



Amino Acid $\delta^{15}\text{N}$ Can Detect Diet Effects on Pollution Risks for Yellow-Legged Gulls Overlooked by Trophic Position

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The use of top-consumers as bioindicators of the health of food webs is hampered by uncertainties in their effective use of resources. In this study, the abundance of stable nitrogen isotopes in amino acids from homogenised eggs of the Yellow-legged Gull (*Larus michahellis*) allowed to identify variations in trophic resource exploitation between geographically adjacent nesting colonies in the Ria de Vigo (NW Spain) that exhibited marked differences in pollutants. Eggs from nests in the Cíes Islands (located in a National Park) showed a large variability in stable carbon and nitrogen isotopes in bulk egg content encompassing that of eggs from Vigo city (a major fishing harbour). However, both colonies differed in the relative concentration and abundance of nitrogen isotopes of lysine, an essential amino acid present in marine prey, but also extensively used in feed stocks for poultry and swine. Notwithstanding the similarity in trophic position for both colonies, gulls from Cíes Islands may have acquired a substantial fraction of lysine from garbage dump sites, while those of the urban colony relied on fish discards. This unexpected conclusion is partly supported by the large variability reported for gull's diet in this region and calls for detailed estimations of diet when assessing the conservation status and pollution risks of marine ecosystems.

Keywords: bioindicator, stable isotopes, amino acids, seagull eggs, food web

INTRODUCTION

Top consumers from oceanic food webs are good indicators of the biogeochemistry and conservation status of marine ecosystems. They integrate processes operating at lower food web levels and thus represent the result of ecosystem functioning at large spatial and temporal scales (Hebert et al., 2016; Ruiz-Cooley et al., 2017). Estimations of their trophic position (*TP*) are key to determine ecosystem properties as food-chain length (Vander Zanden and Fetzer, 2007; Reum et al., 2015) or the biomagnification of pollutants (Storr-Hansen et al., 1995; Dietz et al., 2000; Kelly et al., 2008). For instance, changes in *TP* are indicative of alterations in food web structure (Jenkins and Davoren, 2020) or in nutrient inputs (Ruiz-Cooley et al., 2017). Particularly, seabirds have been extensively used as biomonitors of persistent organic and trace metal contaminants (e.g., OSPAR, 1999), with biomagnification effects critically dependent on their *TP* (Ramos et al., 2013;

Gatt et al., 2020). Methodological uncertainties, however, difficult the accurate determination of *TP* in these organisms.

Direct quantification of diets cannot be achieved for most field populations, as they may be inaccessible for long periods (e.g., Matias and Catry, 2010; Gatt et al., 2020) and the analysis of stomach contents, pellets or regurgitates cannot identify all the consumed prey (González-Solis, 2003; Abdennadher et al., 2010; Moreno et al., 2010; Calado et al., 2018; Méndez et al., 2020). Alternative determinations based on the progressive enrichment in heavy nitrogen isotopes (expressed as $\delta^{15}\text{N}$) through the food web allow for estimations of *TP* and diet composition integrated in space and time, but are highly dependent on factors as the appropriate selection of reference baselines (Post, 2002), the assumptions of the enrichment model (Hussey et al., 2014; Jennings and van der Molen, 2015) and the tissue analysed (Caut et al., 2009; Hebert et al., 2016). Nevertheless, these limitations have not impeded the extensive use of $\delta^{15}\text{N}$ in bulk tissues to infer *TP* and biomagnification of pollutants in many studies (e.g., Abdennadher et al., 2010; Ramos et al., 2013). The application of compound-specific isotopic analyses (e.g., $\delta^{15}\text{N}$ in amino acids) have reduced some of the uncertainties in *TP* estimations, because the changes in the nutrient sources at the base of the food web can be accounted for Chikaraishi et al. (2009). Even when there are still uncertainties in the variability of the isotopic enrichment along the food web (McMahon and McCarthy, 2016; Ohkouchi et al., 2017; Whiteman et al., 2019) the new estimations based on amino acids are being increasingly applied to marine birds (McMahon et al., 2015; Wu et al., 2018; Gatt et al., 2020).

