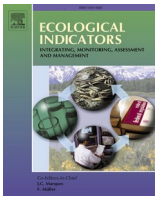
Ecological Indicators 136 (2022) 108649

Contents lists available at ScienceDirect

Ecological Indicators

journal homepage: www.elsevier.com/locate/ecolind



A multi-isotope approach to evaluate the potential of great cormorant eggs for contaminant monitoring

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ARTICLE INFO

Keywords:
Sweden
Hg
Amino acid
Trophic position

ABSTRACT

Contaminant monitoring in biota is important for determining environmental status and to detect or prioritize action on hazardous substances. Predators higher up a food chain are often used for monitoring of contaminants that bioaccumulate. However, it is not always possible to find higher predators that are both abundant and have a wide distribution for national or international contaminant monitoring. Great cormorants (*Phalocrocorax carbo*) are a widespread and increasingly common top predator of fish in fresh, brackish and salt water. We evaluate the suitability of great cormorant eggs as a matrix for contaminant monitoring by using stable isotopes of carbon, nitrogen and sulfur. Despite the fact that cormorants are migratory, egg isotope values showed a significant separation between five breeding colonies in Sweden (1 fresh water lake, 3 Baltic sites and 1 marine site). This high degree of separation indicates that eggs are primarily produced using local resources (not stored body resources) and that contaminants (mercury concentrations in this study) measured in eggs likely reflect levels in fish prey caught close to the breeding area. Compound specific stable isotope analysis was used to estimate cormorant trophic position (TP) and concentrations of mercury in eggs were positively related to TP. The results show that a multi-isotope approach, combined with good ecological diet knowledge allow for meaningful and comparative interpretation of mercury concentrations in biota and that great cormorant eggs appear a suitable matrix to measure locally derived and maternally transferred contaminants.

1. Introduction

Environmental contaminant monitoring programs are crucial to understand the movement, fate and effect of contaminants in biota. A number of contaminant monitoring programs were initiated in the 1960s and 70s, primarily in north America and Europe, to monitor the concentrations and effect of pesticides and their metabolites in biota (Rattner et al., 2011). Target organisms used for monitoring depend on the properties of the contaminant of interest, but generally higher predators are most suitable because many organic pollutants and metals, particularly mercury (Hg), bind to tissues and biomagnify higher up the food chain (Rasmussen et al., 1990). Contaminants at higher concentrations are easier to detect and measure, and more likely to cause biological or ecological effects (Borgå et al., 2012). However, higher level predators are often rare, iconic and patchy in their distribution or have a life history which can limit their use in monitoring programs. This makes it difficult to find, or justify, sampling of a common top predator across diverse habitats or larger geographic areas.

Cormorants are fish eating birds with a worldwide distribution. Of these, the most widely occurring is the great cormorant (*Phalocrocorax carbo*) which is found on all continents except for South America and Antarctica. Two subspecies of great cormorant occur in Europe, *P. carbo carbo* and *P. carbo sinensis*. Due to a combination of hunting (19th century) and environmental contaminants (20th century) the number of cormorants in Europe was low (Herrmann et al., 2019), but in recent decades the number of *P.c. sinensis* has increased substantially; from

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https://doi.org/10.1016/j.ecolind.2022.108649
Received 28 May 2021; Received in revised form 13 January 2022; Accepted 1 February 2022
Available online 7 February 2022

~3500 pairs in the 1960s to ~220 ,000 pairs in 2006 (https://ec.europa.eu/environment/nature/cormorants/numbers-and-distribution.htm accessed 20220111). In Sweden the number of breeding pairs has increased from ~100 in the late 1950s to over 40,000 (Herrmann et al., 2019), and they are now found along the coast throughout the country and on many freshwater lakes (Engström and Wirdheim, 2014). *P.c. sinensis* are generalist piscivores and consume up to 500 g of fish daily (Ovegård, 2017). When nesting they generally feed locally - within a 20 – 30 km radius of the colony (Gremillet, 1997). Their position as a top predator of fish, local feeding habits, abundance and distribution would make it a suitable species for contaminant monitoring. However, like many northerly bird species, *P. carbo sinensis* typically migrates between a summer breeding site and a southerly overwintering area (Fransson et al., 2008). The accumulation of contaminants over large geographical areas would make these migratory birds unsuitable for environmental monitoring, because it would be difficult to assign contaminants to a source or compare contaminant concentrations between areas. This might be overcome by using eggs for contaminant analysis, particularly if collection procedures are documented and standardized (Klein et al., 2012). The impact of collecting eggs on wild populations is potentially low because it is not necessary to kill adult birds and sampling one egg per clutch reduces breeding success only marginally (where multiple eggs are laid). Eggs are relatively easy to sample, process and store and they have a consistent composition and high lipid content (Becker, 1989, Klein et al., 2012). However, for migratory species the amount of local contaminants depend on the relative amounts of endogenous vs exogenous nutrients a female invests in her eggs. Two breeding strategies can be used by birds, making their eggs more or less suitable for local contaminant monitoring. Stephens et al. (2009) summarize the origin and use of the terms income and capital breeding. Briefly, capital breeders use stored energy to finance reproduction whereas income breeders use energy directly from ingested food. Eggs from migratory birds can indicate local contaminants if the females are income breeders (Hobson et al., 1997, Bond and Diamond 2010). However, there appears to be a degree of plasticity in resource allocation for reproduction and individuals usually occur somewhere on a capital – income breeder continuum (Williams et al., 2017). For migrating female birds there is a tradeoff between the costs and benefits of carrying extra nutrient stores to breeding grounds (capital breeding). Delayed laying and the use of external nutrients might be necessary – particularly for females with fewer reserves (Drent et al., 2006). Smaller, long distance migrators rely more heavily on an income strategy (Klaassen et al., 2001). Species of larger migratory birds were thought to be able to allocate more internal resources to breeding (Klaassen 2003), but research has shown a mixed strategy and that locally derived nutrients can be important (Gauthier et al., 2003, Klaassen et al., 2006). The amount of external energy a female invests in eggs depends on her relative weight and reproductive timing (i.e. heavy females use internal resources early in the breeding season) resulting in within population differences in breeding strategy (Jaatinen et al., 2016). In summary, the amount of external:internal nutrients allocated depend on the individual, migratory distance and resource availability at the breeding site.

