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RESEARCH ARTICLE

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Quantifying capital versus income breeding: New promise with stable isotope measurements of individual amino acids

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Abstract

1. Capital breeders accumulate nutrients prior to egg development, then use these stores to support offspring development. In contrast, income breeders rely on local nutrients consumed contemporaneously with offspring development. Understanding such nutrient allocations is critical to assessing life-history strategies and habitat use.
2. Despite the contrast between these strategies, it remains challenging to trace nutrients from endogenous stores or exogenous food intake into offspring. Here, we tested a new solution to this problem.
3. Using tissue samples collected opportunistically from wild emperor penguins *Aptenodytes forsteri*, which exemplify capital breeding, we hypothesized that the stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope values of individual amino acids (AAs) in endogenous stores (e.g. muscle) and in egg yolk and albumen reflect the nutrient sourcing that distinguishes capital versus income breeding. Unlike other methods, this approach does not require untested assumptions or diet sampling.
4. We found that over half of essential AAs had $\delta^{13}C$ values that did not differ between muscle and yolk or albumen, suggesting that most of these AAs were directly routed from muscle into eggs. In contrast, almost all non-essential AAs differed in $\delta^{13}C$ values between muscle and yolk or between muscle and albumen, suggesting de novo synthesis. Over half of AAs that have labile nitrogen atoms (i.e. 'trophic' AA) had higher $\delta^{15}N$ values in yolk and albumen than in muscle, suggesting that they were transaminated during their routing into egg tissue. This effect was smaller for AAs with less labile nitrogen atoms (i.e. 'source' AA).
5. Our results indicate that the $\delta^{15}N$ offset between trophic-source AAs ($\Delta^{15}N_{trophic\text{-}source}$) may provide an index of the extent of capital breeding. The value of emperor penguin $\Delta^{15}N_{Pro\text{-}Phe}$ was higher in yolk and albumen than in muscle, reflecting the mobilization of endogenous stores; in comparison, the value of $\Delta^{15}N_{Pro\text{-}Phe}$ was similar across muscle and egg tissue in previously published data for income-breeding herring gulls *Larus argentatus smithsonianus*. Our results provide a quantitative

basis for using AA $\delta^{13}C$ and $\delta^{15}N$, and isotopic offsets among AAs (e.g. $\Delta^{15}N_{Pro\text{-}Phe}$), to explore the allocation of endogenous versus exogenous nutrients across the capital versus income spectrum of avian reproduction.

KEYWORDS

carbon-13, compound-specific isotope analysis, CSIA, discrimination, fasting, fractionation, nitrogen-15

1 | INTRODUCTION

Allocating stored nutrients to egg formation can alleviate the challenge of accumulating resources at the time or place of reproduction. This 'capital' breeding strategy contrasts with 'income' breeding, in which individuals rely directly on exogenous food sources consumed concomitantly with breeding to build offspring (Drent & Daan, 1980). Understanding such nutrient allocations during reproduction is valuable because it illuminates the evolution of life-history strategies and the relative importance of different habitats and food sources at different points during the annual cycle, a key factor in organismal responses to environmental shifts (Jenouvrier et al., 2014). However, tracing allocation of endogenous and exogenous nutrients to reproduction in wild animals is challenging. In birds, these allocations have been assessed using mass balance (reviewed by Hobson, 2006) and with the measurement of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotope values of diets, endogenous reserves (e.g. skeletal muscle) and egg components (Hobson, 2006; Hupp et al., 2018; Warner et al., 2008). The latter approach requires that endogenous reserves not acquired during reproduction at the breeding grounds have a different isotopic composition than the local diet at the time of egg formation (e.g. Hobson & Jehl, 2010; Hobson et al., 2005), and/or that isotopic discrimination associated with the mobilization of endogenous nutrients and egg synthesis yields unique isotopic patterns in egg components (Bond & Diamond, 2010).

Although stable isotope analysis of bulk tissues has advanced the field, it requires dietary isotopic values from multiple study sites or time points or both. In addition, bulk tissue analysis averages the isotopic composition of all constituent macromolecules. This averaging is problematic because compounds within macromolecules (e.g. amino acids or 'AA' within proteins) participate in different biochemical pathways and can vary widely in isotope values (McMahon & Newsome, 2019; Whiteman et al., 2019). Animals can synthesize non-essential AAs (AA_{NESS}) using carbon from a variety of sources, whereas they cannot synthesize essential AAs (AA_{ESS}). As a result, animal tissue AA_{ESS} $\delta^{13}C$ values reflect primary producers at the base of food webs. Similarly, 'source' AAs retain their amino nitrogen throughout metabolic processing and thus have $\delta^{15}N$ values that remain mostly unaltered among primary producers and consumers (O'Connell, 2017). In contrast, amino nitrogen of 'trophic' AAs can be replaced during metabolic processing; because animals preferentially excrete ^{14}N in their waste products, their pool of available nitrogen usually has a higher $\delta^{15}N$ value than dietary sources (Balter et al., 2006), and therefore this replacement causes a systematic increase in trophic AA $\delta^{15}N$ values with trophic level. Two AAs, Gly and Ser, exhibit $\delta^{15}N$ patterns that fall between trophic and source AAs (O'Connell, 2017). Overall, consumer tissue provides information about their diet and trophic ecology via AA_{NESS} $\delta^{13}C$ and trophic AA $\delta^{15}N$, and about the base of the food web via AA_{ESS} $\delta^{13}C$ and source AA $\delta^{15}N$ (Whiteman et al., 2019).