Eggs of the Yellow-legged Gull (*Larus michahellis*) have been used to monitor organic and trace metal pollutants (Abdennadher et al., 2010; Ramos et al., 2013; Otero et al., 2018; Viñas et al., 2020). The large feeding plasticity reported for this species, however, difficult the identification of the sources of pollutants. For instance, gull colonies near urban areas have been shown to consume more terrestrial and anthropogenic resources than marine prey (Arizaga et al., 2013; Méndez et al., 2020; Zorrozuza et al., 2020). A previous study of two colonies only 10 km apart in the Ria de Vigo (NW Spain) revealed significant differences in the load of pollutants in their eggs, pointing to a rapid adaptation to local feeding resources (Viñas et al., 2020). The colony nesting on the Cíes Islands (located in a marine protected area) showed lower concentrations of pollutants and a wider trophic niche than the colony nesting in the industrialised Vigo city. Furthermore, the estimated diet composition in the region, including colonies in the Cíes Islands, showed marked year to year variations related to the availability of marine vs. anthropogenic resources, the latter including mainly beef, pork, and chicken remains (Moreno et al., 2010; Calado et al., 2020). These findings suggest that major changes in the diet of the Yellow-legged Gull may affect *TP* and therefore *TP* can be used as an indicator of changes in the trophic structure of the food web. Alternatively, pollution loads could be caused by the differential exploitation of trophic resources without a substantial change in *TP*.

The objective of this study is to determine the trophic position of Yellow-legged Gull in two colonies differing in the accumulation of pollutants (Viñas et al., 2020). We applied for

the first time estimations derived from the amino acid stable isotope composition of eggs, allowing to infer differences in trophic position and diet between the gulls in the two colonies. These estimations aimed to assist in the interpretation of the differential impact of contaminants in these colonies by revealing the sensitivity of *TP* estimations to diet variability.

MATERIALS AND METHODS

Sampling

Yellow-legged Gull eggs were collected from nests in the Cíes Islands (11 eggs) and in the city of Vigo (7 eggs) during the breeding season of 2011 (April to early August). First laid eggs in each nest were selected for analysis, weighed and measured (length and width) before opening (**Supplementary Table 1**). Embryonated eggs were discarded and the remaining were individually homogenised, stored frozen (-20°C), and freeze-dried before analysis. Further details on sampling were provided in Viñas et al. (2020).

Analysis

Two types of isotopic determinations were made. First, C:N ratios and natural abundances of carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) stable isotopes were determined in bulk egg samples using an isotope ratio mass spectrometer coupled to an elemental analyzer. Egg samples were analysed in triplicate with precision $< 0.05\%$ for both isotopes. Due to the high lipid content of egg samples, $\delta^{13}\text{C}$ values were corrected using the C:N ratio of each sample

TABLE 1 | Mean and sd trophic position values (TP) estimated for gull species using stable isotopes in this study and in the literature.

Species	Mean	SD	n	Zone	References
<i>Larus michahellis</i> ¹	3.96	0.67	18	Galicia (NE Atlantic)	This study
<i>Larus michahellis</i> ²	3.86	0.47	18	Galicia (NE Atlantic)	This study
<i>Larus michahellis</i> ³	3.70	0.42	60	Tunis (Mediterranean Sea)	Abdennadher et al., 2010
<i>Larus michahellis</i> ⁴	3.91	1.56	30	S Portugal (NE Atlantic)	Lopezosa et al., 2019
<i>Larus atlanticus</i> ³	3.20	0.32	11	Patagonia (SW Atlantic)	Forero et al., 2004
<i>Larus dominicanus</i> ³	3.80	0.14	229	Patagonia (SW Atlantic)	Forero et al., 2004
<i>Larus argentatus</i> ³	3.93	0.51	187	Great Lakes	Hebert et al., 1999
<i>Larus argentatus</i> ³	3.29	0.25	16	Baltic Sea	Sørmo et al., 2011
<i>Larus argentatus</i> ⁵	3.60	0.40	4	Lake Huron	Hebert et al., 2016

N, number of data. Details on the estimation models are given in the section "Materials and Methods."

¹ $\delta^{15}\text{N}_{\text{bulk}}$ in eggs, one baseline, SD = propagated error.

² $\delta^{15}\text{N}_{\text{AA}}$ in eggs, 2 TDF, SD = propagated error.

³ $\delta^{15}\text{N}_{\text{bulk}}$ in feathers, one baseline; SD = reported SD.

⁴Calculated from original $\delta^{15}\text{N}_{\text{bulk}}$ in feathers and diet data, 3 baselines (pelagic fish, demersal fish, terrestrial sources), SD = propagated error.

