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Trace elements and stable isotopes in penguin chicks and eggs: a baseline for monitoring the Ross Sea MPA and trophic transfer studies

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Highlights

- Trace element level in penguin eggs and chick tissues was higher than in past studies
- Opportunistic penguin predators and scavenger may be exposed to high toxicity risks
- Low *vs.* high C and N isotopic variability was found among chick tissues and egg parts
- We provided isotopic and elemental conversion factors between tissues and egg parts

Trace elements and stable isotopes in penguin chicks and eggs: a baseline for monitoring the Ross Sea MPA and trophic transfer studies

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ABSTRACT

Multi-tissue trace elements (TEs), C, N concentrations and stable isotopes ($\delta^{13}C$, $\delta^{15}N$) of chick carcasses and eggs of Adélie and Emperor penguins were studied to i) provide reference data before the recent institution of the Ross Sea Marine Protected Area (Antarctica); ii) assess the potential TE exposure to predators/scavengers and the release into the environment; iii) provide conversion factors that allow estimating C, N, $\delta^{13}C$ and $\delta^{15}N$ in edible tissues from non-edible ones. Higher concentrations of As, Cd, Cr, Cu, Hg, Mn and Pb were found in chick carcasses than in eggs, suggesting increasing contamination in recent decades and high toxicity risks for penguin consumers. Isotopic conversion factors highlighted small differences among body tissues and conspecifics. These values can be useful in trophic transfer studies and suggest that chick carcasses are reliable indicators of the energy pathways underlying the two penguin species and their trophic position in the food web.

KEYWORDS

Antarctica, *Pygoscelis adeliae*, *Aptenodytes forsteri*, contamination, internal tissues, food web

INTRODUCTION

Trace element contamination is a pressing problem worldwide, mainly due to anthropic activities and the consequent pressure on natural systems. Not even the most remote Antarctica area is free from contamination, as proved by scientific evidence (e.g. Bargagli et al., 1998; Calle et al., 2015; Corsolini et al., 2011; Signa et al., 2019; Sun and Xie, 2001). Within this framework, the recent institution (December 2017) of the Ross Sea Marine Protected Area (MPA) represents a steppingstone for protecting Antarctic marine living resources and habitats, preventing further contamination and promoting scientific research. Further studies aiming to identify the current baseline scenario are extremely needed for long-term monitoring, as it will allow to record future changes and assess the MPA effectiveness.

Penguins, likewise many seabirds, are deemed sentinels of environmental contamination and contaminant availability in marine ecosystems (Blévin et al., 2013; Brasso et al., 2014; Carravieri et al., 2020, 2013; Metcheva et al., 2006) chiefly because they are widely distributed with abundant populations, they are generally long-lived and hence prone to bioaccumulation in various tissues, and they occupy a high position in the food webs, which makes them prone to biomagnification (Burger and Gochfeld, 2004). Moreover, penguins are relatively sedentary, but highly specialized diving seabirds and therefore integrate contamination over time and space (Carravieri et al., 2013; Jerez et al., 2013). Among others, Adèlie (*Pygoscelis adeliae*) and Emperor penguin (*Aptenodytes forsteri*) spend their whole life in the Southern Ocean (Corsolini et al., 2011), but have different feeding strategies (Carravieri et al., 2020). Therefore, both species are valuable bioindicators for long-term monitoring, as they reflect trace element contamination at regional scale, while providing also additional insights about the contamination of dietary sources. In particular, pre-fledging

penguin chicks are suitable bioindicators, because contaminant levels in their tissues mirror the parental dietary sources that are exploited in the recent period and predictable foraging areas, related to the foraging behaviour of the two penguin species (Burger and Gochfeld, 2004). Logistic constraints and ethical standards make penguin feathers and guano commonly collected samples to infer overall environmental contamination, as they imply simple and non-invasive collection methods according to the Antarctic Treaty signed in 1959. Although both feathers and guano play an important role in trace elements detoxification (Ancora et al., 2002; Becker et al., 2016; Signa et al., 2013), there is no evidence that they reflect accumulation patterns in internal tissues (Finger et al., 2015), as trace elements may also follow differing detoxification routes (Espejo et al., 2017). Indeed, if feathers and guano contamination levels may help to understand the role of penguins as secondary contaminant source in the environment, they fail to inform about toxicological risks for the health of penguins and their predators. Similarly, eggs represent another biogenic material that can be used to reveal specific information about the environment where seabirds live (Brasso et al., 2012a; Burger, 1994; Montanari, 2018) and, at the same time, represent a potential food item for opportunistic predators. Nevertheless, while eggshells and associated membranes can be commonly found on the ground, abandoned intact eggs providing sufficient internal tissue for contamination and trophic studies may not be easy to find in adequate number. In the Ross Sea, Southern Hemisphere skua (*Catharacta* sp.) are known as opportunistic predators and scavengers that preferentially rely on Emperor and Adélie penguin eggs and chicks (Carravieri et al., 2013; Mund and Miller, 1995). Consequently, critical levels of inorganic and organic contaminants have been found in the feathers of skua (Bearhop et al., 2000; Calle et al., 2015; Metcheva et al., 2014), suggesting they may suffer from negative effects on fitness, development, reproductive performances and survival. However, when studies aim at understanding the transfer of contaminants along food chains, the analysis of prey' non-edible parts own limitations. This is particularly true for studies based on stable isotopes of carbon and nitrogen, commonly applied to trace the transfer of elements from resources to their consumers (Polito et al., 2011; Rossi et al.,

2019; Signa et al., 2019). Indeed, the isotopic signature of a consumer's tissues (measured as the ratio between the heavier to the lighter isotope of an element) reflects that of the food assimilated (Post, 2002). Nevertheless, consumers are relatively enriched in the heavier isotope with respect to their food sources. Such increase in isotopic values among trophic levels (referred to as isotopic enrichment, or fractionation) can vary among tissues of the same organism due to differences in metabolic pathways (Polito et al., 2011; Post, 2002). In addition, C and N stable isotopes in penguins are used to obtain information on food chain length and productivity in Antarctic food webs (Emslie et al., 2013; Jaeger and Cherel, 2011; Lorenzini et al., 2010). Accordingly, species- and tissue-specific conversion factors are needed to allow the isotopic comparison of edible and non-edible parts and obtain reliable diet information for trophic transfer and food web studies.

The first goal of this study was to provide both essential and non-essential trace element data in body tissues (feathers, muscles, and internal organs) of penguin chicks and in eggs, as to provide useful reference data of the baseline conditions in the Ross Sea before the recent institution in the region of the world's largest MPA. The second goal was to assess the role of penguins as a contaminant source i) to opportunistic predators and scavengers that rely on penguin chick carcasses and eggs as food items and ii) to the environment. Lastly, the third goal was to provide conversion factors that allow estimating the carbon and nitrogen stable isotope values of soft tissues that can be eaten by consumers (internal body tissues, organs and egg content, hereafter referred to as edible tissues) based on the stable isotope values of feathers and eggshell membranes and thus assess the reliability of non-edible tissues in trophic transfer studies. These represent the most common samples collectable; they are generally found in good conservation status and their sampling avoids decreasing the availability of edible parts of carcasses and eggs in the nutrient-limited Antarctic environment.

MATERIALS AND METHODS

Study area

Samples were collected in one Emperor penguin colony, Cape Washington, and in one Adélie penguin colony, Cape Hallet (Fig. 1). Both colonies are located in the Ross Sea and are included in the recently established Ross Sea MPA and the Antarctic Specially Protected Areas (ASPAs). Cape Washington represents one of the biggest Emperor penguin colonies in the Ross Sea, and it hosts up to 25000 breeding pairs (Barber-Meyer et al., 2008). Emperor penguins lay eggs in August, while hatching typically occurs in September. During its reproductive and chick growing period (between September and January), this species colonises the sea ice close to the shore, and both abandoned eggs and chick carcasses can be commonly found in the colony area before the seasonal sea-ice break-up. In the study area, this typically occurs between the end of December and January. Although there are not Skua colonies in the area of Cape Washington, but nearby, Antarctic Skua are commonly observed nearby the Emperor colony before the sea ice breaks up. Specifically, one Skua colony is located in the area of Edmonson Point, 36 Km northward, and one in the area of Adélie Cove, 44 Km south-westward. In addition, other Skua are often observed to nest in the area of Tethys Bay, 39 km on the west of Cape Washington. Further North, Cape Hallet hosts around 42500 Adélie penguin breeding pairs (Harris et al., 2015). Differently to the Emperor penguins, this species builds rocky nests on the ground near the shore. Egg-laying occurs in October, while hatching typically occurs in November. Eggs and chick carcasses are commonly found in the colony area, which also hosts a Skua colony.