Stable isotopes are commonly used to determine breeding strategy in birds (Hobson, 2006). Chemical elements have naturally occurring and stable light and heavy forms (isotopes), which differ in the number of protons in the nucleus of an atom. This small difference does not markedly effect an elements structure or function but results in distinctive ratios of light to heavy forms that can be used in ecological studies (Fry, 2006). For example, it is common to measure the ratio of heavy to light forms of hydrogen, oxygen, sulfur and especially carbon and nitrogen to understand food web dynamics. Heavy nitrogen (^{15}N) accumulates with increasing trophic level (i.e. the light form, ^{14}N, is metabolized more rapidly than the heavy form), known as fractionation, but the ratio of heavy to light carbon (^{13}C:^{12}C) and sulfur (^{34}S:^{32}S) is relatively stable and predictable throughout a food web. Stable isotopes are expressed in δ notations and as ‰ deviations from standards. Hence, δ^{15}N isotopes can be used to calculate an individual's position in a food web (Post, 2002), and δ^{13}C and δ^{34}S isotopes as "natural dyes" to trace the source of food. Differences in photosynthetic pathways and inorganic carbon substrates mean that primary producers have different δ^{13}C (DeNiro and Epstein, 1978), which are assimilated into the food web and can be used to determine predator prey dynamics. Differences in δ^{13}C also occur between marine and terrestrial habitats, most likely due to the higher amount of δ^{13}C in oceanic dissolved inorganic carbon (Boutton, 1991). Similarly, there are differences in δ^{34}S between marine and terrestrial environments but also between benthic and pelagic food sources (due to sulfur oxidizing and reducing bacteria found in sediments; Michener and Lajtha (2008)) which has proved useful in disentangling food sources in wading birds (Morkūnė et al., 2016).

Cormorants have low winter site fidelity (Frederiksen et al., 2002). Winter populations consist of mixtures of birds from different breeding origins – with mixtures variable over time (Frederiksen et al., 2018) (presumably because of low site fidelity). Even within winter sites, high mobility has been demonstrated and δ^{13}C and δ^{15}N for marine and freshwater roosting cormorants show a high degree of overlap, with marine roosting cormorants apparently moving to freshwater bodies to feed (Farinós-Celdrán et al., 2019). Feeding can be widespread in winter, but is localized and limited to ~30 km round the roosting site when breeding. If cormorants are income breeders the isotope signal of their eggs should mirror local fish prey with greater differences between than within colonies. In contrast, if they are capital breeders differences in egg isotopes between colonies should be small reflecting winter behavior.

An aspect important to the interpretation of biomagnifying contaminants is trophic position (TP) commonly assessed from δ^{15}N. Traditionally TP has been estimated by comparing bulk nitrogen isotope of predators with bulk isotopes in prey (Post, 2002). However, compound specific isotope analyses (CSIA) where δ^{15}N in source and trophic amino acids are analyzed in the same sample has become increasingly popular (Chikaraishi et al., 2009) for interpreting contaminants in predators (Dolgova et al., 2018, Thébault et al., 2021). Apart from providing more reliable TP estimates in food webs, which is essential for comparing contaminant accumulation between sites (Dolgova et al., 2018) and over time (Hebert and Popp, 2018), the CSIA approach enables one to separate the diet aspect from the ultimate N sources which can vary due to for e.g. eutrophication status causing elevated bulk δ^{15}N values.

This study had 2 primary aims: i) evaluate the suitability of great cormorant eggs as a matrix for measuring locally derived contaminants by determining breeding strategy and ii) use compound specific isotope analysis to calculate δ^{15}N at the base of a food web, consumer TP and interpret contaminant concentrations.

Cormorant eggs were collected from geographically separate colonies from fresh, brackish and marine locations throughout Sweden and the bulk δ^{13}C, δ^{15}N and δ^{34}S as well as δ^{15}N in specific amino acids were measured. It was expected that this multi-isotope approach would reveal that great cormorants are largely income breeders by showing complete site separation in multivariate isotope-space meaning that contaminants found in eggs should be derived from prey close to the breeding colony. We measured and compared mercury (Hg) concentrations, a contaminant of concern due to its toxicity and biomagnification potential. Furthermore, it was expected that individuals (eggs) with higher TP, assessed from δ^{15}N in amino acids, would have higher concentrations of Hg reflecting their position in the food chain (e.g. Thébault et al., 2021). Compound specific isotope analysis should give an accurate estimate of TP and was hypothesized to improve comparisons of Hg loading between sites and among individuals within a site, reflecting individual differences in diet (e.g. piscivorous fish prey vs planktivorous fish prey).

2. Methods

2.1. Egg collection, processing and storage

Eggs ($n = 10$) from five cormorant breeding colonies (total $n = 50$) were collected from four locations around the Swedish coast and from one freshwater lake (Roxen) (Fig. 1). The coastal locations show a salt gradient from site 1 (~20 ppm) on the Swedish west coast to site 4 (~3 ppm) in the northern Baltic Sea. Sites 2 and 3 were in the southern and mid Baltic respectively. Breeding times vary according to latitude and egg collection occurred from 28th March (site 2, southern Baltic) until the 6th June (site 4, northern Baltic) 2017. One egg per nest was collected early in the breeding season (i.e. within 10 days of egg laying). Eggs were checked for embryo development by candling (Fig. 2) to ensure they were freshly laid. The eggs were kept refrigerated (~5 °C) and transported to the Swedish Museum of Natural History (SMNH) for processing. Egg collection was approved by the Swedish Environmental Protection Agency (Naturvårdsverket) and carried out in strict accordance with the terms included in the permit NV-03970–16.

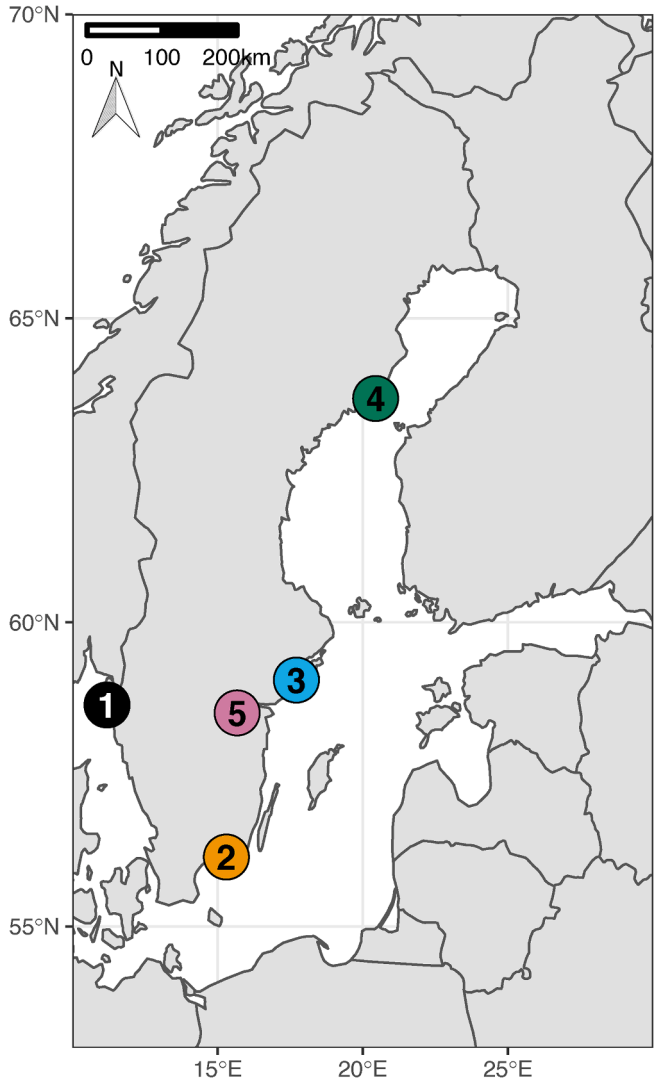

Fig. 1. Map showing the location of 5 sites where cormorant eggs were collected. Site 1 (black) was from the west coast of Sweden (Bohuslän; marine), sites 2–4 (yellow, blue and green) from the brackish Baltic Sea (2 = Blekinge, 3 = Södermanland, 4 = Västerbotten) and site 5 (pink) from a freshwater lake (Roxen). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Candling to check for embryo development. No sign of embryo development confirms that the egg was sampled within a few days of being laid.

In the laboratory eggs were weighed (g) and length and breadth (mm) measurements taken. A small hole was drilled into the equator of each egg and the contents blown into a sterile pyrex jar. The contents of each egg were homogenized using a mixer (IKA Ultraturrax T 25 with a stainless steel shaft dispersion tool), placed into sterile glass jars and frozen at −25C.

2.2. Stable isotope analysis

Fifty eggs were analyzed for bulk $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$; 25 at the Department of Geological Sciences, Stockholm University (5 per site) and 25 at the Center for Physical Science and Technology (CPST), Vilnius (the other 5 eggs per site). A small sample of homogenate (~1–2 g) from each egg were freeze dried overnight and stored in a desiccator. For analysis of bulk $\delta^{13}C$ and $\delta^{15}N$, 0.5 – 1.0 mg of dry material from each egg was weighed in tin capsules. Analyses were carried out using an elemental analyzer interfaced to a continuous flow isotope ratio mass spectrometer. The results are given in per mill (‰) units as $\delta^{13}C$ vs. Vienna Pee Dee Belemnite and $\delta^{15}N$ vs. air. For analysis of bulk $\delta^{34}S$, ~2.5 mg of dry egg material was weighed into tin capsules with ~ 2.0 mg V_2O_5. The tin capsules were combusted with an elemental analyzer connected to a continuous flow isotope ratio mass spectrometer. $\delta^{34}S$ are given in per mill (‰) units vs Vienna Canyon Diablo Troilite. Standards used for calibration of the reference gases during the measurement period of the samples in this study were for CO_2, IAEA-CO-1 and an in house acetanilide standard. For N_2, IAEA-N-1 and IAEA-NO-3 were used. Calibration of the reference gas for SO_2 was NBS127 and an in-house standard, genuine CDT ($\delta^{34}S = 0$ ‰, measured to + 0.07 ± 0.26 ‰) and SSS-2 (SU). At CPTS, IAEA-S-1 ($\delta^{34}S = -0.30$ ‰VCDT) and IAEA-S-2 ($\delta^{34}S = +22.62$ ‰VCDT) were used. Precision was better than ± 0.2‰ for $\delta^{15}N$ and $\delta^{13}C$ isotope analyses and ± 0.5‰ for $\delta^{34}S$ at both labs.