In this study, we tested AA isotope analysis of body and egg tissues as a novel method to quantify allocation of nutrient sources to eggs in the context of capital versus income reproduction. This method offers the potential benefits of not requiring diet sampling and of having expectations based on well-described anabolic and catabolic pathways for individual AAs. Using samples collected from emperor penguins *Aptenodytes forsteri*, we compared AA $\delta^{13}C$ and $\delta^{15}N$ values among the primary endogenous protein store (skeletal muscle; Robin et al., 1988) and eggs (yolk and albumen). Emperor penguin females fast at their colony for approximately 3 weeks (Ancel et al., 2013; Groscolas, 1990; Groscolas et al., 1986) then synthesize yolk for their single egg over ~21 days, with albumen synthesis on the final day (Adams, 1992; Ancel et al., 2013). Yolk protein is primarily derived from liver vitellogenin (Deeley et al., 1975), and albumen protein from the oviduct (Muramatsu et al., 1991). Both sites of protein production likely draw their AAs from the body pool of free AAs, which is primarily maintained during fasting by the input of mobilized AAs from skeletal muscle (Cherel et al., 1994; Spargo et al., 1979). A capital breeder such as emperor penguins may therefore have similar isotope values for yolk and albumen. In contrast, in income breeding, variation in protein isotope values between yolk and albumen can reflect diet items that were consumed during the different time periods in which these tissues were synthesized (Hobson & Jehl, 2010).

We hypothesized that the physiological processes distinguishing capital from income breeding have differential effects on AA $\delta^{13}C$ and $\delta^{15}N$ values. Dietary trophic AAs should be subjected to nitrogen discrimination twice in capital breeders. The first instance occurs during assimilation from diet into body tissue, and the second occurs when these AAs are mobilized from body tissue into the endogenous pool of free AAs where they can be used for incorporation into eggs or for biochemical processes such as gluconeogenesis to maintain blood glucose levels during fasting (Castellini & Rea, 1992; Cherel et al., 1988, 1992). This second round of discrimination, during mobilization from endogenous stores (e.g. as fasting penguins 'feed on themselves'; Cherel et al., 2005), should be unique to capital breeders and result in

higher $\delta^{15}N$ values for trophic AAs in their eggs relative to their endogenous stores than in income breeders. For carbon, income breeders likely source AA_{NESS} via the same pathway for both their own tissues and for their eggs, whether routing them intact from the diet or synthesizing them de novo from exogenous carbon. In contrast, capital breeders should be more likely to synthesize AA_{NESS} for their egg tissues using carbon from stored lipids, which provide the largest labile carbon pool in the body and which are ^{13}C depleted compared to protein and carbohydrates (DeNiro & Epstein, 1977). In comparison to these expected trends for trophic AAs and AA_{NESS}, capital breeding should cause minimal isotopic discrimination for the $\delta^{13}C$ and $\delta^{15}N$ values of AAs classified as both essential and source (i.e. Phe and Lys).

The general patterns summarized above lead to specific predictions of how AA isotope values may differ in capital versus income breeding species:

1. In capital breeding, AA_{ESS} will be routed from muscle to yolk and albumen without significant isotopic discrimination, resulting in similar $\delta^{13}C$ values across these tissues. In income breeding, these tissues may or may not have similar $\delta^{13}C$ values, depending on the $\delta^{13}C$ values of the dietary sources consumed during egg growth.
2. In capital breeding, AA_{NESS} will either be routed from muscle (similar to AA_{ESS}) or synthesized de novo for eggs with a combination of protein- and lipid-derived carbon, resulting in similar or lower $\delta^{13}C$ values in yolk and albumen as compared to muscle. In income breeding, the relationship between $\delta^{13}C$ values in yolk, albumen and muscle will be unpredictable because AAs may be routed from endogenous tissue or synthesized de novo from a variety of dietary carbon sources.
3. In capital breeding, source AAs will have similar $\delta^{15}N$ values across muscle and egg tissues if their $\delta^{13}C$ values suggest direct routing from muscle. In income breeding, source AAs in yolk, albumen and muscle will have $\delta^{15}N$ values that reflect dietary intake during the period of tissue synthesis.
4. In capital breeding, trophic AAs will have higher $\delta^{15}N$ values in yolk and albumen than in muscle because of the exchange of their amino nitrogen with the enriched endogenous nitrogen pool. In income breeding, trophic AAs will have similar $\delta^{15}N$ values across yolk, albumen and muscle because the amino nitrogen in AAs will be subjected to similar metabolic processing in all of these tissues.

As a result of predictions three and four above, we also predict that the offset in $\delta^{15}N$ values between trophic and source AAs will be higher in egg tissue than in muscle during capital breeding. The $\Delta^{15}N_{Glx\text{-}Phe}$ offset ('Glx' refers to both glutamine and glutamic acid) in consumer tissue is commonly used as a proxy for trophic level (Chikaraishi et al., 2009) because $\delta^{15}N_{Glx}$ values increase during metabolic processing by consumers at each trophic level, whereas $\delta^{15}N_{Phe}$ values typically do not (O'Connell, 2017). Recent studies suggest that $\Delta^{15}N_{Pro\text{-}Phe}$ may exhibit a similar trend (McMahon & McCarthy, 2016; Whiteman et al., 2019). Here, instead of trophic level, we interpret these offsets in yolk and albumen (as compared to offsets in muscle) as proxies for capital breeding that reflect the extent to which muscle AAs provide the raw materials to synthesize AAs in egg tissues. We compare the offsets in emperor penguins to data previously reported for free-ranging herring gulls *Larus argentatus smithsonianus*, an income-breeding species that feeds throughout egg development (Hebert et al., 2016).

2 | MATERIALS AND METHODS

2.1 | Field sampling

Eggs and skeletal muscle were opportunistically collected in four different years from female penguin carcasses after natural mortalities during egg laying in the breeding colony of Pointe Géologie (66°40′S, 140°01′E), Adélie Land, Antarctica (Prévost, 1961). It was possible in only one instance to collect an egg that was clearly associated with a carcass that was also sampled for skeletal muscle; the data for these paired yolk, albumen and muscle samples are highlighted throughout the results and are presented in combination with data pooled from multiple eggs and muscle samples. Total sample sizes included: yolk and albumen samples from eggs collected in 2011 ($N = 3$) and 2014 ($N = 5$); and muscle samples collected in 2008 ($N = 3$), 2010 ($N = 2$) and 2011 ($N = 3$).