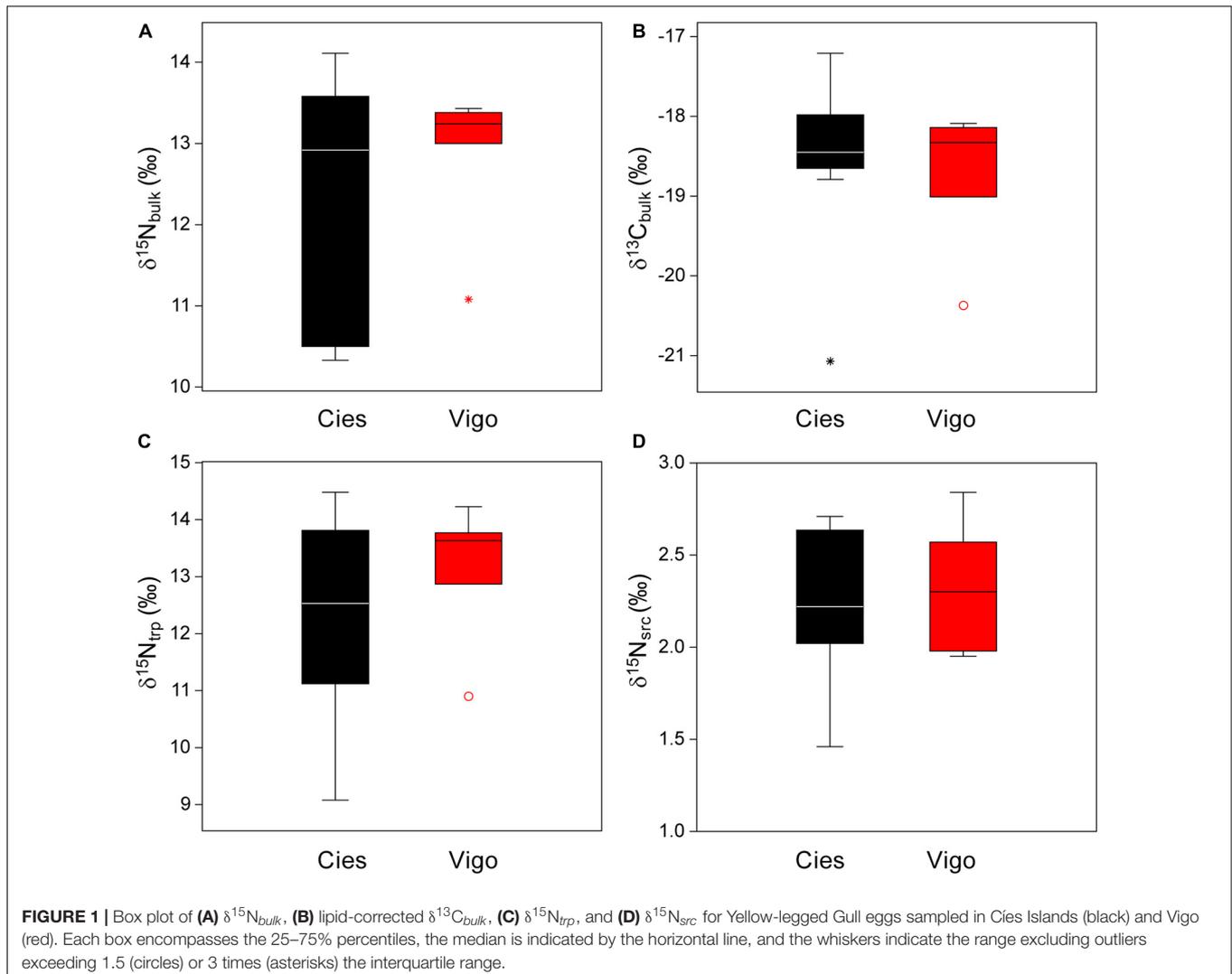
⁵ $\delta^{15}\text{N}_{\text{AA}}$ in muscle, 2 TDF (terrestrial, aquatic), SD = reported SD.

(Elliott and Elliott, 2016). These analyses were described in more detail in Viñas et al. (2020).