Amino-acids from a subset of the same egg homogenates were extracted and analyzed for N isotopes at the stable isotope facility UC Davis, California. $\delta^{15}N$ analysis of amino acids were carried out on 24 of the 50 eggs analyzed for bulk isotopes ($n = 5$ per site except for site 4 where only 4 eggs were analyzed). 8 – 10 mg of freeze dried homogenate

per egg were weighed into glass vials. The samples were sent for analysis where they were first hydrolyzed (using HCl) to breakdown the protein structure, and derivatized. Analyses of the derivatives were carried out using a gas chromatograph coupled to an isotope ratio mass spectrometer. Samples were analyzed in duplicate and the mean of 2 readings per amino acid were used in further analyses. Precision of the duplicates and the reference samples in the lab was ± 0.45‰.

2.3. Mercury analysis

The eggs ($n = 24$) sent for CSIA analysis (and bulk isotopes) were also analyzed for mercury concentrations at Stockholm University, Dept Ecology, Environment and Plant Science. Samples of freeze dried homogenate were first digested in a mix of concentrated $HClO_4$:HNO_3 (3:7; v:v) at 180 °C for 19 h (TECATOR Digester System 40 with 1012 Autostep Controller), together with samples of Reference Material BCR No. 60 from the European Community. Analysis was carried out using a cold AAS-Vapor generator (Equipment; Agilent 240 AA + GTA-120; Varian SpectrAA 55b + VGA-77). All samples were analyzed with standard addition using standards from BDH (Spectrosol Mercury Standard).

2.4. Statistical analysis

Two eggs from the marine site (site 1) had sulfur isotopes indicating a diet strongly influenced by freshwater fish ($\delta^{34}S$: 6.6 and 7.5, all other individuals from site 1 between 11.5 and 17.3). These two eggs were not included in further analyses. Because of the high fat content in eggs, $\delta^{13}C$ values (depleted in lipids) were adjusted according to Elliott et al. (2014). We carried out a multivariate analysis of variance (MANOVA) to test if significant differences in C, N and S isotopes in eggs occurred between sites. Univariate analysis of variance (ANOVA) and Tukey's post-hoc tests were used to see if and where significant differences occurred between sites for egg C, N and S isotopes. Following this, linear discriminant analysis (LDA) was used to find combinations of 2 (C and N) and 3 (C, N and S) isotopes that best separated the 5 locations and calculate the amount of separation assigned to each isotope (i.e. how much carbon, nitrogen and sulfur contributes to separating sites). The data was randomly split 80:20 into training and test sets. LDA on the training sets established the degree of separation and was then used to predict site of origin on the test data. To visualize and further analyze the data we calculated and plotted site standard ellipse areas, shape, centroid locations and degree of overlap, based on C and N isotopes (Jackson et al., 2011), and site standard ellipsoid volumes, shape, centroid location and degree of overlap (Rossman et al., 2016) for C, N and S isotopes.

ANOVA and Tukey's post-hoc tests were used to see if and where significant differences occurred between sites for egg phenylalanine (a source amino acid indicative of baseline N differences) and trophic position estimates. The magnitude of difference in Hg concentrations between sites were quantified using Cohen's d (Cohen 2013). Trophic position (TP) was calculated using $\delta^{15}N$ values in amino acids (glutamic acid and phenylalanine) measured in eggs according to Chikaraishi et al. (2009), but with a trophic discriminant factor of 5.4 (Hebert et al., 2016). The relationship between egg log Hg concentrations, site and TP was tested using linear models (LM). All analyses and plots were carried out in R version 3.6.1 (R Core team 2019) using packages car (Fox and Weisberg 2018), ggplot2 (Wickham, 2016), dplyr (Wickham et al., 2015), tidyverse (Wickham et al., 2019), caret (Kuhn et al., 2019), MASS (Venables and Ripley 2013), JagsUI (Kellner et al., 2019), rgl (Adler et al., 2019), SIBER (Jackson et al., 2011) and gridExtra (Auguie et al., 2017).

3. Results

The multivariate analysis of variance (MANOVA) revealed that mean cormorant egg stable isotope values differed significantly between sites (Pillai's Trace = 1.35, $F(9, 102) = 9.24$, $p < 0.001$). Univariate analysis of variance (ANOVA) showed that significant differences in mean values among sites occurred for carbon ($F(4,43) = 64.30$, $p < 0.001$), nitrogen ($F(4,43) = 87.88$, $p < 0.001$) and sulfur ($F(4,43) = 172.50$, $p < 0.001$). Pairwise site comparisons using Tukey's post-hoc tests for ANOVAs can be found in the appendix (Tables A.3. – A.5.).

Linear discriminant analysis (LDA) was able to separate the sites accurately using 2 or 3 isotopes in the eggs (in site variation lower than between site variation). Misclassification of eggs from the test datasets was low using 2 or 3 isotopes. In both cases 1 egg from the northern Baltic site was misclassified to the southern Baltic (see also Fig. 4). Using 2 isotopes, 2 discriminant functions described 62.3 and 37.7 % of separation. Using 3 isotopes, the 3 discriminant functions described 63.8, 34.66 and 1.6 % of separation. C and N isotopes both had high loadings in the LDA using 2 isotopes (i.e. account for separation between sites). Using 3 isotopes, the largest loadings for coefficients were found for C and S isotopes (Table 2).

A high degree of separation was also observed between 2 and 3 dimensional standard ellipses/ellipsoids with sites having different centroid locations, a range of ellipse/ellipsoid areas and volumes and a low degree of overlap (Fig. 4, Appendix Tables A.1. and A.2.). The southern Baltic site (site 2) had the largest Standard Ellipse Area (SEA) and Standard Ellipsoid Volume (SEV). Two sites showed a small degree of SEA overlap (southern and northern Baltic sites; Fig. 4a, Appendix Tables A.1. and A.2.). The amount of overlap was 3 % of the combined SEA area for these 2 sites (Appendix Table A.1.). The same two sites showed some overlap in SEV (Fig. 4b). The degree of overlap was less than 1% of the combined site volumes respectively (Appendix Table A.2.). The freshwater lake Roxen (site 5) was extremely different to the other 4, brackish and marine, locations (Table 1, Fig. 4).

The range in trophic position (TP) estimates for all eggs was 4.6 – 5.6 (mean ± SD/SE in Table 1, Fig. 3b). ANOVA showed that significant differences in TP estimates occurred between sites ($F(4,19) = 4.44$, $p = 0.01$) and Tukey's post hoc tests revealed that TP estimates from site 3 were significantly higher than all other sites. The range in egg $\delta^{15}N$ for the source amino acid phenylalanine ($\delta^{15}N$-Phe) was 3.5 – 10.8 (mean ± SD/SE in Table 1, Fig. 3c). ANOVA showed that significant differences in egg $\delta^{15}N$-Phe occurred between sites ($F(4,19) = 30.14$, $p < 0.001$) and Tukey's post-hoc tests revealed that mean $\delta^{15}N$-Phe values differed significantly between all sites except sites 1:3 and sites 2:3 (Fig. 3b). Concentrations of Hg ranged from 0.57 to 4.18 μg/g dw (mean ± SD / SE in Table 1, Fig. 3a). The magnitude of difference (effect size, Cohen's d) in Hg between site 5, the lake (lowest Hg) and all the other sites was large ($d > 0.5$). Pairwise comparisons of effect size between all other sites revealed small ($d < 0.2$), or in 1 case (site 3 vs site 4) medium ($d = 0.32$) differences in Hg concentrations. The linear regression showed a positive and nearly significant relationship between log Hg and TP (β coefficient = 0.67, 95% CI [−0.02, 0.30], $p = 0.057$) and no significant differences in Hg concentrations between sites (Table 3, model $R^2 = 0.31$, adjusted $R^2 = 0.12$). The model was deemed acceptable based on visual inspection of the residuals against fitted values and leverage, its quantile plot and a histogram of residuals (Appendix A Fig A.1.).

4. Discussion

Bird eggs are commonly used to measure environmental contaminants (Helander et al., 1982, Elliott et al., 1989, Hobson et al., 1997, Eriksson et al., 2016). This study provides the first evidence of the possibility of using great cormorant eggs to monitor local contaminants. A good degree of separation between sites, based on isotope values in the eggs, indicate a higher degree of income breeding so that maternally transferred contaminants measured in eggs should reflect local contaminant levels in prey. Furthermore, cormorants generally occupied similar trophic positions (TP) across sites, enabling contaminant comparisons between habitat types (freshwater, marine and brackish)

Table 1
Mean (±SD) bulk $\delta^{13}C$ (corrected for lipids), $\delta^{34}S$, $\delta^{15}N$, Standard ellipsoid volume (SEV; median values), $\delta^{15}N$ in trophic (glutamic acid) and source (phenylalanine) amino acids, trophic position (TP_{AA}) and mercury concentrations (Hg; μg/g dw) in cormorant eggs from 5 sites in Sweden. Site 1 is marine on the west coast of Sweden, 2 – 4 brackish from the southern, mid and northern Baltic Sea and 5 a freshwater lake. Number of eggs used differ because fewer eggs were analyzed for TP_{AA}, $\delta^{15}N$-Glu, $\delta^{15}N$-Phe and Hg (5 or 4 eggs per site) compared to bulk $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$ and SEV (10 eggs per site except for site 1 where n = 8, see text).

Site	$\delta^{13}C$	$\delta^{34}S$	$\delta^{15}N$	SEV median (‰^{3})	$\delta^{15}N$-Glu	$\delta^{15}N$-Phe	TP_{AA}	Hgμg/g dw
1 (Marine) (n = 8/5)	−19.30 ± 0.93	15.51 ± 2.14	16.44 ± 0.32	4.1	25.81± 0.87	8.00± 1.06	4.93± 0.07	1.98± 1.28
2 (s. Baltic) (n = 10/5)	−22.14 ± 1.30	14.62 ± 1.99	13.64 ± 0.81	5.4	23.75± 0.58	6.32± 0.97	4.86± 0.15	2.06± 0.85
3 (mid Baltic) (n = 10/5)	−21.16 ± 0.67	16.08 ± 0.80	15.83 ± 0.94	2.5	26.92± 0.62	7.46± 0.93	5.24± 0.23	2.25± 0.99
4 (n. Baltic) (n = 10/5)	−22.43 ± 0.41	16.21 ± 1.48	12.48 ± 0.94	2.3	21.57± 0.32	3.71± 0.25	4.95± 0.09	1.89± 1.13
5 (Fresh) (n = 10/4)	−25.31 ± 0.52	1.33 ± 0.80	17.38 ± 0.42	1.6	27.67± 0.39	9.86± 0.79	4.93± 0.17	1.23± 0.63

Table 2
Results from linear discriminant analysis showing the amount of separation achieved between sites using a) 2 isotopes ($\delta^{13}C$ and $\delta^{15}N$) and b) 3 isotopes ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$). The contribution of each isotope to the discriminant functions (loadings) are given. Note large negative or positive numbers indicate greatest loadings.

	Discriminant function	Separation (%)	Loadings		
			$\delta^{13}C$	$\delta^{15}N$	$\delta^{34}S$
a)	LD 1	62.3	2.01	2.58	NA
	LD 2	37.7	−1.91	1.41	NA
b)	LD 1	63.8	1.39	0.91	3.40
	LD 2	34.6	−1.50	−2.67	0.46
	LD 3	1.6	−1.87	1.57	2.39

making it interesting for national (or international) contaminant monitoring programs. Mercury (Hg) concentrations in eggs were within the expected range for fish eating birds (Jackson et al., 2016) but lower from the freshwater lake (site 5) compared with the 4 coastal locations.

4.1. Assessing migration and breeding strategy using isotopes

If females primarily used stored (body) reserves for egg formation large in-site variation in egg isotope values would be expected resulting in an inability to group eggs by site. This is because cormorants show low winter site fidelity and are known to move between freshwater and marine sites (Frederiksen et al., 2002). Furthermore, individuals in summer breeding colonies do not overwinter together on common winter sites (Frederiksen et al., 2018). Grouping found in egg stable isotopes are likely the result of a high degree of income breeding and a reflection of the local fish population isotopes. There was a small overlap in Standard Ellipsoid Volumes (SEV, isotope niche size) between the southern and northern Baltic sites, and the LDA also misclassified 1 egg between these sites. This overlap is an artifact of each sites combination of TP, ^{15}N fractionation, and baseline $\delta^{15}N$ values. $\delta^{15}N$ entering food webs in the Bothnian Sea (northern Baltic) is known to be low (Voss et al., 2005, Kiljunen et al., 2020, Jones et al., 2021) and $\delta^{15}N$ in source amino acids in eggs from the northern Baltic were much lower than all other sites (including the southern Baltic) in our study, giving further support to the separation of sites (despite an overlap in bulk nitrogen isotope signal with site 2) and an income breeding strategy. Interestingly, the most southern population (site 2) had the largest isotope niche size (area and volume). This could reflect a broader feeding niche and a previous study has indicated relatively varied diet in cormorants from this location (Ovegård et al., 2016). Alternatively, this was the most southern site (with the earliest breeding start) in our study and individuals might migrate shorter distances so that females have more stored (body) reserves which they invest in eggs (Jaatinen et al., 2016), resulting in a larger isotopic niche. If this is the case not all contaminants measured in eggs from this site are derived locally (i.e. from fish close to the breeding colony). In order to quantify the degree of stored reserves cormorants invest in eggs, $\delta^{15}N$ in amino acids would have to be compared between mothers (e.g. blood liver, muscle) and eggs (Whiteman et al., 2021). This would be a logical next step and would provide

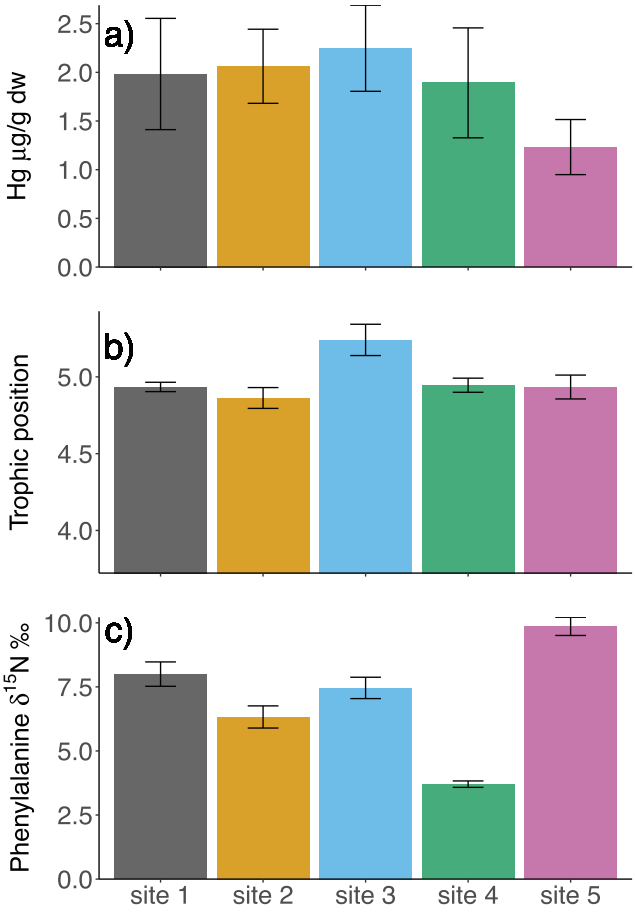

Fig. 3. Mean (±SE, n = 5) mercury concentration (a), trophic position (b) and phenylalanine $\delta^{15}N$ (c) in cormorant eggs from 5 sites in Sweden. Site 1 (black) is marine on the west coast of Sweden, 2 – 4 brackish from southern (yellow), mid (blue) and northern (green) Baltic Sea and site 5 freshwater (lake Roxen, pink), see Fig. 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

valuable insights relevant to contaminant interpretation.

There are a number of reasons great cormorants are likely to be income breeders. Firstly, they are closely related to double crested cormorants which are known to invest heavily in eggs using external resources (Hobson et al., 1997). Secondly, cormorants have a large body size which increases blood volume and oxygen and is beneficial for fish foraging, but which also increases wing loading and the energy costs of flight (Lovvorn and Jones 1994). Energetic costs of flying are shown to be high in cormorants (Watanabe et al., 2011, Elliott et al., 2013) meaning that migration might use more stored energy than expected. Thirdly, cormorants have wettable plumage (i.e. there is no layer of insulating air trapped in feathers when diving) resulting in high

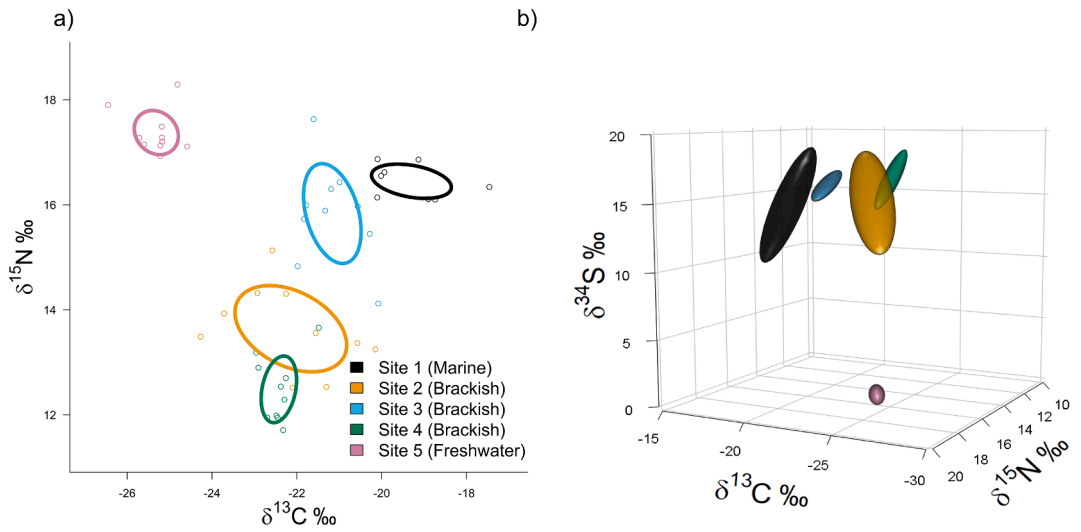

Fig. 4. Cormorant egg standard ellipses (a) and ellipsoids (b) for 5 sites in Sweden. Ellipses and ellipsoids are based on isotope values of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ from 48 eggs (10 per site except site 1 where 8 eggs were used, see methods). Site 1 (black) is marine on the west coast of Sweden, 2 – 4 brackish from southern (yellow), mid (blue) and northern (green) Baltic Sea and site 5 freshwater (lake Roxen, pink). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Results from the linear regression estimating the relationship between mercury concentration (logHg) in eggs and trophic position (estimate is numerical and represent the slope) and differences in logHg between sites (estimates are categorical and represent intercepts). Eggs from the lake (site 5) had the lowest Hg values and this site was used to compare Hg concentrations from sites 1–4 (T and *p* values for sites 1–4 are based on comparisons with site 5). Site 1 is marine on the west coast of Sweden, 2 – 4 brackish from the southern, mid and northern Baltic Sea and site 5 a freshwater lake.

Effect	Lower 95% CI	Estimate	Higher 95% CI	T-value	*p* - value
Trophic position	−0.025	0.665	1.352	2.035	0.057
Site 1 (marine)	−0.111	0.272	0.481	1.312	0.206
Site 2 (s. Baltic)	−0.039	0.349	0.561	1.829	0.084
Site 3 (mid Baltic)	−0.301	0.149	0.425	0.358	0.724
Site 4 (n. Baltic)	−0.178	0.223	0.450	0.908	0.376
Site 5 (freshwater)	−0.125	0.088	0.300	0.866	0.398

thermoregulatory costs when foraging in cold water (Grémillet and Wilson 1999), which will further deplete stored reserves on arrival at breeding colonies in early spring. Income breeding is thought to be favored in predictable environments with high resource availability (Jönsson, 1997). Apart from indicating an income breeding strategy the data showed some other interesting patterns relevant to contaminant loading and monitoring. Despite the fact that cormorants in our study appear to be primarily income breeders, isotope analysis on individual eggs revealed freshwater feeding habits for some coastally breeding females (at the marine site; particularly sulfur isotopes helped to identify this). One of the benefits of using a widely abundant and generalist higher level predator is the ability to compare contaminant concentrations spatially and between habitat types (here fresh, marine and brackish water). However, this also means that careful consideration / investigation should be carried out when selecting colonies for monitoring. Where colonies have access to separate food webs (here freshwater lakes close to the coast) there is a risk that contaminant monitoring will not fully represent the intended habitat. Another issue is that fish can be highly mobile so that contaminants accumulated in prey represent contaminants from a wider area than the breeding colony. These issues are not unique to cormorants and can be accounted for using a combination of ecological knowledge and a multi-stable isotope approach.

Our study suggests an income breeding strategy for great cormorants and that contaminants measured in eggs are derived primarily from local (~30 km radius from breeding colony) fish prey. However, it is important to consider the biochemical pathway and transfer of contaminant types in order to relate or monitor concentrations measured in eggs. The occurrence of a contaminant depends both on biological factors (clutch size, egg mass, yolk content, maternal resources, energetic demands) and physico-chemical properties of the contaminant (i.e. molecular structure lipid solubility, rate of metabolism, affinity to circulating and tissue macromolecules; Verreault et al., 2006). Even if eggs are commonly used for biomonitoring (see Klein et al., 2012), to establish if they are a suitable matrix to measure a contaminant of interest one needs to understand the biotransformation, accumulation and transfer of individual contaminants by measuring concentrations in mothers and eggs (see for example Jouanneau et al., 2021, Knudtzon et al., 2021). We measured Hg concentrations which are known to bind to proteins, and are transferred from mother to egg (Ackerman et al., 2016) – notably in the albumen via serum proteins (Kennamer et al., 2005).

4.2. *Interpreting mercury concentrations using compound specific isotopes and ecological knowledge*

Trophic position was high, biologically plausible for this species and generally similar across all sites. However, the mid Baltic site (site 3) did have a significantly higher TP than the other sites. Previous investigations on diet at this site have shown that cormorants feed on proportionally large amounts of perch (*Perca fluviatilis*) and especially pike (*Esox lucius*) (Ovegård, M. unpublished), which would explain the higher TP. The generally high TP across all sites suggest that cormorant eggs are suitable for measuring biomagnifying contaminants and the relatively low between site variations in TP makes it easier to compare contaminant concentrations between geographic areas despite a varied and generalist maternal diet - if toxicodynamics of the compound of interest is known. We found a general positive relationship between Hg and TP in cormorant eggs, not detected in bulk $\delta^{15}N$ (not shown), supporting the use of amino acid specific $\delta^{15}N$ analyses. Concentrations of biomagnifying contaminants, such as Hg, are expected to increase in organisms higher up in a food chain including marine seabirds (Lavoie et al., 2013). Whilst bulk $\delta^{15}N$ is related to TP, comparisons between

consumers from different sites using bulk $\delta^{15}N$ requires a constant baseline $\delta^{15}N$ value. This is unlikely since source $\delta^{15}N$ entering the base of a food web varies over time and space and knowledge of baseline $\delta^{15}N$ (or baselines organisms) is generally not available within monitoring programs. Furthermore, it is difficult to estimate a baseline $\delta^{15}N$ for predators with diverse feeding habits (i.e. feeding from both pelagic and littoral zones within a lake or the sea). However, these obstacles are largely overcome using the amino acid method and relevant trophic discriminant factors (Hebert et al., 2016).

We found an expected positive relationship between TP and Hg, but no statistically significant difference in egg Hg concentrations between sites even though TP was included in our model (i.e. potential site differences when accounting for TP). This could be due to inter-individual variability in Hg biomagnification (within sites). It is well known that Hg is highly variable in aquatic organisms (Lavoie et al., 2013) requiring large dissimilarities between populations or large sample sizes to detect differences at a significant level. However, the magnitudes of difference in Hg concentrations (Cohens *d*) between the freshwater (site 5) and coastal sites were large, suggesting meaningful differences exist. The egg contaminant burden in coastal sites were between 54 and 83 % higher than the freshwater site. Site 5 is situated on a relatively shallow and nutrient rich lake, Roxen. High nutrient loading in lakes causes elevated $\delta^{15}N$ values (Vander Zanden et al., 2005) which was evident from the significantly higher egg phenylalanine $\delta^{15}N$ values from this site. Elevated bulk $\delta^{15}N$ has also been observed in fish from other eutrophic lakes within the Swedish freshwater monitoring program (data publically available at www.sgu.se). In highly productive lakes concentrations of methylmercury (the dominant form of mercury in aquatic food webs) at the base of the food web can be diluted because of high algal growth (Pickhardt et al., 2002) and trophic magnification of Hg decreases in freshwater sites with high total phosphorus (Lavoie et al., 2013). Also, if conditions for growth are good, bioaccumulation of contaminants might be lower (growth dilution hypothesis, see Trudel and Rasmussen, 2006) resulting in lower Hg in cormorant eggs. In addition, site 3, which had the highest TP estimates but not highest Hg concentrations, is located in a eutrophied bay where large cyanobacterial blooms occur (Zakrisson et al., 2014) - which may counteract biomagnification through biodilution. A combination of species and site specific ecological data (in this case cormorant diet analysis) and correct TP estimates allow for meaningful interpretation of contaminants in biota.

In conclusion, great cormorant eggs (*P. c. sinensis*) from different locations can be separated according to their stable isotope values. Adding a third isotope, $\delta^{34}S$, was not necessary for statistical separation among sites but helped to identify outlier samples (eggs from birds that fed in freshwater but bred coastally). The clear site separation suggests an income breeding strategy and a potential common and widespread matrix for contaminant analysis of a top predator of fish. Cormorant eggs are abundant, relatively easy to collect, process and store. In addition, they provide a geographically specific matrix that is uniform in age and collection time (season). The study also shows the value of amino-acid stable nitrogen isotope analysis and relevant biological data for the ecological interpretation of mercury loading in eggs.

CRediT authorship contribution statement

Douglas Jones: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Maria Ovegård:** Investigation, Writing – original draft, Writing – review & editing. **Henrik Dahlgren:** Conceptualization, Methodology, Investigation, Writing – review & editing. **Sara Danielsson:** Conceptualization, Methodology, Writing – review & editing. **Maria Greger:** Investigation, Resources, Writing – review & editing. **Tommy Landberg:** Investigation, Resources, Writing – review & editing. **Andrius Garbaras:** Investigation, Resources, Writing – review & editing. **Agnes ML Karlson:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

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

Acknowledgements

Special thanks for help collecting eggs to Claes Kyrk, Sven-Gunnar Lunneryd, Anders Nilsson, and Tomas Brodin. Thanks also to Eva Kylberg for laboratory help preparing and storing eggs and Carl-Magnus Mörth and Heike Siegmund for making time to analyse 34S. Funding for isotope analyses was available to AK from the Stockholm University Baltic Sea Centre.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2022.108649.

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