Eggs and pectoral muscle from carcasses were stored at −20°C before sample processing. Egg albumen and yolk were separated after thawing the egg. All tissues were freeze-dried then powdered. Lipids were removed from muscle samples using cyclohexane and from yolk using 2:1 chloroform:methanol (6 ml of solution per 100 mg tissue; 3 × 24 hr). Albumen has a low lipid content and did not require treatment.

2.2 | Stable isotope analysis

Samples were analysed at the Center for Stable Isotopes (University of New Mexico, Albuquerque, NM, USA). Compound-specific isotopic analysis of individual AAs followed Whiteman et al. (2019). Briefly, samples were weighed out to ~5–20 mg and hydrolysed in 1 ml of 6 N HCl for 20 hr at 110°C. During hydrolysis, asparagine was converted to aspartic acid (Asx) and glutamine was converted to glutamic acid (Glx). The resulting hydrolysate was derivatized to *N*-trifluoracetic acid isopropyl esters. Samples were injected (1 μl) into a gas chromatograph (Thermo Scientific Trace 1300 GC; column BPx5, 60 m, ID 0.32 mm, film thickness 1.0 μm) and separated AAs were then combusted to CO_2 or reduced to N_2 at 1,000°C and analysed on an isotope ratio mass spectrometer (Thermo Scientific Delta V Plus).

We injected each sample twice, interspersed with standard injections (AAs of known isotopic values; Sigma-Aldrich Co.) that bracketed either two samples ($\delta^{13}C$) or one sample ($\delta^{15}N$). Replicate sample injections had mean *SD* of 0.2 ‰ for $\delta^{13}C$ and 0.3 ‰ for $\delta^{15}N$

values. Standardization across runs was achieved with intermittent pulses of CO_2 or N_2 gases of known isotopic value. To account for the addition of carbon to AAs and for the fractionation associated with derivatization, we corrected $\delta^{13}C$ values as follows:

$$\delta^{13}C_{USA} = \left(\delta^{13}C_{DSA} - \delta^{13}C_{DST} + \left(\delta^{13}C_{UST} \times P\right)\right) \times P^{-1}, \quad (1)$$

$\delta^{13}C_{USA}$ is the calculated value for the underivatized sample; $\delta^{13}C_{DSA}$ is the measured value for the derivatized sample; $\delta^{13}C_{UST}$ is the known value of the underivatized standard; and P is the known proportion of carbon in the AAs from the original sample. Correction of $\delta^{15}N$ values was less complex because derivatization does not add nitrogen atoms to AA:

$$\delta^{15}N_{USA} = \left(\delta^{15}N_{DSA} + \left(\delta^{15}N_{DST} - \delta^{15}N_{UST}\right)\right). \quad (2)$$

For data analysis (henceforth: yolk and muscle refer to lipid-free samples), we first compared the $\delta^{13}C$ and $\delta^{15}N$ values of each AA between yolk and albumen with paired *t* tests because these tissues were sampled from the same egg. Second, for each AA, we compared $\delta^{13}C$ and $\delta^{15}N$ values in muscle among 2008, 2010 and 2011 using a Kruskal–Wallis rank sum test followed by the Dunn's pairwise test. Third, for each AA, we pooled samples from different years and compared $\delta^{13}C$ and $\delta^{15}N$ values among muscle, yolk and albumen using a one-way ANOVA followed by a Tukey's HSD. In this step, we compared the distribution of the pooled data to the data points of the muscle, yolk and albumen that were collected from a single individual. Fourth, we calculated the offsets of $\Delta^{15}N_{Glx\text{-}Phe}$ and $\Delta^{15}N_{Pro\text{-}Phe}$ for muscle, yolk and albumen, then compared offsets among tissues with a one-way ANOVA followed by a Tukey's HSD pairwise test. We calculated the same offsets for herring gulls after extracting $\delta^{15}N$ values for AAs in muscle and egg homogenate (yolk and albumen combined) from figure 3 in Hebert et al. (2016). We compared herring $\Delta^{15}N_{Glx\text{-}Phe}$ and $\Delta^{15}N_{Pro\text{-}Phe}$ between muscle and egg homogenate using two-tailed *t*-tests. We set $\alpha = 0.05$ and analysed statistics in Program R (R Core Team, 2018).

3 | RESULTS

Table 1 summarizes the predicted and observed differences among isotope values for emperor penguin muscle, yolk and albumen.

3.1 | $\delta^{13}C$ values

Most AA $\delta^{13}C$ values did not differ between years: across all tissues, 33 of 39 comparisons between years were not significant (Tables S1–S3). In muscle, no AA $\delta^{13}C$ values differed among 2008, 2010 and 2011; in yolk, $\delta^{13}C$ differed between 2011 and 2014 for Ser, Lys, Pro and Val; and in albumen, $\delta^{13}C$ differed between 2011 and 2014 for Phe and Asp. For the AAs that differed between years, three were higher in 2011 and three were higher in 2014. Based on the general consistency of $\delta^{13}C$ values across years, and that yolk and albumen samples were paired and came from the same egg, we pooled data across years. Using these pooled data, yolk and albumen differed in how their AA_{ESS} $\delta^{13}C$ values compared to muscle

TABLE 1 Predicted and observed differences in $\delta^{13}C$ and $\delta^{15}N$ values of individual AAs for egg yolk and egg albumen versus skeletal muscle, in emperor penguins. Each AA is classified based on nitrogen and carbon dynamics; for carbon, NESS is non-essential and ESS is essential. Because tyrosine is synthesized from the essential AA phenylalanine, here it is classified as essential. The 'Predicted' columns indicate the expected direction of the difference between egg tissues and muscle (↔ indicates no difference). The 'Yolk' and 'Albumen' columns indicate the observed direction and magnitude of this difference (three arrows: >6‰, two arrows: 3–6 ‰, one arrow: <3‰); '↔' indicates no significant difference

Classification			$\delta^{13}C$			$\delta^{15}N$		
$\delta^{13}C$	$\delta^{15}N$	AA	Predicted	Yolk	Albumen	Predicted	Yolk	Albumen
NESS	Trophic	Pro	↓	↑	↔	↑	↑↑	↑↑
NESS	Trophic	Ala	↓	↔	↓	↑	↑	↑↑
NESS	Trophic	Glx	↓	↔	↓	↑	↑	↔
NESS	Trophic	Asx	↓	↔	↓↓	↑	↔	↔
ESS	Trophic	Val	↔	↑	↔	↑	↑↑	↑
ESS	Trophic	Leu	↔	↔	↔	↑	↑	↑
ESS	Trophic	Ile	↔	↔	↔	↑	↔	↔
ESS	Metabolic	Thr	↔	↑↑	↑	↓	↔	↔
NESS	Unclassified	Gly	↓	↓↓	↓	↑/↔	↑↑↑	↑↑↑
NESS	Unclassified	Ser	↓	↓↓↓	↓↓	↑/↔	↑↑↑	↑↑↑
ESS	Source	Phe	↔	↑↑	↔	↔	↑	↑
ESS	Source	Tyr	↔	↔	↔	↔	↔	↑↑
ESS	Source	Lys	↔	↔	↔	↔	↑	↑

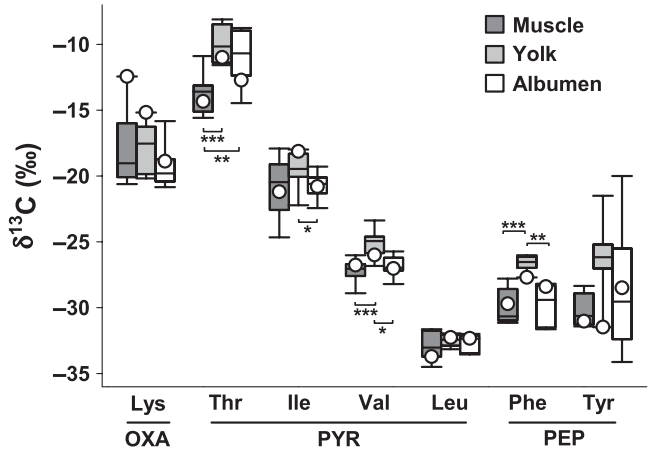

FIGURE 1 $\delta^{13}C$ values for essential AAs in skeletal muscle, egg yolk and egg albumen from emperor penguins. AAs are grouped by precursor molecule for de novo synthesis: OXA = oxaloacetate, PYR = pyruvate, PEP = phosphoenolpyruvate. Yolk and albumen samples were from the same egg ($N = 8$ eggs). Muscle samples ($N = 8$ penguins) were not paired to yolk and albumen samples (i.e. muscle samples did not come from the penguin that laid the egg) with one exception: white circles represent the data for a single penguin from which both muscle and an egg were collected. All samples were collected in 2008, 2010, 2011 and 2014. For rectangles: bars, boxes and error bars indicate the median, 25th and 5th percentiles respectively. Asterisks indicate significance of comparisons: ***$p < 0.001$, **$0.001 < p < 0.01$, *$0.01 < p < 0.05$. For each AA, yolk and albumen were compared with a paired *t* test, and muscle was compared to yolk and albumen with Tukey's HSD pairwise comparisons after a one-way ANOVA

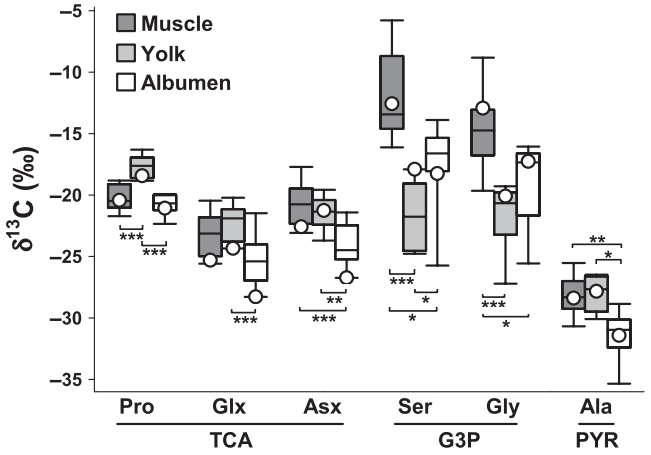

FIGURE 2 $\delta^{13}C$ values for non-essential AAs in skeletal muscle, egg yolk and egg albumen from emperor penguins. AAs are grouped by precursor molecule for de novo synthesis: TCA = tricarboxylic acid cycle, G3P = glyceraldehyde-3-phosphate, PYR = pyruvate. Yolk and albumen samples were from the same egg ($N = 8$ eggs). Muscle samples ($N = 8$ penguins) were not paired to yolk and albumen samples (i.e. muscle samples did not come from the penguin that laid the egg) with one exception: white circles represent the data for a single penguin from which both muscle and an egg were collected. All samples were collected in 2008, 2010, 2011 and 2014. For rectangles: bars, boxes and error bars indicate the median, 25th and 5th percentiles respectively. Asterisks indicate significance of comparisons: ***$p < 0.001$, **$0.001 < p < 0.01$, *$0.01 < p < 0.05$. For each AA, yolk and albumen were compared with a paired *t* test, and muscle was compared to yolk and albumen with Tukey's HSD pairwise comparisons after a one-way ANOVA

(Figure 1). For yolk, four AA_{ESS} (Lys, Ile, Leu and Tyr) had $\delta^{13}C$ values that were similar to muscle, consistent with routing of these AA_{ESS} from muscle to yolk. Surprisingly, three AA_{ESS} (Thr, Val and Phe) had higher $\delta^{13}C$ values in yolk than in muscle, indicating that these AA_{ESS} either experienced fractionation during their routing from muscle, or were sourced from other endogenous tissue. In contrast to yolk, almost all AA_{ESS} (except Thr) in albumen had $\delta^{13}C$ values that were similar to muscle, suggesting their routing from muscle to albumen.

Yolk and albumen also differed in how their AA_{NESS} $\delta^{13}C$ values compared to muscle (Figure 2). For yolk, three AA_{NESS} (Glx, Asx and Ala) had $\delta^{13}C$ values that were similar to muscle, suggesting that they were routed between these two tissues. Three AA_{NESS} had $\delta^{13}C$ values that differed between muscle and yolk, indicating that in yolk they had been synthesized with carbon that was either a) lower in $\delta^{13}C$ value than in muscle and thus potentially lipid-derived (Ser and Gly); or b) higher in $\delta^{13}C$ value and thus from an unknown source or subjected to fractionation (Pro). For albumen, two AA_{NESS} (Pro and Glx) had $\delta^{13}C$ values that were similar to muscle and therefore appeared to be routed between tissues. The remaining four AA_{NESS} (Asx, Ser, Gly and Ala) had lower $\delta^{13}C$ values in albumen than in muscle, likely reflecting that they had been synthesized with carbon from endogenous lipid stores (e.g. adipose tissue).

3.2 | $\delta^{15}N$ values

Most AA $\delta^{15}N$ values did not differ between years: across all tissues, 34 of 39 comparisons between years were not significant (Tables S4–S6). In muscle, $\delta^{15}N$ differed between 2008 and 2010 for Phe; in yolk, $\delta^{15}N$ differed between 2011 and 2014 for Glu and Thr; and in albumen, $\delta^{15}N$ differed between 2011 and 2014 for Lys and Ala. For the AAs that differed between years, one was highest in 2008, two were highest in 2011 and two were highest in 2014. Based on the general consistency of $\delta^{15}N$ values across years, and that yolk and albumen samples were paired, we pooled data across years, similar to $\delta^{13}C$. Using these pooled data, both yolk and albumen had substantially higher $\delta^{15}N$ values than muscle for Gly and Ser (Figure 3), suggesting that the nitrogen of these two AAs came from an endogenous pool with a high $\delta^{15}N$ value. This origin would be consistent with extensive de novo synthesis of Gly and Ser for yolk and albumen as suggested by their low $\delta^{13}C$ values relative to that of muscle.

Surprisingly, $\delta^{15}N$ values of the source AAs (Phe, Tyr, Lys) were also higher in yolk and albumen than in muscle, except for Tyr in yolk (Figure 3). Four trophic AAs (Pro, Ala, Val and Leu) had higher $\delta^{15}N$ values in yolk and albumen than in muscle (Figure 4), suggesting that their amino nitrogen originated from an enriched endogenous pool; Glx exhibited a higher $\delta^{15}N$ value in yolk than in muscle but not in

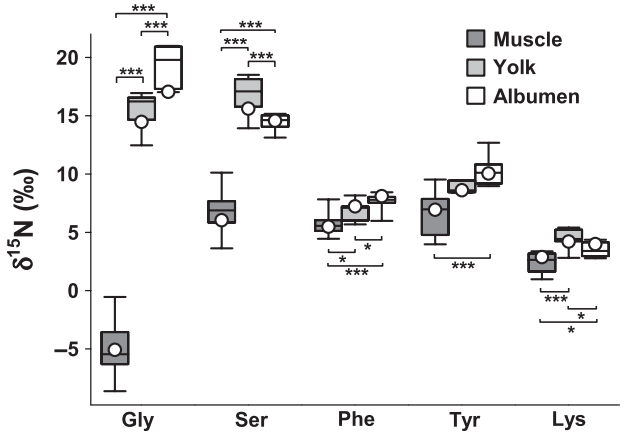

FIGURE 3 $\delta^{15}N$ values for Gly, Ser and source AAs (Phe, Tyr, Lys) in samples of skeletal muscle, egg yolk and egg albumen from emperor penguins. Yolk and albumen samples were from the same egg ($N = 8$ eggs, except $N = 3$ for Tyr yolk). Muscle samples ($N = 8$ penguins) were not paired to yolk and albumen samples (i.e. muscle samples did not come from the penguin that laid the egg) with one exception: white circles represent the data for a single penguin from which both muscle and an egg were collected. All samples were collected in 2008, 2010, 2011 and 2014. For rectangles: bars, boxes and error bars indicate the median, 25th and 5th percentiles respectively. Asterisks indicate significance of comparisons: ***$p < 0.001$, **$0.001 < p < 0.01$, *$0.01 < p < 0.05$. For each AA, yolk and albumen were compared with a paired t test, and muscle was compared to yolk and albumen with Tukey's HSD pairwise comparisons after a one-way ANOVA

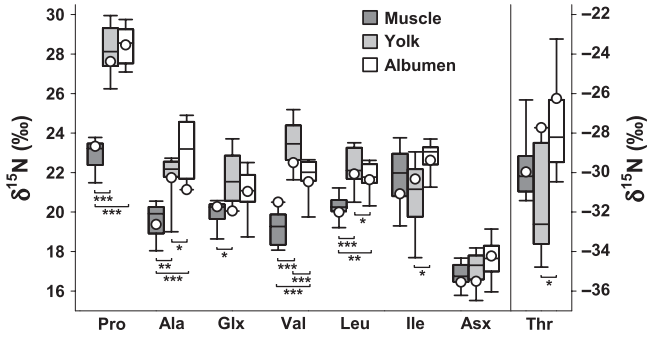

FIGURE 4 $\delta^{15}N$ values of trophic AAs, and separately of Threonine (Thr), in samples of skeletal muscle, egg yolk and egg albumen from emperor penguins. Yolk and albumen samples were from the same egg ($N = 8$ eggs). Muscle samples ($N = 8$ penguins) were not linked to yolk and albumen samples (i.e. they did not come from the penguin that laid the egg) with one exception: white circles represent the data for a single penguin from which both muscle and an egg were collected. All samples were collected in 2008, 2010, 2011 and 2014. For rectangles: bars, boxes and error bars indicate the median, 25th and 5th percentiles respectively. Asterisks indicate significance of comparisons: ***$p < 0.001$, **$0.001 < p < 0.01$, *$0.01 < p < 0.05$. For each AA, yolk and albumen were compared with a paired t test, and muscle was compared to yolk and albumen with Tukey's HSD pairwise comparisons after a one-way ANOVA

albumen. For the remaining trophic AAs (Ile and Asx) and Thr, yolk and albumen did not differ from muscle, consistent with routing of these AAs from muscle into the egg. For Glx and Asx, the similarity in

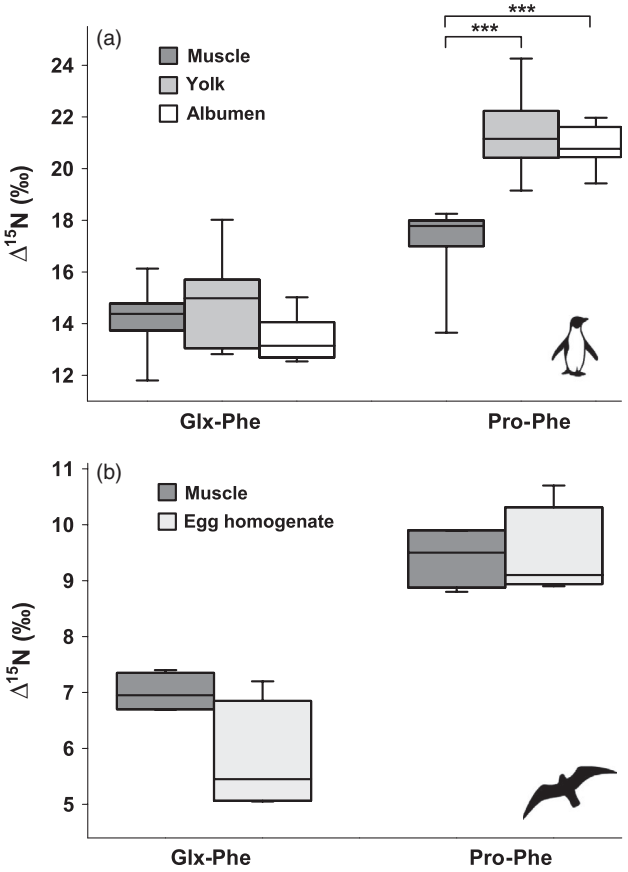

FIGURE 5 Offsets in $\delta^{15}N$ values ($\Delta^{15}N$) between trophic AAs (Glx, Pro) and source AAs (Phe). Bars, boxes and error bars indicate the median, 25th and 5th percentiles respectively. (a) Offsets in skeletal muscle, egg yolk and egg albumen in emperor penguins ($N = 8$ samples for each tissue type). Asterisks indicate significance of comparisons within Glx-Phe and Pro-Phe groups via Tukey's HSD pairwise comparison (***$p < 0.001$) after a one-way ANOVA. (b) Offsets in skeletal muscle and egg homogenate in herring gulls ($N = 4$); data are from Hebert et al. (2016)

their $\delta^{15}N$ values between muscle and albumen is surprising because their low $\delta^{13}C$ values relative to muscle suggest that these AAs are synthesized de novo for albumen from ^{13}C-depleted lipid-derived carbon.

In emperor penguins, $\Delta^{15}N_{Glx\text{-}Phe}$ offsets were similar across muscle, yolk and albumen (one-way ANOVA, $p = 0.135$, $F = 2.205$) but $\Delta^{15}N_{Pro\text{-}Phe}$ offsets were larger in yolk and albumen than in muscle (one-way ANOVA, $p < 0.001$, $F = 23.300$; Figure 5a). In contrast, published data from herring gulls (Hebert et al., 2016) show that both $\Delta^{15}N_{Glx\text{-}Phe}$ and $\Delta^{15}N_{Pro\text{-}Phe}$ offsets were similar in muscle and in egg homogenate ($\Delta^{15}N_{Glx\text{-}Phe}$, t test, $p = 0.089$; $\Delta^{15}N_{Pro\text{-}Phe}$, t test, $p = 0.962$; Figure 5b). In other words, for a capital breeder (emperor penguins), $\Delta^{15}N_{Pro\text{-}Phe}$ offsets were greater in egg tissue than in muscle, whereas for an income breeder (herring gulls), $\Delta^{15}N_{Pro\text{-}Phe}$ offsets were similar in egg tissue and in muscle. This result reflected the general trend for $\delta^{15}N$ values to be higher in egg tissues than in muscle for trophic AAs (as well as Gly and Ser) in penguins but not in gulls (Figure 6).

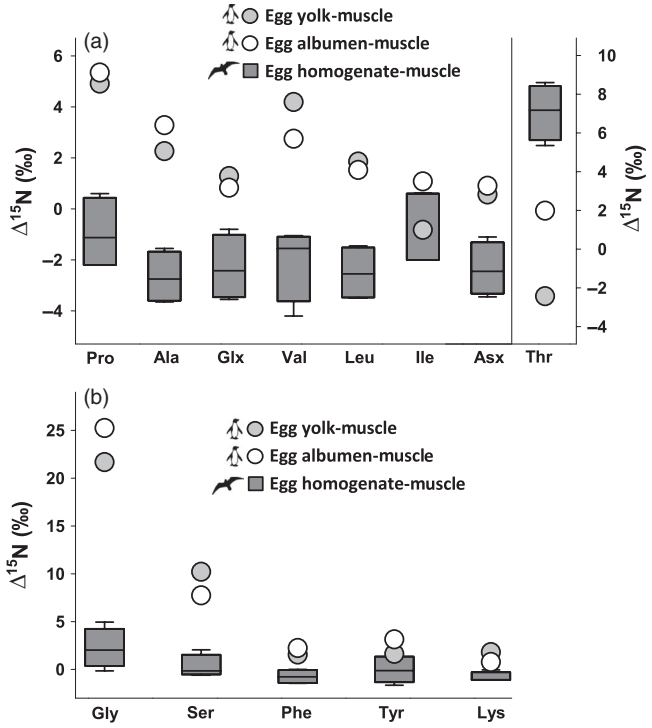

FIGURE 6 Differences in $\delta^{15}N$ values ($\Delta^{15}N$) between egg tissues and muscle, in an income breeder (herring gull; Hebert et al., 2016) and a capital breeder (emperor penguin), for (a) trophic AAs and threonine and for (b) Gly and Ser and source AAs (Phe, Tyr, Lys). For gulls, egg homogenate and muscle $\delta^{15}N$ values were compared from the same individual ($N = 4$). For penguins, the median $\delta^{15}N$ value was compared between egg yolk or albumen ($N = 8$) and muscle from unrelated individuals ($N = 8$), except for one individual which was sampled for both muscle and egg (see text for details). For gulls, bars are medians and boxes and error bars indicate 25th and 5th percentiles. In general, values near 0‰ are expected to be consistent with routing nutrients from the diet to egg tissue, whereas positive values are expected to be consistent with routing endogenous nutrients to egg tissue

4 | DISCUSSION

Overall, our results were consistent with many of our predictions and broadly support the use of differences in AA isotope values between endogenous protein stores and egg tissues to trace nutrient sourcing in the context of capital and income breeding (Table 1). Our data must be interpreted with some caution because samples of emperor penguin eggs and muscle were primarily collected from different individuals in different years. However, results were similar between a) pooled data representing eggs that were not associated with muscle samples from specific penguins, and b) data from egg and muscle samples collected from a single individual. This consistency, in combination with relatively small variation in isotope values across sampling years, suggests that our inferences based on pooled data are robust.

Over half of AA_{ESS} had similar $\delta^{13}C$ values across muscle, yolk and albumen, suggesting that they were routed from endogenous proteins to egg tissues, as expected because emperor penguins cannot synthesize these AAs. Several AA_{ESS} differed in $\delta^{13}C$ values across tissues, which is surprising because these AAs must be obtained from dietary sources; below we discuss the possibility that catabolism-driven fractionation caused this discrimination. In contrast to AA_{ESS}, all AA_{NESS} except for Glx differed in $\delta^{13}C$ values between muscle and yolk or between muscle and albumen, as would be expected when these AAs are synthesized de novo to build egg tissue. While source AAs unexpectedly had higher $\delta^{15}N$ values in yolk and albumen than in muscle, these differences were small in comparison to the differences among tissues in $\delta^{15}N$ values for trophic AAs, which more readily exchange their nitrogen with the ^{15}N-enriched body pool.

Our data suggest that overall, the AAs deposited during yolk production experienced more fractionation than the AAs in albumen. Conversely, the AAs in albumen were more likely to have been routed from muscle or synthesized de novo from lipid-derived carbon. These inferences indicate that even for a species that is exclusively capital breeding, the sourcing pathways of AAs for protein synthesis in various egg components or fetal tissues can vary. The synthesis of AAs with lipid-derived carbon emphasizes that studies of nutrient allocation during reproduction and fasting should consider carbon transfer between endogenous stores of lipid and protein (McMahon et al., 2015; Newsome et al., 2014).

We found that $\Delta^{15}N_{Pro\text{-}Phe}$ offsets were substantially larger in egg tissues than in muscle, reflecting the isotopic discrimination that occurs when mobilizing endogenous resources. In comparison, $\Delta^{15}N_{Pro\text{-}Phe}$ offsets were similar across egg tissue and muscle in herring gulls (Hebert et al., 2016). Emperor penguins are representative of capital breeding because their reproductive fast precludes income contribution to egg tissue (Ancel et al., 2013). Although the reproductive strategy of herring gulls is less well-documented, they feed during egg development and are thought to be primarily income breeders (Bond & Diamond, 2010; Hebert et al., 2016). Differences in $\Delta^{15}N_{Pro\text{-}Phe}$ between gulls and the emperor penguins in this study therefore suggest that nitrogen isotope offsets between this trophic-source pair of AAs provides an index of capital breeding. Further validation of this index is important because use of endogenous stores for reproduction is a fundamental aspect of animal life history, and can constrain how wildlife responds to environmental change (Hupp et al., 2018).

4.1 | Yolk AA origins

Of the 13 AAs we investigated, seven appeared to be routed from muscle directly into yolk without carbon isotope discrimination. Four of these were likely directly routed because they are AA_{ESS} (Ile, Leu, Lys and Tyr), which penguins cannot synthesize de novo. The remaining three were AA_{NESS} (Glx, Asx and Ala) that have carbon skeletons which are frequently interconverted with important metabolites (e.g. TCA cycle intermediates). This frequent interconversion could have mixed the carbon in these AAs with larger pools of carbon in other molecules, buffering these AAs from measurable isotope discrimination.

Trophic AAs that were routed directly from muscle to yolk had variable nitrogen dynamics. Ala and Leu appeared to exchange their amino nitrogen with an endogenous pool, resulting in ^{15}N enrichment of these AAs. This enrichment also occurred for Glx, although the effect size was smaller, perhaps because it was partially buffered by the relatively large pool size of Glx. Asx and Ile did not exhibit ^{15}N enrichment. Asx may have been buffered from enrichment because frequent transamination caused by its participation in the urea cycle (Nelson & Cox, 2008) constantly exchanges its amino nitrogen. Ile may have avoided enrichment because its physiological importance as a branched-chain AA can spare it from extensive metabolic processing (Holeček et al., 2001). It is unclear why the other two branched-chain AA that we measured, Leu and Val, would not have been similarly spared, although it is noteworthy that they share a precursor (alpha-ketoisovalerate; Nelson & Cox, 2008).

Source AAs that were routed directly from muscle into yolk also had variable nitrogen dynamics. As expected for a source AA, Tyr did not differ between muscle and yolk. However, Phe and Lys were ^{15}N enriched in yolk. Because emperor penguins had no access to exogenous AAs, we suggest that this enrichment reflected catabolism of Phe and Lys for use as oxidative fuel. Emperor penguins primarily oxidize stored lipids during their breeding fast (Robin et al., 1988); however, some AA catabolism is unavoidable (Owen et al., 1998). The complete oxidation of free AAs into CO_2 via processes like gluconeogenesis may favour AAs containing the lighter nitrogen isotope. This process would leave AAs with higher $\delta^{15}N$ values available for deposition into new tissue. It is unclear why this process would not similarly affect the source AA Tyr, although perhaps the ability of animals to synthesize Tyr from Phe (Nelson & Cox, 2008) reduces enrichment caused by catabolism.

Of the 13 AAs we investigated, six differed in $\delta^{13}C$ values between muscle and yolk. Of these six, Ser and Gly, both AA_{NESS}, had the lowest $\delta^{13}C$ values and the highest $\delta^{15}N$ values, suggesting extensive de novo synthesis using lipid-derived carbon and endogenous recycled nitrogen. Widespread de novo synthesis of Gly is plausible because of the high demand for this AA in birds for incorporation into (a) uric acid, their primary nitrogenous waste product (Taylor et al., 1994) and (b) eggshell membranes (Zhao & Chi, 2009). Because Gly and Ser are readily interconverted via a single reaction, their isotope values tend to be similar (O'Connell, 2017). Our results here support the continued distinction of Gly and Ser from other AAs, as their isotope values appear to be sensitive to nitrogen balance and to the primary nitrogen excretionary pathway of the study organism (McMahon & McCarthy, 2016; O'Connell, 2017; Whiteman et al., 2019).

Of the six AAs that differed in $\delta^{13}C$ values between muscle and yolk, four had higher $\delta^{13}C$ values in yolk: the AA_{NESS} Pro and the AA_{ESS} of Thr, Val and Phe. It is possible that these AAs were used by emperor penguins as fuel for aerobic oxidation resulting in isotopic enrichment as explained above. This explanation would be consistent with the enrichment of both ^{13}C and ^{15}N in yolk relative to muscle for Pro, Val and Phe. Intriguingly, higher $\delta^{15}N$ values of Pro were a sensitive indicator of fasting in southern elephant seals *Mirounga leonina* (Lübcker et al., 2020), suggesting that this AA may be particularly responsive to changes in endogenous nitrogen flux. Catabolism-driven discrimination may also explain the enrichment of ^{13}C in yolk Thr, although the processes governing nitrogen isotopic fractionation for this AA remain unclear (Fuller & Petzke, 2017).

Other potential mechanisms to explain higher $\delta^{13}C$ values in yolk than in muscle for Pro, Thr, Val and Phe seem less likely. First, these AAs could have been routed into yolk from exogenous diet items that were ^{13}C enriched relative to endogenous muscle. However, in the Southern Ocean primary producer $\delta^{13}C$ values decline with increasing latitude (Lorrain et al., 2009), therefore the last food items consumed by penguins before their breeding fast were likely relatively low in ^{13}C. In addition, penguins fast for 3 weeks before the initiation of yolk formation (Ancel et al., 2013), long enough to fully metabolize all food items. Second, AAs could have been mobilized from an endogenous tissue that had a different $\delta^{13}C$ value than muscle. However, major proteinaceous tissues other than muscle (e.g. liver) tend to have more rapid carbon turnover (Bauchinger & McWilliams, 2009), which means that the most recently consumed food items should have led to relatively lower $\delta^{13}C$ values, as explained above. Regardless, skeletal muscle is by far the largest protein reservoir in the body (Cherel et al., 1994; Spargo et al., 1979). A third possibility is that AAs were synthesized by gut microbes using alternative carbon sources (Newsome et al., 2011), a phenomenon that appears to occur even in hypercarnivores (Whiteman et al., 2018). However, this explanation would require routing of new AAs from gut microbes exclusively to yolk, a pathway which we are not aware of.

4.2 | Albumen AA origins

In contrast to the 3 weeks required for synthesis of yolk, albumen is produced over a single day (Ancel et al., 2013) shortly before egg laying. This rapid production may cause a high demand for routing of AA_{ESS} from muscle to albumen, which would explain the similar $\delta^{13}C$ values between these two tissues for all measured AA_{ESS} except Thr. As expected for trophic AAs that were mobilized from muscle, Val and Leu exhibited ^{15}N enrichment in albumen relative to muscle. The trophic AAs Ile had surprisingly similar $\delta^{15}N$ values in albumen and muscle, a pattern also observed in the yolk–muscle comparison. As described above for yolk, we speculate that Ile in albumen was spared from metabolic processing because of its role as a branched-chain AA (Holeček et al., 2001).

Despite the expectation that source AAs would be routed from muscle with minimal exchange of nitrogen with endogenous pools, Phe, Lys and Tyr had higher $\delta^{15}N$ values in albumen relative to muscle. We hypothesize that these AAs were enriched in albumen because of fractionation during catabolism for gluconeogenesis, as suggested for yolk. However, unlike yolk, there was no concomitant enrichment in their $\delta^{13}C$ values, potentially because

the time window for catabolism and fractionation is shorter during synthesis of albumen (~1 day) than yolk (~21 days). It is also possible that AA carbon generally experiences less isotopic discrimination than nitrogen because AAs contain several carbon atoms (2–9), whereas most forms only contain a single nitrogen in the amine group. Regardless of the exact mechanisms causing isotopic fractionation, the resulting nitrogen discrimination tended to be smaller for source AAs (0.8–3.1‰) than for trophic AAs (0.8–5.3‰; see Table 1), which is consistent with previous explanations of the biochemical processes that produce patterns in AA $\delta^{15}N$ values (O'Connell, 2017).

The rapid synthesis of albumen may also create a high demand for de novo synthesis of AA_{NESS} using the most plentiful endogenous source of carbon: lipids in adipose tissue. Use of ^{13}C-depleted carbon from lipids would explain why four of six AA_{NESS} (Asx, Ser, