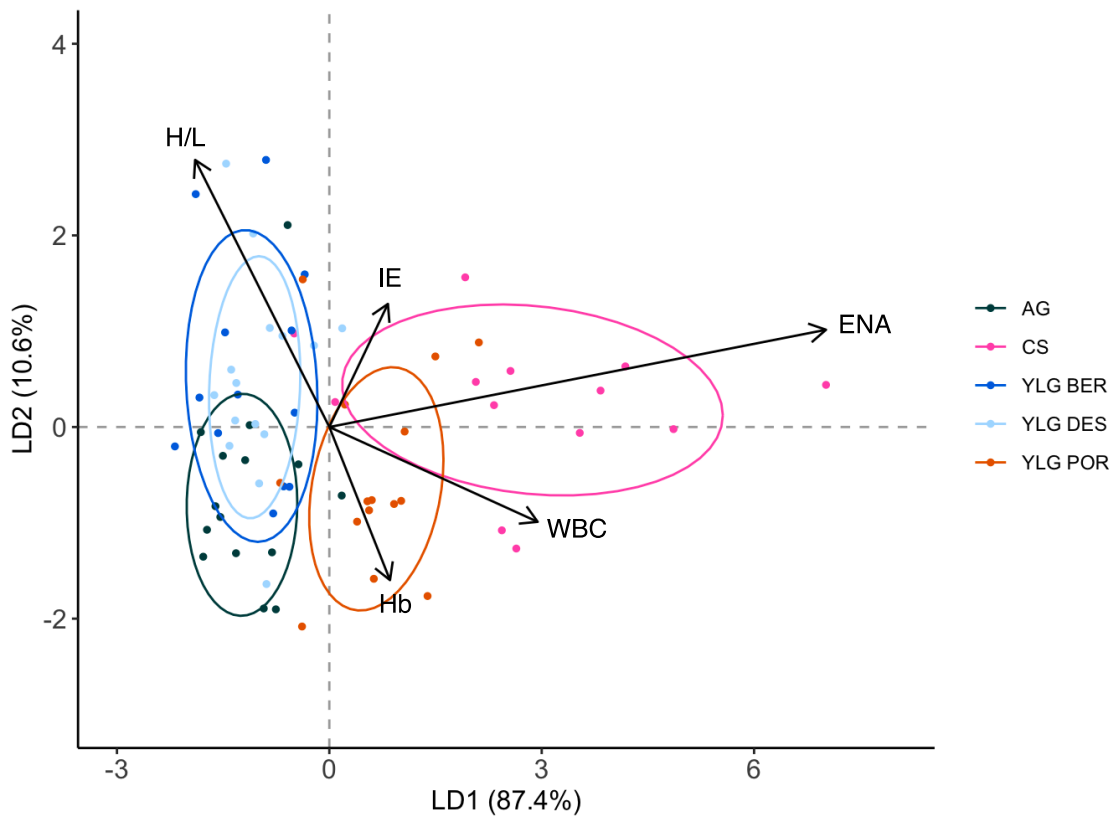


Fig. S2. Biplot of adult scores extracted by LDA on the two principal axes (LD1 and LD2) with normalised and scaled immuno-haematological parameters measured in adults' blood (see Table S2) of gulls and shearwaters breeding along the West and South coast of Portugal. Ellipses include 70% of data. Dashed lines indicate the axes where LD1 and LD2 are equal to zero. Abbreviations: AG, Audouin's gull; CS, Cory's shearwater; YLG: yellow-legged gull; BER, Berlenga Island; DES, Deserta Island; POR: Porto; Hb, haemoglobin concentration; H/L, heterophil/lymphocyte ratio; WBC, IE, and ENA counts, number of white blood cells, immature erythrocytes, and erythrocyte nuclear abnormalities, respectively.

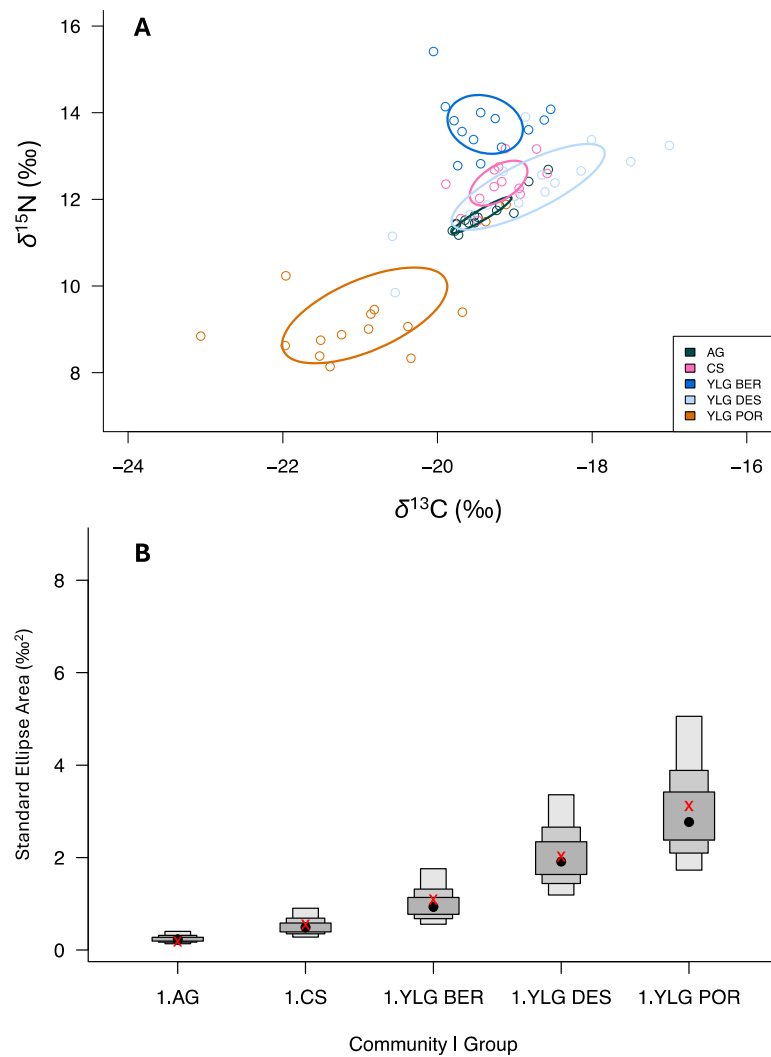


Fig. S3. A) Isotopic niche space computed using blood $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures of adult gulls and shearwaters breeding along the West and South coast of Portugal. Ellipses include 40% of the points. B) Standard ellipse area (SEA), Bayesian SEA (SEA_B) represented by the black point and the SEA corrected for small sample sizes (SEA_C) represented by the red cross. Abbreviations: AG, Audouin's gull; CS, Cory's shearwater; YLG: yellow-legged gull; BER, Berlenga Island; DES, Deserta Island; POR: Porto.

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