Skua can be seen feeding on Emperor and Adélie penguin chicks and eggs during the spring and the summer seasons (E. Calizza, personal observation), both as active predators or as scavengers, as commonly observed in other Antarctic areas (Pezzo et al., 2001; and literature cited therein).

Samples collection and laboratory procedures

Within each colony, intact abandoned eggs (5 at each colony) and chick carcasses (4 and 6 for Emperor and Adélie penguins respectively) were collected by hand in November 2016 (for Emperor penguins) and January 2017 (for Adélie penguins). A lower variation coefficient of the

concentrations of contaminants has been observed in seabirds compared to other organisms (i.e. fish or mammals), indicating similar accumulation/detoxification pathways, and hence suggesting that the analysis of a low or high number of specimens should give comparable and reliable results (Espejo et al., 2017).

For Adélie penguin chicks, dissection occurred in the field in Antarctica. Before dissection, carcasses were carefully cleaned from impurities. Samples of axillary feathers, abdominal muscle, heart and liver were collected from each carcass and conserved frozen (-20°C) in sterile Petri dishes. Carcasses were left at the collection site after dissection.

For Emperor penguin chicks, carcasses were dissected in Italy and were conserved at -20 °C during transportation. For the collection of body tissues, carcasses were partially defrosted to enable the collection of feathers, muscles, and internal organs. For each carcass, samples of axillary and dorsal feathers, abdominal and dorsal muscle, heart, liver, and kidney were collected and conserved frozen (-20°C) in sterile Petri dishes.

In addition to penguins' tissues and eggs, we had the opportunity to collect three Krill samples (*Euphasia* spp.) at each colony. Samples were recovered from the remains of food regurgitated by adults and not consumed by chicks. The muscle of Krill was analysed with the only scope of obtaining isotopic values indicative of the isotopic baseline characterizing each colony area. Indeed, potential differences in $\delta^{15}N$ of Krill should be taken into account to calculate differences in the trophic position of the two penguin species based on their isotopic values (see below).

Once collected and before the transportation to Italy, samples were conserved frozen (-20°C) at the Italian Research Station Mario Zucchelli. Once in Italy, eggshells were carefully cleaned to remove impurities, eggs were dissected and samples of egg membrane and egg content (a mix of yolk and albumen, not distinguishable within the egg in most cases) were collected. For each egg, three fragments of the membrane and three fragments of the egg content were taken and conserved frozen (-20 °C) in sterile Petri dishes until analysis.

Elemental and isotopic analyses

Trace elements

Ground samples of body tissues (feathers, muscles and internal organs) of penguin chicks, as well as of egg parts were analysed for trace elements (As, Cd, Cr, Cu, Hg, Mn and Pb) using an ICP-OES (Optima 8000, PerkinElmer) after mineralisation in a microwave system (MARS 5, CEM) with 67-70% HNO_3, 30% H_2O_2 and Milli-Q water. Concentrations of As and Hg were determined using a hydride generation system linked to the ICP-OES with a reductant solution, consisting of 0.2% Na borohydride and 0.05% Na hydroxide. Certified Reference Materials (CRM) Fish protein DORM-4 (National Research Council of Canada) was used for the analytical quality control. The recovery was comprised between 82 and 105%. The detection limit was calculated as three times the standard deviation for digestion blanks ($n > 20$) and was 0.003 mg kg^{-1} dw for all analysed TEs. All results are given in mg kg^{-1} dw.

Stable isotopes

Eggshell samples were excluded from stable isotope and elemental analyses, and only egg membranes were considered to obtain conversion factors among edible (i.e., internal content) and non-edible (i.e., shell membrane) egg parts. Egg membranes were always found in association with eggshells, their isotopic values reflect the same dietary signature of the organic fraction of penguin eggshells (Polito et al., 2009), while they do not need preliminary acidification, which produces a small yet unavoidable bias in $\delta^{15}N$ values and it is necessary for the analysis of eggshells (Jacob et al., 2005; Polito et al., 2009). In addition, penguin egg membranes are commonly used in isotopic studies (Brasso et al., 2012b; Emslie et al., 2013).

Before isotopic analysis, samples were stored at -80°C for 24 hours and subsequently freeze-dried for 24 hours. Then, each sample was pulverised in a ball mill (Mini-Mill Fritsh Pulverisette 23: Fritsh Instruments, Idar-Oberstein, Germany). Aliquots of 2 ± 0.2 mg of powder were pressed into tin capsules. Samples were analysed in two replicates using an Elementar Vario Micro-Cube

elemental analyser (Elementar Analysensysteme GmbH, Germany) coupled with an IsoPrime100 continuous flow mass spectrometer (Isoprime Ltd., Cheadle Hulme, UK). Carbon (C) and Nitrogen (N) isotopic values were expressed in δ units ($\delta^{13}C$; $\delta^{15}N$) as parts per-thousand (‰) deviations from international standards: Vienna Pee Dee Belemnite (PDB) for C and atmospheric N_2 for N. Isotopic ratios were computed according to the equation: δX (‰) = $[(R_{sample} - R_{standard})/R_{standard}] \times 10^3$, where X is the C or N isotope and R is the heavy-to-light isotope ratio of the respective element ($^{13}C/^{12}C$; $^{15}N/^{14}N$). The internal laboratory standard used was IAEA600 Caffeine. $\delta^{13}C$ and $\delta^{15}N$ measurement errors were typically smaller than ± 0.05 ‰.

Data analysis

Trace element data

Differences in trace element (TE) concentration were investigated through univariate permutational analysis of variance (PERMANOVA - PRIMER 6 v6.1.10 & PERMANOVA+ β20; Anderson et al. 2008) based on the Euclidean distance matrix obtained by normalised TE data, followed by pairwise tests. A two-factor design was adopted, separately for body tissues and egg parts, with Species as a fixed factor with two levels: Emperor and Adélie penguins, and Tissue, as a fixed and orthogonal factor with a) four levels in the case of body tissues: axillary feathers, abdominal muscle, heart and liver, and b) three levels in the case of egg parts: shell, membrane, and content. TE concentration of internal body tissues was also compared with that of the egg content to test for differences in TE exposure for opportunistic predators and scavengers that rely on penguin carcasses or eggs. A separate PERMANOVA was run for each TE, with a two-factor design: Species was set as a fixed factor with two levels (Emperor and Adélie), and Food Item as a fixed and orthogonal factor with two levels (body tissues and egg content). Lastly, TE concentration of all internal compartments (body tissues and egg content) was compared with that of the external compartments (feathers, eggshell and egg membrane) to highlight patterns of TE allocation (internal accumulation vs. elimination) and hence to assess the role of penguins as potential contaminant

source in the environment. To do this, separate PERMANOVA for each TE was run with a two-factor design: Species, as a fixed factor with two levels (Emperor and Adélie) and Compartment, as a fixed and orthogonal factor with two levels (internal and external).

Stable isotope data

One-way ANOVA was used to test differences in isotopic values ($\delta^{13}C$; $\delta^{15}N$) and elemental (C, N) concentrations between the different sample typologies (i.e., feathers, muscles, internal organs and egg parts). To test differences between penguin species, a one-way ANOVA for repeated measures was used, and the tissues analysed were considered as repeated observations.