Second, 10 mg aliquots of the homogenised eggs were also used for determinations of $\delta^{15}\text{N}$ in individual amino acids (Chikaraishi et al., 2009). Samples were hydrolysed with 6N HCl, esterified with acetyl chloride:2-propanol, and treated with a mixture of 3:1 diclormethane:trifluoroacetic anhydride (McCarthy et al., 2013; Mompeán et al., 2016). Derivatised amino acids were purified by solvent extraction in 1:2 chloroform:phosphate buffer and centrifugation (Loick et al., 2019), evaporated at room temperature under N_2 , and stored at -20°C in 3:1 diclormethane:trifluoroacetic anhydride for up to 6 months until isotope analysis. The individual amino acids were separated in a chromatography column (TraceGOLD TG-5MS, 60 m, 0.32 mm ID, 1.0 μm film) using a gas chromatograph (Trace1310GC, Thermo Fisher Scientific), and subsequently injected into a mass spectrometer (DeltaV Advantage, Thermo Fisher Scientific) via a continuous flow interface and combustion module (GC Isolink, Thermo Fisher Scientific). Amino acid $\delta^{15}\text{N}$ values were

calibrated with the values obtained for isolated standards (Shoko Science) analysed by combustion as described for bulk analysis, and further corrected using an internal L-norleucine standard (SIGMA) of known isotopic composition added to each sample. The molar fraction of each amino acid (% molar) was also determined in the same analytical run by calibration of the spectrometric signals with amino acid standards (McCarthy et al., 2013). Precision ($\pm\text{SE}$) of triplicate samples (two injections per sample) was $<0.6\text{‰}$ per individual amino acid.

Values of $\delta^{15}\text{N}$ and %molar were obtained for trophic and source amino acids (McClelland and Montoya, 2002; McCarthy et al., 2013; McMahan and McCarthy, 2016). Trophic amino acids included alanine (Ala), leucine (Leu), isoleucine (Ile), proline (Pro), valine (Val), and the mixtures of glutamine (Gln) and glutamic acid (Glu), and of aspartamine (Asn) and aspartic acid (Asp). The latter mixtures were caused by the acid hydrolysis and were termed as Glx and Asx, respectively. Source amino acids included glycine (Gly), threonine (Thr), serine (Ser), methionine (Met), phenylalanine (Phe), and lysine



(Lys). The representation of these amino acids in the bulk protein was assessed by correlation of their molar-weighted average $\delta^{15}\text{N}$ ($\delta^{15}\text{N}_{THAA}$) with $\delta^{15}\text{N}$ values for bulk samples ($\delta^{15}\text{N}_{bulk}$). Similarly, the correlations between molar-weighted averages of trophic ($\delta^{15}\text{N}_{trp}$) or source amino acids ($\delta^{15}\text{N}_{src}$) with $\delta^{15}\text{N}_{bulk}$ were used to determine the relative importance of variations in trophic vs. nitrogen source factors for the interpretation of $\delta^{15}\text{N}_{bulk}$.

Diet Estimations

The potential contribution of marine and anthropogenic food sources to the diet of the gulls of each colony was estimated from the $\delta^{15}\text{N}$ signature of source amino acids. Reference values for marine sources were provided by sardines (*Sardina pilchardus*) and anchovies (*Engraulis encrasicolus*) collected in spring (April 2017 and April 2019) near the Ria de Vigo as part of a regular fish survey (see Bode et al., 2018). Anthropogenic sources were provided by samples of beef, pork and chicken meat obtained from local markets. Four individuals of each fish species and three replicates of each meat type were analysed as described for the gull eggs. The potential contribution of these sources to the gulls

diet were estimated using the Mix SIAR Bayesian mixing model (SIAR v4.1.3) of Stock and Semmens (2013) by using only the $\delta^{15}\text{N}$ values of individual amino acids that varied significantly between colonies and assuming no isotopic fractionation between sources and eggs. This simplified approach was intended only to highlight the potential implications of the marked differences in $\delta^{15}\text{N}$ among the sources.

Trophic Position

Estimations of the trophic position of Yellow-legged Gull individuals were made using the $\delta^{15}\text{N}$ values of representative trophic (Glx) and source (Phe) amino acids (TP_{AA}) and a model considering two trophic discrimination factors (McMahon and McCarthy, 2016):

$$TP_{AA} = 2 + \left(\frac{\delta^{15}\text{N}_{\text{Glx}} - \delta^{15}\text{N}_{\text{Phe}} - TDF_p - \beta}{TDF_b} \right) \quad (1)$$

Where, β is the difference between Glx and Phe in primary producers, and TDF_p and TDF_b are the trophic discrimination factors for plankton and marine birds, respectively. Mean (\pm SE) values were $3.4 \pm 0.9\text{‰}$ for β (Chikaraishi et al., 2009),

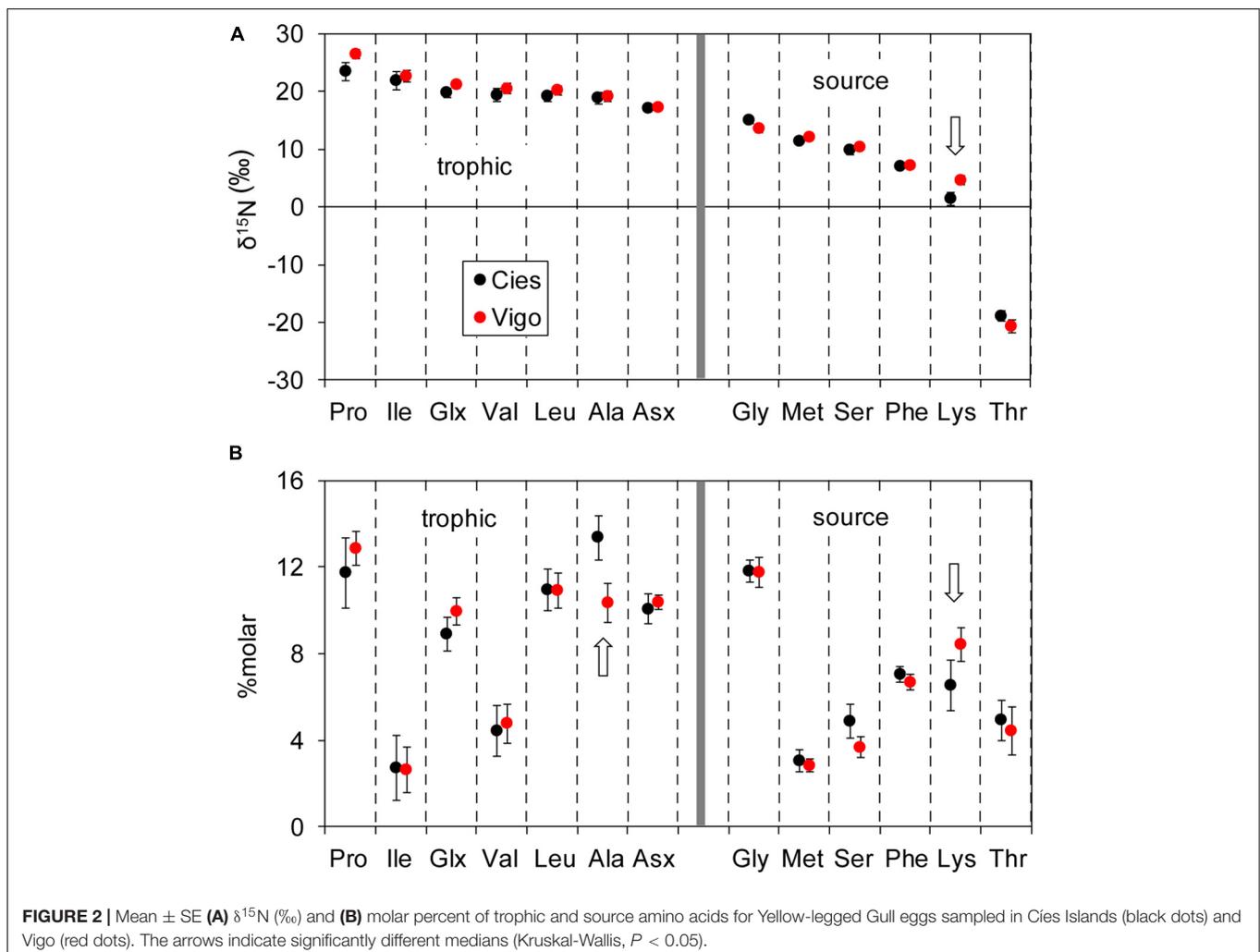


FIGURE 2 | Mean \pm SE (A) $\delta^{15}\text{N}$ (‰) and (B) molar percent of trophic and source amino acids for Yellow-legged Gull eggs sampled in Cies Islands (black dots) and Vigo (red dots). The arrows indicate significantly different medians (Kruskal-Wallis, $P < 0.05$).

$7.6 \pm 1.2\text{‰}$ for *TDFp* and $3.5 \pm 0.4\text{‰}$ for *TDFb* (McMahon et al., 2015; Wu et al., 2018; Gatt et al., 2020). As there was isotopic fractionation between the different tissues, increments of 2.5‰ and 0.9‰ were applied to the measured values of $\delta^{15}\text{N}_{\text{Glx}}$ and $\delta^{15}\text{N}_{\text{Phe}}$, respectively, to produce *TP* estimations equivalent to those obtained for the muscle of adult gulls (Hebert et al., 2016).

For comparative purposes, additional *TP* estimations were made using the measured $\delta^{15}\text{N}_{\text{bulk}}$ in eggs using the classical model (Post, 2002):

$$TP_{\text{bulk}} = 2 + \left(\frac{\delta^{15}\text{N}_{\text{bulk}} - \delta^{15}\text{N}_{\text{zoo}}}{TDF_z} \right)$$

where, $\delta^{15}\text{N}_{\text{zoo}}$ was the value for zooplankton samples collected in the nearby shelf during spring 2011 (mean \pm se = $5.97 \pm 0.92\text{‰}$, $n = 14$) as described in Bode et al. (2018), and $TDF_z = 3.4\text{‰}$ (Post, 2002). The error for all *TP* estimations was computed by error propagation by taking into account the analytical errors in $\delta^{15}\text{N}$ measurements in bulk or trophic and source amino acids, as well as the errors reported for *TDF* and β values employed (Post, 2002; Ohkouchi et al., 2017).

Further comparisons of *TP* were made with those reported in the literature for Yellow-legged Gull and similar species (Table 1). In the case of data reported in Lopezosa et al. (2019), *TP* was estimated from the reported $\delta^{15}\text{N}_{\text{bulk}}$ in body feathers and diet data using the model described in Hebert et al. (2016) by considering the consumption of pelagic fish, demersal fish, and garbage.

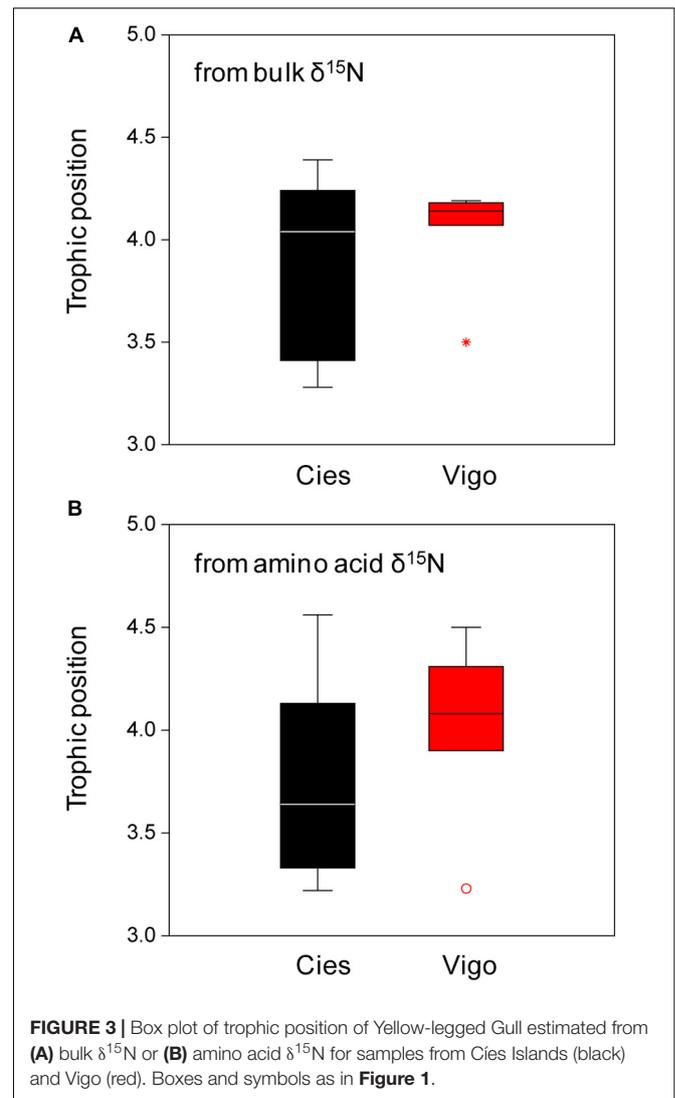
Statistics

Differences in isotopic measurements between eggs from Cies and Vigo colonies were analysed by means of non-parametric ANOVA (Kruskal-Wallis). Regressions between variables were computed using reduced major axis regression, as there were large differences in the measurement error of the variables. Statistical analyses were performed using SPSS 17.0 (SPSS Inc.) and Past 4.0 (Hammer et al., 2001).

RESULTS

There were no significant differences in median bulk $\delta^{15}\text{N}$ or $\delta^{13}\text{C}$ between both colonies, nor in averaged $\delta^{15}\text{N}$ values for trophic or source amino acids (Kruskal-Wallis test, $P > 0.05$, Figure 1). However, the weighted means of $\delta^{15}\text{N}$ for total amino acids ($\delta^{15}\text{N}_{\text{THAA}}$) and trophic amino acids ($\delta^{15}\text{N}_{\text{trp}}$) were linearly correlated with bulk $\delta^{15}\text{N}$ ($P < 0.001$, $n = 16$), while the means for source amino acids showed no correlation ($P > 0.05$, Supplementary Figure 1).

Values of $\delta^{15}\text{N}$ values for most amino acids were similar for the two colonies (Figure 2A), except in the case of lysine that showed lower values for the Vigo colony (Kruskal-Wallis test, $P < 0.05$). Mean \pm SD $\delta^{15}\text{N}$ values for lysine in eggs from the Cies Islands were $1.50 \pm 1.18\text{‰}$ ($n = 9$) while in those from Vigo were $4.68 \pm 0.77\text{‰}$ ($n = 7$). Even when adjusted for variations in the $\delta^{15}\text{N}$ of the canonical source amino acid phenylalanine, the differences in isotopic composition of Lys between colonies persisted, and were indicative of differences in the use of marine



resources (Supplementary Table 2). The eggs from the Cies Islands had also lower mean relative molar concentration of lysine and higher concentration of alanine than those from Vigo (Kruskal-Wallis tests, $P < 0.05$, Figure 2B).

As for gull eggs, there were also significant differences in Lys $\delta^{15}\text{N}$ among food sources (Mann-Whitney test, $P < 0.05$, Supplementary Figure 2A). Using these values, the estimations of diet suggested that, notwithstanding marine sources had a major contribution potential for both colonies, the gulls from Cies had a higher contribution of anthropogenic sources than those from Vigo (Supplementary Figure 2B).

The estimations of *TP* (Table 1) indicated that the studied Yellow-legged Gulls were almost tertiary consumers (i.e., *TP* ca. 3) and revealed no significant differences using either bulk or amino acid $\delta^{15}\text{N}$ (Kruskal-Wallis test, $P > 0.05$). Besides, there were no significant differences in *TP* between colonies (Figure 3, Kruskal-Wallis test, $P > 0.05$).

DISCUSSION

The results of this study represent the first estimation of Yellow-legged Gulls *TP* obtained with amino acid $\delta^{15}\text{N}$. These estimations are comparable to those obtained using bulk $\delta^{15}\text{N}$ in this and previous studies in the same and related gull species (Table 1), and in other marine birds (Gatt et al., 2020). Furthermore, the significant correlation between $\delta^{15}\text{N}$ of trophic amino acids and bulk $\delta^{15}\text{N}$ (and the lack of correlation for $\delta^{15}\text{N}$ in source amino acids) supports previous assumptions using bulk $\delta^{15}\text{N}$ as an index of *TP* (Abdennadher et al., 2010; Muñoz-Arnanz et al., 2012; Pedro et al., 2013; Calado et al., 2018). However, the determination of $\delta^{15}\text{N}$ in individual amino acids provided additional information on the variability of feeding among individuals that complements *TP* estimations. For instance, the similarity in *TP* between the studied colonies supports that the differences in pollution loads reported in Viñas et al. (2020) were caused by changes in diet unrelated to *TP*, even when detailed diet composition was not available for the individuals studied. Similar conclusions were reached in other studies where diet and *TP* could be determined (Muñoz-Arnanz et al., 2012; Ramos et al., 2013).

The low $\delta^{15}\text{N}$ in lysine found in eggs from the Cíes Island colony suggest the consumption of non-marine prey. First, because the mean difference between $\delta^{15}\text{N}$ of Lys and Phe for the Cíes eggs was significantly lower than values typical of phytoplankton (Nielsen et al., 2015). Second, because the estimated contributions of anthropogenic sources to the diet in Cíes were generally higher than those in Vigo (Supplementary Figure 2). This is an unexpected result, as the gulls from the islands would have, in principle, more access to marine prey than those nesting in the city. Lysine is an essential amino acid in the diet of animals and is one of the major additives to feed stocks, notably for poultry and swine raising (Toride, 2002). It is produced industrially through fermentation with the addition of sugars and inorganic nitrogen (Ikeda, 2017). Industrial lysine is likely to have low $\delta^{15}\text{N}$, since ammonium derived from atmospheric nitrogen represents the most economic source for fertilizers (Smil, 2001). For instance, the values of Lys $\delta^{15}\text{N}$ measured in beef, pork and chicken meat in this study were lower than those measured in fish. These results agree with those reported for bulk $\delta^{15}\text{N}$ from chick gull regurgitates in the Bay of Biscay (Arizaga et al., 2013). Therefore, a substantial consumption of rearing animals remains by gulls from the Cíes Island colony cannot be discarded, as noted by previous studies (Moreno et al., 2010). Given the large variability reported in the diet of the Yellow-legged Gull, it would be not surprising that the Cíes Islands colony partly relied on garbage, at least during the time frame considered in this study. This is supported by the year-to-year reduction in the consumption of fish by this species along the NW Spanish coast and attributed to the decline in sardine populations (Calado et al., 2020). Furthermore, the reported mean composition of the gull diet in this region indicated the dominance of anthropogenic over marine food items during 2010, an exceptional contribution compared to the series for the whole recording period (Supplementary

Table 3). In this regard, it must be noted that the isotopic composition of eggs is considered representative of both recent (ca. 1 month) and past (ca. 1 year) diet of the breeding adult females (Hebert et al., 2016). The high consumption of marine resources in Vigo agrees with the general pattern reported for the region and could be favoured by the proximity of the fishing harbour, one of the largest in Europe with 84,000 metric tons of fresh marine products landed in 2011 (Port Authority of Vigo, 2012). Fish offal would be a favoured resource for gulls nesting in Vigo, as reported for similar species elsewhere (Hebert et al., 1999; González-Solís, 2003; Calado et al., 2018).

Differences in diet, thus, were the likely cause of the high levels of pollutants in eggs from the Vigo colony (Viñas et al., 2020). Marine fishes of high *TP* were reported to bioaccumulate organic and inorganic pollutants. For instance, Hg compounds in tuna (e.g., Méndez et al., 2001) can reach concentrations equivalent to those found in the gull eggs from Vigo (Viñas et al., 2020). These results imply that biomagnification does not only depend on *TP* but also on the diet of the consumer species. Therefore, future assessments of pollution risks in food webs would require coupled studies of diet and *TP*. Gull diet can be reconstructed from observations of feeding remains or stomach contents, but also from instrumental techniques as the analysis of marker fatty acids (Hebert et al., 2016). The latter can be very sensitive when coupled to stable isotope determinations (Twining et al., 2020). The application of these techniques will help to determine the appropriate biomagnification factors when the monitored species, as the Yellow-legged Gull, feeds on different habitats or food webs.

Conclusion

Our study shows that reliable *TP* estimations for the yellow-legged gull can be obtained using $\delta^{15}\text{N}$ either in bulk homogenised eggs or in individual source and trophic amino acids. However, the latter technique would be preferred because data or assumptions about the baseline reference values from other species are not required, and because it can provide valuable information on the variability in diet among individuals. Indeed, diet, rather than *TP*, appears as the dominant factor affecting the bioaccumulation of pollutants in the Yellow-legged Gull.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because gull eggs for this study were collected as part of the

pollution monitoring program of the IEO and the Ministerio para la Transición Ecológica (Spain).

AUTHOR CONTRIBUTIONS

LV and VB designed the project. AB designed the research reported here, analysed the data, and wrote the manuscript with comments from coauthors. LV, BP-F, and AB analysed the samples. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2021.657131/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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