The relative difference in the trophic position of the two species (ΔTP) was calculated according to the following equation:

$$\Delta TP = (\delta^{15}N_{X\_Emperor} - \delta^{15}N_{X\_Adélie})/TEF - \Delta^{15}N_{Krill}/TEF$$

where X represents the specific body tissue (i.e., axillary feathers, abdominal muscle, heart or liver) for which a comparison among the two species was possible, and TEF represents the expected trophic enrichment factor (in ‰) among $\delta^{15}N$ values in penguins and that in their food sources. $\Delta^{15}N_{Krill}$ represents the difference in $\delta^{15}N$ values of Krill samples collected at the two colonies. Specifically, $\delta^{15}N$ of Krill was 6.1 ± 1.1‰ and 5.4 ± 0.4‰ at Cape Washington and Cape Hallett respectively, which implies a $\Delta^{15}N_{Krill} = 0.7‰$.

A TEF = 3.5‰ was applied. This value has been measured in feathers of the penguin species *Aptenodytes patagonicus* (congeneric of the Emperor penguin) and *Pygoscelis papua* (congeneric of the Adélie penguin) (Polito et al., 2011; and literature cited therein). It has been shown as not affected by the eventual reduction in food intake and to not differ between adults and chicks (Cherel et al., 2005; Polito et al., 2011). Hence, a ΔTP of 3.5‰ between Emperor and Adélie penguins would imply that Emperor penguins are 1 trophic position higher in the food web than Adélie penguins. Here, we were interested in comparing ΔTP values obtained through the analysis of different body tissues. To do this, for each body tissue, a distance matrix based on N isotopic

distances (i.e., Euclidean distances) between specimens was created, obtaining a total of 24 pairwise comparisons (4 Emperor penguins x 6 Adélie penguins).

Paired T-tests were used to compare (i) isotopic values among Adelie and Emperor penguins, with values belonging to the same kind of tissue treated as paired observations in the comparison of the two species, and (ii) C and N conversion factors for each of the two penguin species, with values belonging to the same specimen or egg treated as paired observations in the comparison of tissues and egg parts respectively.

RESULTS

Trace elements in body tissues and eggs

Two main patterns arose in the analysis of trace elements (TEs) of the body tissues (feathers, muscles and internal organs) of the chicks of Emperor and Adélie penguins. Overall trace elements were concentrated mainly in liver (As, Hg, Cu, Mn) and axillary feathers (Cd, Pb, Cr) (Fig. 2). In more detail, permutational analysis of variance (PERMANOVA) on As and Cu concentration in the body tissues showed significant differences among tissues but not between species (Table 1) with both TEs peaking in the liver (max As: 2.3 mg kg^{-1}, max Cu: 146.8 mg kg^{-1}) of both species. All the other TEs analysed showed significant differences for the interaction of the factors species and tissues. The highest Cd concentration (max Cd: 1.1 mg kg^{-1}) was recorded in the axillary feathers of both species, while interspecific differences were evident for heart and abdominal muscle (higher in Emperor than Adélie penguin) and liver (higher in Adélie than Emperor penguin). Interspecific differences were much more evident for Hg, whose concentration was significantly higher in liver, heart and abdominal muscle of Emperor penguin (max Hg: 5.3 mg kg^{-1}) than in Adélie. Cr and Pb showed comparable body tissue accumulation patterns, with significantly higher levels in the axillary feather of Adélie penguin (max Cr: 11.3 mg kg^{-1}, max Pb: 8.3 mg kg^{-1}), than in those of the Emperor penguin, and vice-versa for the abdominal muscle. Similarly, heart and liver showed the lowest Cr and Pb levels in both species. Finally, Mn pattern highlighted the highest concentration in

the liver of both species (max Mn: 12.0 mg kg^{-1}), while interspecific differences, with significantly higher concentrations in Emperor than in Adélie penguin, were evident in both abdominal muscle and axillary feathers.

Egg parts of Emperor and Adélie penguins showed overall similar levels and patterns for almost all TEs (Fig. 3). PERMANOVA revealed indeed significant differences only among egg parts, and not between species and the interaction species x egg parts for most TEs (As, Hg, Pb, Cr and Cu). The eggshell showed always the lowest TE concentration than egg membrane and content, which, in turn, showed similar concentrations (As and Pb), lower concentration in the egg content than in the membrane (Cr and Cu), and vice-versa only for Hg. In contrast, higher Cd and Mn concentrations were measured in the eggshell (only Cd) and membrane of the Emperor penguin, compared with the same egg parts of Adélie penguin (Fig. 3).

Higher Cd, Cu and Mn concentrations were detected in the body tissues of penguin chicks than in the egg content, both potential food items for Antarctic scavengers, regardless of the species (Cd = F: 4.6, $p \leq 0.05$; Cu = F: 6.0, $p \leq 0.05$; Mn = F: 13.4, $p \leq 0.001$). Cr, Hg and Pb showed significant differences for the interaction of the factors species and food item with i) higher concentration of all the three elements in the body tissues of Emperor than in those of the Adélie chicks (Cr = t: 4.68, $p \leq 0.001$; Hg = t: 3.75, $p \leq 0.001$; Pb = t: 4.68, $p \leq 0.001$) and no interspecific differences in the egg content; ii) higher concentrations in the body tissues than in the egg content of both species for Cr and Hg (Adélie: Cr = t: 3.08, $p \leq 0.01$; Hg = t: 3.25, $p \leq 0.05$; Emperor: Cr = t: 3.98, $p \leq 0.5$; Hg = t: 3.54, $p \leq 0.05$) and only in the emperor penguin for Pb (t: 2.46, $p \leq 0.05$). Arsenic did not show significant differences between species, nor between trophic items or their interaction. On the other hand, significantly higher Cu, Hg and Mn concentrations were recorded in the internal compartment (I: muscle, internal organs, and egg content) than in the external compartment (E: feathers, eggshell and egg membranes) (Cu = F: 5.0, $p \leq 0.05$; Hg = F: 10.8, $p \leq 0.01$; Mn = F: 7.4, $p \leq 0.05$) and also higher Hg concentrations in Emperor than Adélie penguin (F: 9.2; $p \leq 0.01$) (Fig. 3). An opposite pattern between compartments was highlighted for Cd (I < E: F: 6.4; $p \leq 0.05$) and no differences

for As. Lastly, Cr and Pb concentrations were significantly lower in the internal compartment of Adélie, than in both the external compartment of the same species (Cr = t: 2.7, $p \leq 0.01$; Pb = t: 3.2, $p \leq 0.05$), and the internal compartment of Emperor penguin (Cr = t: 3.7, $p \leq 0.001$; Pb = t: 4.1, $p \leq 0.001$) (Fig. 4).

Isotopic and elemental composition of body tissues and eggs

Overall, $\delta^{15}N$ values differed between Emperor and Adélie penguins (paired T-test, $t = 7.3$, $p < 0.001$), while $\delta^{13}C$ values overlapped ($p > 0.05$) (Table 2 and Fig. 5). In both species, the egg membrane had a markedly lower $\delta^{13}C$ value than all the other samples, which showed small yet significant differences among them (Fig. 5, one-way ANOVA Emperor: $F = 70.7$, $p < 0.0001$; Adélie: $F = 196.4$, $p < 0.0001$). In Emperor penguins, the egg parts had generally lower $\delta^{15}N$ values than body tissues (one-way ANOVA $F = 8.2$, $p < 0.0001$, and Tukey's pairwise comparisons, $p < 0.05$), which did not differ among them ($p > 0.05$) (Fig. 5). In contrast, in Adélie penguins, the egg membrane and the muscle had the highest and the lowest $\delta^{15}N$ value respectively (one-way ANOVA, $F = 10.7$, $p < 0.0001$, and Tukey's pairwise comparisons, $p < 0.05$), while the other samples did not differ among them ($p > 0.05$) (Table 2). In both species, the relative content of N (N%) was generally higher in feathers, followed by muscles, internal organs, and egg parts, which had the lowest N% (Table 2, one-way ANOVA, Emperor: $F = 22.7$, $p < 0.0001$, Adélie: $F = 131.1$, $p < 0.0001$, and Tukey's pairwise comparisons, $p < 0.05$).

The isotopic and elemental variability of body tissues, expressed as the coefficient of variation (C.V.) among the specimens analysed, was generally low, and never exceeded 7% (Table 2). In contrast, the variability of egg membranes and egg contents was higher, with the highest variability observed in the N% of the egg membrane of Emperor penguins (C.V.= 23.3%) (Table 2).

Isotopic and elemental discrimination among body tissues and egg parts

By mean, the isotopic difference (Δ) among feathers (our reference tissue), muscles and internal organs did never exceed 2‰ and was characterized by a low inter-individual variability (Table 3). Nevertheless, small yet significant differences in Δ values were observed among body tissues and between the two penguin species (two-way ANOVA, Factor: Tissue, $F = 37.4$, $p < 0.0001$; Factor: Species, $F = 23.9$, $p < 0.0001$; Interaction: $F= 39.5$, $p < 0.0001$). In Emperor penguins, $\Delta^{13}C$ ranged from -1.92 ± 0.15 ‰ in the liver to 1.60 ± 0.16 ‰ in heart, while $\Delta^{15}N$ ranged from -0.74 ± 0.33 ‰ in heart to 1.11 ± 0.25 ‰ in the liver. In Adelie penguins, $\Delta^{13}C$ ranged from -1.61 ‰ in the liver to -1.02 ‰ in muscle, while $\Delta^{15}N$ ranged from -1.16 ‰ in muscle to 0.51 ‰ in the liver. Similarly, differences in N% among feathers and the other body tissues (ΔN%) were generally small, with the highest difference observed between feathers and liver in Adélie penguins (ΔN% = -3.6%) (Table 3). Considering the two penguin species together, $\Delta^{13}C$ values were strongly correlated with ΔN% values ($\Delta^{13}C = 0.64$, ΔN% = + 0.06, $r^2 = 0.97$, $p < 0.0001$; data provided in Table 3), while no significant correlation was observed between $\Delta^{15}N$, ΔC% and ΔN% (p always > 0.05).

Regarding the isotopic difference among egg parts, $\Delta^{15}N$ was low and similar to what observed among tissues (Table 3), while $\Delta^{13}C$ was markedly higher (paired T-test, Emperor: $t = 11.4$, $p < 0.001$; Adelie: $t = 3.4$, $p < 0.05$).

Independently on the tissue analysed, Emperor penguins occupied a higher TP than Adélie penguins. The relative difference in the TP (ΔTP) ranged from +1.3 ± 0.1 when comparing feathers, to +1.6 ± 0.2 when comparing muscles. While small, these differences were statistically significant (one-way ANOVA, $F= 32.3$, $p < 0.0001$).

DISCUSSION

Although penguins are acknowledged bioindicators of environmental contamination (e.g. Carravieri et al., 2013; Catán et al., 2017; Finger et al., 2015; Metcheva et al., 2006), this is one of the few investigations on trace element (TE) accumulation and elimination patterns focusing on both internal and external body tissues of penguin chicks and eggs. Moreover, while Adélie (*Pygoscelis*

adeliae) is one of the most studied species (e.g. Ancora et al., 2002; Celis et al., 2015; Jerez et al., 2013; Metcheva et al., 2006; Smichowski et al., 2006) and among the most abundant Antarctic penguins, investigations about Emperor (*Aptenodytes forsteri*) penguins as valuable sentinels of environmental contamination are less frequent, while very promising (Carravieri et al., 2020; Pilcher et al., 2020). In this direction, here we provided trace element data in body tissues (feathers, muscles and internal organs) of penguin chicks and eggs, which represent reference data of the baseline conditions in the Ross Sea before the institution of the Ross Sea Marine Protected Area. Furthermore, we provided isotopic conversion factors between soft tissues that can be consumed by predators and scavenger but are difficult to collect, and non-edible yet easily collectable penguin feathers and egg membranes. This will help to support trophic transfer studies dealing with TE transfer along food chains as well as the use of stable isotopes in penguins as proxies for food chain length and productivity in Antarctic food webs (Jaeger and Cherel, 2011; Lorenzini et al., 2010).

Chicks' tissues

Higher TE levels were overall recorded in the penguin tissues than in past (Bargagli et al., 1998; Honda et al., 1986; Yamamoto et al., 1996) and recent studies on penguin chicks from various Antarctic sites (Blévin et al., 2013; Catán et al., 2017; Jerez et al., 2013; Smichowski et al., 2006), indicating a general increase in TE contamination in the Antarctic marine system over the last decades. In more detail, the concentration of both essential (Cr, Cu and Mn) and non-essential TEs (As, Cd, Hg and Pb) recorded in feathers was alarmingly more comparable to published data on adults (Ancora et al., 2002; Bargagli et al., 1998; Brasso et al., 2014; Jerez et al., 2011) than to those on chicks (Jerez et al., 2013; Smichowski et al., 2006).

The most striking TE levels found in this study were attributable to Cr and Pb in the feathers of Adélie chicks, with values more than 20-fold higher than in other penguin chicks (Catán et al., 2017; Jerez et al., 2013). Although feathers are the main target tissue by which birds sequester and eliminate trace elements through moulting (adults) or body growth (chicks) (Ancora et al., 2002;

Carravieri et al., 2014), TE concentration in chicks is expected to be lower than that observed in adults, due to the different exposure time (weeks or months *vs*. years respectively) (Blévin et al., 2013). High TE levels were found also in the liver of penguin chicks: Cu was notably high in both species, while Hg peaked in Emperor showing concentration much higher than what the recent literature reports for chicks (Smichowski et al., 2006). Trace elements are generally absorbed from dietary sources and then are transferred trough the circulatory system to the target tissues (Catán et al., 2017), among which liver is involved in several biochemical processes (e.g. storage, redistribution and detoxification through transformation and/or inactivation) to cope with contaminant toxicity (Burger and Gochfeld, 2004).

Although the TE allocation patterns in the body tissues were overall comparable between the two penguin species, there was evidence of interspecific differences in TE burden, which are likely attributable to the different trophic habit of the two species. Adélie and Emperor penguin chicks are fed with prey collected by adults from the surrounding neritic waters (Carravieri et al., 2020), but if Adélie penguins preferentially feed on zooplankton, Emperor penguins rely mostly on fish and cephalopods (Cherel, 2008). While the description of penguins' diet was behind the scope of the present research, our isotopic data confirm the higher trophic position occupied by Emperor than Adélie penguins, as generally reported for these species (Cherel, 2008). The higher Hg levels found in the tissues of the Emperor penguin chick can thus be related to interspecific dietary differences, as preys at high trophic levels convey Hg to top-predators due to biomagnification (Signa et al., 2017, 2019). Similarly, the higher TE level recorded in the Adélie liver (Cd) and feathers (Cr, Cu and Pb) than in the Emperor tissues, is likely due to the high concentration of these elements in krill (Metcheva et al., 2014; Pilcher et al., 2020; Signa et al., 2019).

Measured differences in the trophic position of the two penguin species were consistent among the tissues analysed. This can be ascribed to the low variations in the values of $\delta^{15}N$ conversion factors among tissues and conspecifics. Indeed, the maximum isotopic difference observed between feathers and the other tissues (i.e., $\Delta^{15}N$, equal to -1.16‰ between feathers and muscle in Adélie

penguins) was equivalent to -0.3 TP only. Notably, tracking changes in the trophic position of these Antarctic predators is considered a good approach for understanding changes in the productivity of the Antarctic pelagic food web over space and time associated to human pressure and climate change (Jaeger and Cherel, 2011; Lorenzini et al., 2010). Our results suggest that comparisons of TPs among the two species and conspecifics across Antarctic areas based on chick samples may provide reliable results even when the comparison of the same tissue is not feasible.

Similarly, variations in $\delta^{13}C$ conversion factors among tissues (i.e. $\Delta^{13}C$) of the same species were relatively low. Nevertheless, differences in $\Delta^{13}C$ were directly dependent on differences in N% content among tissues (i.e., ΔN%). Based on C and N stable isotope analysis, Podlesak and McWilliams (2006) demonstrated that C used for the synthesis of proteinaceous tissues in birds may be obtained from poorly proteinaceous food sources, being subject to a longer metabolic routing than N allocated in the same tissues, which mainly derives from proteinaceous food sources. Such longer metabolic routing may be expected to enrich the tissue in ^{13}C, which is proportionally more retained in tissues than its lighter isotope (^{12}C) at each metabolic step. This can explain the observed positive correlation between the difference in N% (as a proxy of protein content in tissues) and $\delta^{13}C$ among the tissues analysed.

In the Antarctic coastal environment, basal food sources, i.e., sea-ice associated algae, phytoplankton and benthic producers, are characterized by markedly different $\delta^{13}C$ values (Norkko et al., 2007; Rossi et al., 2019; Signa et al., 2019). These differences seem to be broadly conserved across Antarctic areas and over time (Calizza et al., 2018; Norkko et al., 2007). Thus, the comparison of the carbon isotopic composition of penguin chicks may provide useful information on the relative importance of distinct production pathways in the secondary productivity of Antarctic coastal food webs.

Eggs

As in body internal tissues, there is very little information on TEs in penguin eggs, but for Hg. There is evidence, indeed, that females can transfer Hg to the eggs (Bargagli et al., 1998; Brasso et al., 2012a, 2014), and that eggs may reach comparable Hg concentration as the egg-laying mother (Honda et al., 1986). Here we found a high interspecific similarity, indicating similar maternal allocation mechanisms to eggs in the two studied species, with significant higher content in the egg membrane and the egg content (yolk + albumen) than in the eggshell. Moreover, we found higher TE content in both eggshell and membrane than reported in the few published studies on penguin eggs from Antarctica (Bargagli et al., 1998; Brasso et al., 2014, 2012a; Honda et al., 1986; Metcheva et al., 2011), indicating a high maternal dietary exposure to TEs during the pre-breeding seasons (Brasso et al., 2012a, 2014).

Looking at the potentially negative effects for bird predators and scavengers, among which skua is one of the most abundant in the region, the significantly higher concentration of almost all the TEs analysed in the body tissues than in the egg content suggests a higher potential exposure risk for skua that rely on chick carcasses than on eggs. Skua have high trophic plasticity, although preferentially prey upon carcasses and eggs to feed their chicks during the nesting and chick-rearing season (Carravieri et al., 2017). Very high Hg concentrations were found in skua chick blood (Carravieri et al., 2017) and adult feathers from the Antarctic peninsula (Calle et al., 2015) and were explained by the Hg trophic transfer and biomagnification from penguins to skua. Hg-induced breeding failure, potentially leading to population decline, was also documented in south polar skua (Goutte et al., 2014). Similarly, high Cd, Cu and Pb concentrations were found in brown skua feather and were related to a diet rich in these TEs (Metcheva et al., 2014). Non-essential TEs, among which As, Cd, Hg and Pb, do not have any physiological function and may trigger toxicity over certain thresholds. On the other hands, although essential elements (Cr, Cu, and Mn) are needed to play several vital functions, they may also produce toxicity at high concentration. Although we did not analyse TE concentration in the skua tissues, we may expect that the dietary ingestion of chick tissues with high TE level, as those found in this study, may lead to a high TE

bioaccumulation with potential high toxicity risks for skua populations. Moreover, we expect that biomagnification and synergistic interactions among TEs may occur further increasing the toxic effects of individual TEs. It is documented that Cu exposure, for instance, can further increase the toxic effects led by Pb in birds (Espejo et al., 2017), both elements whose concentration in the chick tissues was very high.

Lastly, the two distinct patterns of TE internal accumulation vs. TE elimination through feathers and eggs (shell + membranes) highlighted in this study give also useful insights about the role of penguins as secondary contaminant sources in the environment. Notably, Adélie chicks seemed to allocate most of the Cd, Cr and Pb burden in the external tissues, potentially contributing to local contamination of terrestrial ecosystems. Alongside guano, feathers and eggs are important routes of toxic elimination by penguins (Burger, 1994; Perfetti-Bolaño et al., 2018; Pilcher et al., 2020) and contribute to the high levels of inorganic and organic contamination of the ornithogenic soils near the breeding colonies (Cipro et al., 2019; Roosens et al., 2007).

With reference to the isotopic composition of eggs, $\delta^{13}C$ values were markedly different between edible (i.e., internal organic content) and non-edible (i.e., egg membrane) parts. Conversion factors among egg parts had values as high as 5.7‰ and 4.3‰ in Emperor and Adélie penguins respectively. In addition, eggs of both species had a similar and higher C/N than those measured in chicks' body tissues, suggesting a higher nutritional value of chicks' carcasses than eggs. Our results are only partially similar to a previous isotopic study based on penguin eggs by Polito et al. (2009). Although Polito et al. (2009) measured lower $\delta^{13}C$ values in the yolk and albumen than in egg membranes of captive Gentoo penguins (*Pygoscelis papua*), isotopic differences were smaller (around 2.8‰) than what measured in this study. Also, C/N values were much lower in eggs of both captive and wild Gentoo penguins from the northernmost sector of the Antarctic peninsula than in our study. Lower C/N values in this region were also measured in Adelie penguin' eggs (Polito et al., 2009). In this case, differences could be explained by the high consumption of fish, a higher-quality food source than krill, which constitutes more than half of the diet of Adélie penguins. The

contribution of fish to penguins' diet is expected to decrease with sea ice coverage (Ainley et al., 1998). Higher sea ice extent and persistence characterize the Ross Sea (our study region) in comparison to the Antarctic Peninsula and are expected to increase the metabolic costs of foraging trips for penguins (Watanabe et al., 2020; Wienecke et al., 2000). Consequently, increased sea ice may also decrease nutrients allocated for egg production as well as the amount and nutritional value of food provided by adults to chicks (Clarke et al., 2002).

CONCLUSION

Trace element (TE) levels in the tissues of penguin chicks were highly suitable to reveal high baseline conditions for monitoring the Ross Sea MPA contamination as they reflect only the recent and local TE availability, and, at the same time, they provided relevant information about the risk which predators and scavengers are exposed to. In general, the concentration of both non-essential (As, Cd, Hg, Pb) and essential (Cr, Cu, Mn) TEs in the chick tissues and in eggs of Adélie and Emperor penguins from the Ross Sea was sensibly higher than in previous studies, revealing an increasing contamination trend in the last decades and potential toxicity risks for skua, one of the most abundant penguin predators in the area. At the same time, the high TE concentration eliminated by penguins through feathers and eggs may contribute to the contaminant subsidy and accumulation in the terrestrial environment.

Isotopic analysis indicated that chick carcasses can be reliable indicators of the energy pathways that support the two penguin species, as well as their trophic position in the Antarctic food web, with low confounding effects related to the tissues analysed. Thus, data shown here represent useful baseline information to track changes within the Ross Sea MPA and to compare areas outside the MPA. In parallel, the separated analysis of the egg membrane, useful to track food inputs assimilated by adult penguins, and the internal egg content, necessary to track egg consumption by predators (e.g., skua), should be carefully taken into account. Lastly, the combined analysis of TEs and nutrient content suggests that, while chicks of the two species have a similar and higher

nutritional value than eggs (based on the C to N ratio), they expose predators/scavengers to higher TE concentrations, which may also vary depending on the penguin species consumed. Indeed, exposure risk differed among Emperor and Adélie chicks based on the trophic position occupied by their parents and the specific behaviour of different TEs within the Antarctic food web (Signa et al., 2019).

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TABLES

Table 1. Results of univariate PERMANOVA (main test and pairwise tests) testing for differences in trace element concentrations between species and among common body tissues (axillary feathers AF, abdominal muscle AM, hearth H, liver L) to both species.

Main test		**As**			**Cu**		
Source of variation	**df**	**MS**	**Pseudo-F**	**p**	**MS**	**Pseudo-F**	**p**
Species (Sp)	1	2.11	3.34	0.067	0.22	2.16	0.182
Tissue (Ti)	3	5.00	7.93	**0.001**	11.12	107.67	**0.001**
Sp x Ti	3	0.09	0.14	0.943	0.17	1.65	0.189
Residuals	32	0.63			0.10		
Pairwise tests							
between tissues	across species	H < AM = AF ≤ L			AM < H < AF < L		

Main test		**Cd**			**Hg**		
Source of variation	**df**	**MS**	**Pseudo-F**	**p**	**MS**	**Pseudo-F**	**p**
Species (Sp)	1	0.16	0.90	0.358	13.67	36.98	**0.001**
Tissue (Ti)	3	9.12	50.10	**0.001**	3.86	10.44	**0.001**
Sp x Ti	3	0.76	4.16	**0.016**	1.33	3.61	**0.019**
Residuals	32	0.18			0.37		
Pairwise tests			**t**	**p**		**t**	**p**
between species within tissues	Heart	A < E	5.36	**0.004**	A < E	3.89	**0.004**
	Liver	E < A	2.80	**0.019**	A < E	3.95	**0.003**
	Muscle A	A < E	4.22	**0.010**	A < E	2.09	**0.044**
	Feathers A	A = E	1.15	0.241	A = E	1.94	0.111
between tissues within species	Adelie	H < AM < L < AF			H = AM = AF ≤ L		
	Emperor	H = L ≤ AM < AF			AF = AM = H ≤ L		

Main test		**Pb**			**Cr**			**Mn**		
Source of variation	**df**	**MS**	**Pseudo-F**	**p**	**MS**	**Pseudo-F**	**p**	**MS**	**Pseudo-F**	**p**
Species (Sp)	1	0.01	0.07	0.788	0.00	0.02	0.892	1.57	19.94	**0.001**

Tissue (Ti)	3	6.66	36.53	**0.001**	6.31	26.12	**0.001**	9.50	120.74	**0.001**
Sp x Ti	3	2.93	16.07	**0.001**	2.48	10.27	**0.001**	0.84	10.68	**0.001**
Residuals	32	0.18			0.24			0.08		
Pairwise tests			**t**	**p**		**t**	**p**		**t**	**p**
between species	Heart	A < E	3.25	**0.010**	A = E	1.74	0.118	A = E	1.98	0.084
within tissues	Liver	A = E	1.74	0.121	A = E	1.98	0.067	A = E	1.29	0.229
	Muscle A	A < E	6.83	**0.011**	A < E	5.56	**0.001**	A < E	10.95	**0.002**
	Feathers A	E < A	2.64	**0.031**	E < A	2.33	**0.048**	A < E	3.71	**0.013**
between tissues	Adelie	L = H= AM < AF			H = L = AM < AF			AM < AF = H < L		
within species	Emperor	L < H < AM ≤ AF			H = L < AM = AF			H < AM = AF < L		

Table 2. C and N isotopic and elemental concentration in tissues and eggs of Emperor and Adélie penguins. For each parameter, the mean value and the variability (i.e. coefficient of variations, CV) among specimens are shown. Feathers A and D: axillary and dorsal feathers respectively; Muscle A and D: abdominal and dorsal muscle respectively.

Emperor penguins	**$\delta^{13}C$ (‰)**	**$\delta^{15}N$ (‰)**	**C%**	**N%**	**C/N**	**CV $\delta^{13}C$**	**CV $\delta^{15}N$**	**CV C%**	**CV N%**	**CV C/N**
Feather A	-24.3	13.3	51.0	15.9	3.2	1.2	1.9	4.9	2.5	3.2
Feather D	-24.1	13.8	50.2	16.1	3.1	2.2	3.2	0.9	1.9	1.1
Muscle A	-25.0	13.4	48.7	14.6	3.3	3.0	3.2	1.3	5.8	7.0
Muscle D	-25.2	13.5	48.0	14.6	3.3	1.8	3.2	1.7	3.3	1.9
Heart	-25.9	14.1	50.7	13.9	3.7	1.3	3.4	0.3	2.0	1.8
Liver	-26.2	14.4	52.6	12.4	4.3	0.9	2.2	2.3	2.8	3.7
Kidney	-25.8	14.4	50.9	13.4	3.8	2.5	6.2	3.1	2.8	4.2
Egg membrane	-25.5	12.0	40.8	11.9	3.5	3.2	11.7	17.4	23.3	7.6
Egg content	-31.2	12.6	60.5	7.8	7.8	1.4	6.3	1.6	5.0	4.7
Average tissues	-25.2	13.8	50.3	14.4	3.5	1.8	3.3	2.1	3.0	3.3
Average eggs	-28.3	12.3	50.6	9.8	5.6	2.3	9.0	9.5	14.2	6.1
Adélie penguins	**$\delta^{13}C$ (‰)**	**$\delta^{15}N$ (‰)**	**C%**	**N%**	**C/N**	**CV $\delta^{13}C$**	**CV $\delta^{15}N$**	**CV C%**	**CV N%**	**CV C/N**
Feather A	-24.8	8.2	51.8	15.8	3.3	1.0	5.5	3.5	3.0	2.2
Muscle D	-25.8	7.1	48.0	14.6	3.3	0.9	4.7	2.1	2.2	1.2
Heart	-26.4	8.2	49.4	13.4	3.7	1.0	3.6	1.9	2.2	1.6
Liver	-26.4	8.8	50.7	13.1	3.9	1.0	3.3	1.3	2.3	1.2
Egg membrane	-25.4	10.1	46.4	14.3	3.2	1.6	14.3	3.0	4.8	1.9
Egg content	-29.7	8.3	54.1	9.0	7.4	0.7	10.0	12.5	5.8	7.6
Average tissues	-25.9	8.1	50.0	14.2	3.5	1.0	4.3	2.2	2.4	1.6
Average eggs	-27.5	9.2	50.3	11.6	5.3	1.2	12.1	7.7	5.3	4.8

Table 3. Isotopic and elemental differences (Δ, i.e. conversion AFctors) among axillary feathers (our reference tissue) and the remaining tissues analysed in Emperor and Adélie penguin chicks, as well as among the egg membrane (our reference egg part) and the egg internal content.

Emperor penguins	**$\Delta^{13}C$ (‰)**	**$\Delta^{15}N$ (‰)**	**ΔC (%)**	**ΔN (%)**	**ΔC/N**
Feather D	0.13 ± 0.03	0.38 ± 0.14	-1.03 ± 1.56	0.08 ± 0.14	-0.08 ± 0.07
Muscle A	-0.73 ± 0.33	0.09 ± 0.32	-2.36 ± 1.30	-1.31 ± 0.48	0.14 ± 0.16
Muscle D	-0.91 ± 0.14	0.16 ± 0.25	-3.02 ±0.98	-1.33 ± 0.25	0.09 ± 0.07
Heart	1.60 ± 0.16	-0.74 ± 0.33	0.34 ± 1.25	2.08 ± 0.30	-0.46 ± 0.03
Liver	-1.92 ± 0.16	1.11 ± 0.25	1.54 ± 1.30	-3.58 ± 0.36	1.06 ± 0.05
Kidney	-1.52 ± 0.23	1.02 ± 0.54	-0.14 ± 0.74	-2.57 ± 0.11	0.61 ± 0.07
Egg content	5.74 ± 0.27	-0.61 ± 0.49	-19.67 ± 3.76	4.09 ± 1.41	-4.31 ± 0.23
Adelie penguins	**$\Delta^{13}C$ (‰)**	**$\Delta^{15}N$ (‰)**	**ΔC (%)**	**ΔN (%)**	**ΔC/N**
Muscle A	-1.01 ± 0.18	-1.16 ± 0.22	-3.63 ± 0.80	-1.10 ± 0.35	-0.01 ± 0.03
Heart	-1.59 ± 0.19	-0.05 ± 0.23	-2.15 ± 0.96	-2.36 ± 0.33	0.42 ± 0.04
Liver	-1.61 ± 0.19	0.51 ± 0.15	-0.61 ± 0.77	-2.62 ± 0.32	0.61 ± 0.04
Egg content	4.33 ± 0.16	1.74 ± 0.61	-7.62 ± 3.10	5.34 ± 0.39	-4.19 ± 0.24

FIGURES

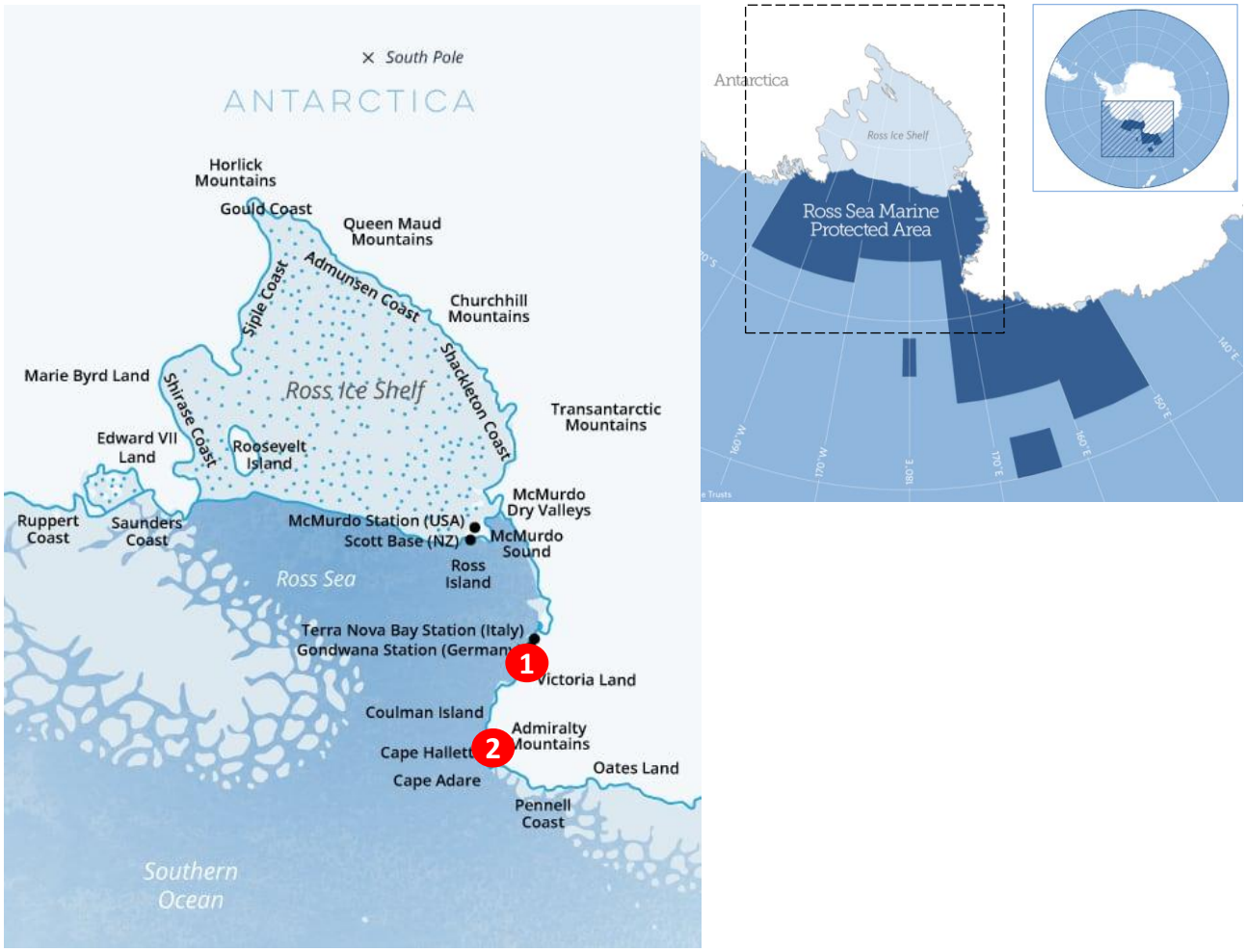

Fig. 1. Map of the study area. 1 and 2 in the biggest panel indicate the position of Cape Washington (1) and Cape Hallett (2). The smaller panel shows the position of our study area in Antarctica and depicts the borders of the Ross Sea Marine Protected Area (dark blue polygon).

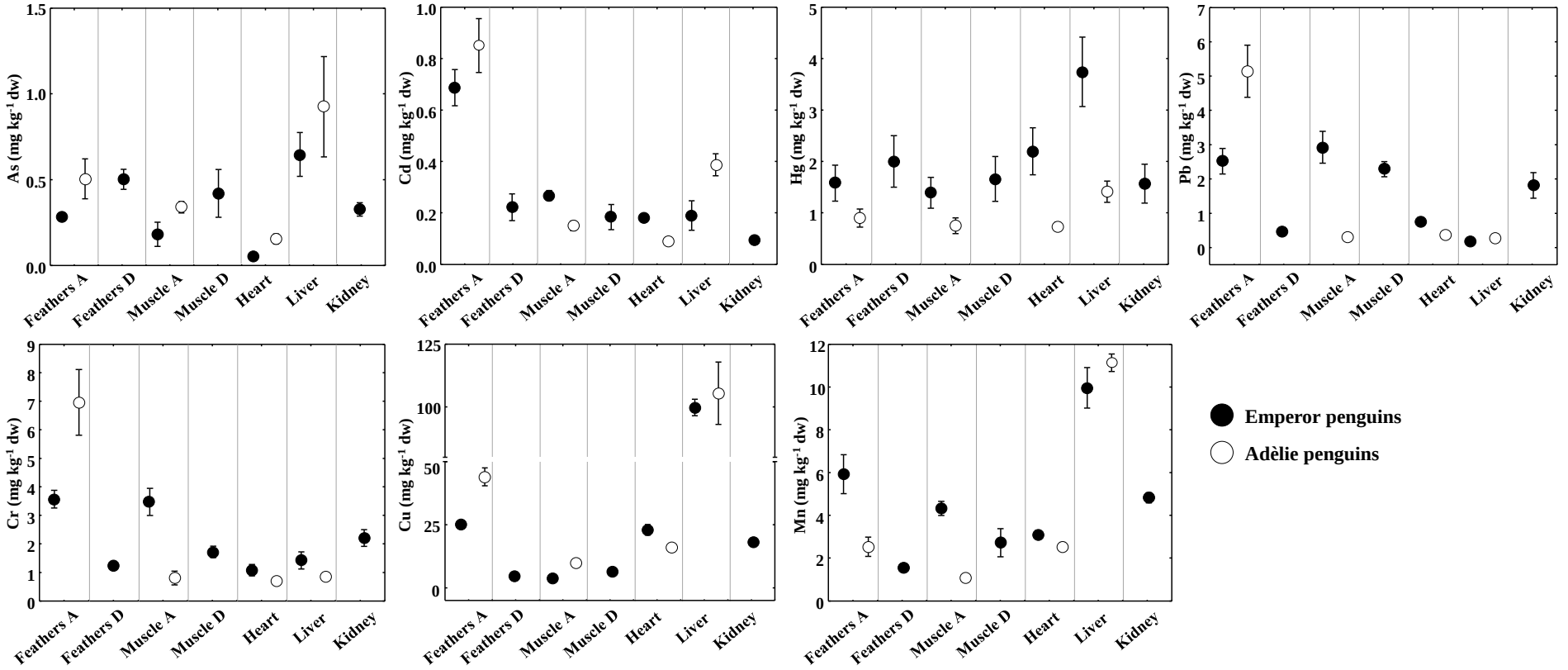

Fig. 2. Mean (± standard error) concentration of trace elements analysed in the different body tissues of the Emperor (black circles) and Adélie penguin (white circles) chicks. Feathers A and D: axillary and dorsal feathers respectively; Muscle A and D: abdominal and dorsal muscle respectively

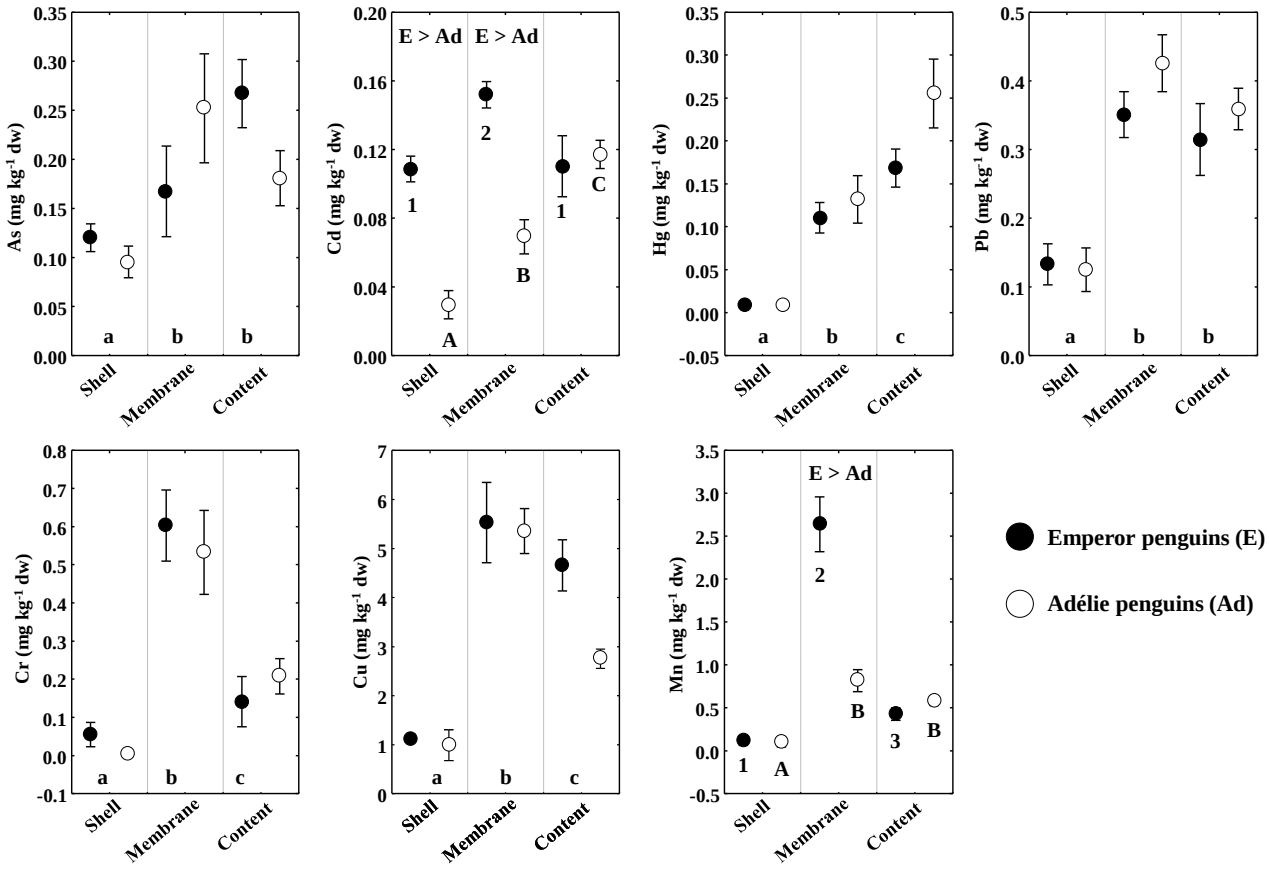

Fig. 3. Mean (± standard error) concentration of trace elements analysed in the different egg parts of Emperor (black circles) and Adélie penguins (white circles). Results of the univariate PERMANOVA pair-wise tests are also showed: small letters indicate significant differences among egg parts, numbers and capital letters indicate significant differences among egg parts within species (Emperor and Adélie penguins respectively). E > Ad indicates significant differences between species within egg parts.

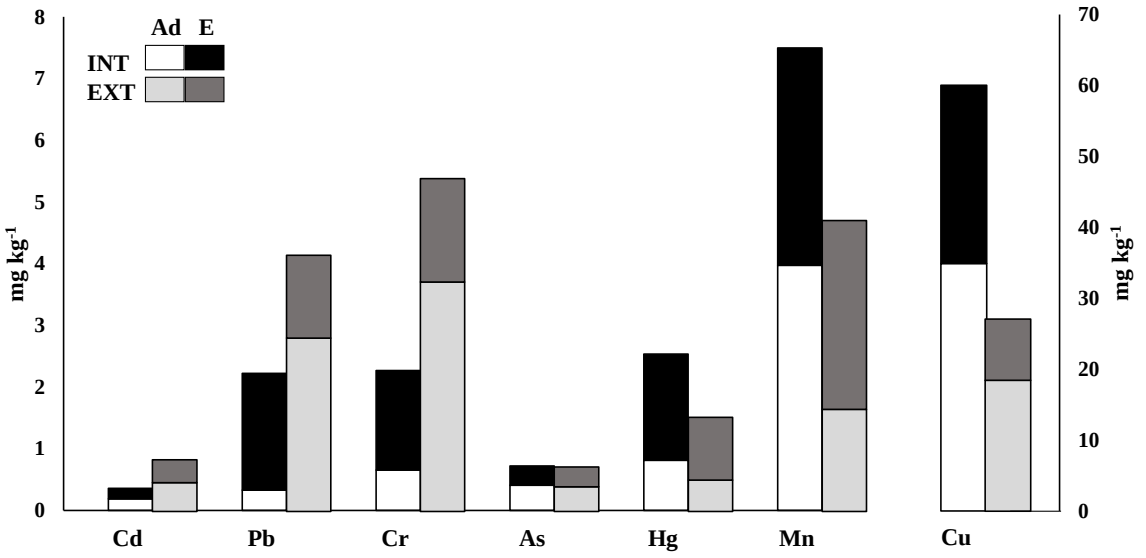

Fig. 4. Mean concentration of trace elements in the internal (muscle, internal organs and egg content) *vs*. external (feathers, eggshell and membrane) compartments of Emperor (E; black and dark grey bars) and Adélie penguins (Ad; white and light grey bars).

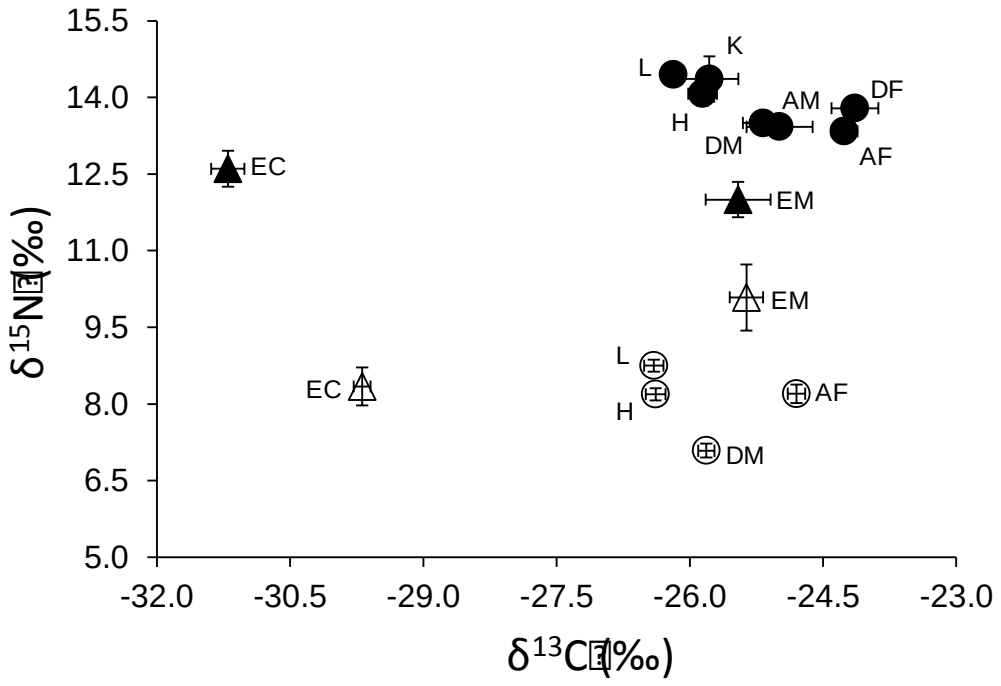

Fig. 5. C and N stable isotopes (mean ± standard error among specimens) of body tissues (circles) and egg parts (triangles) of Emperor (black symbols) and Adélie (empty symbols) penguins. EC and EM: egg content and egg membrane respectively. L: Liver, H: Heart, K: Kidney, AM and DM: abdominal and dorsal muscle respectively, AF and DF: axillary and dorsal feathers respectively. Details on isotopic values can be found in Table 1.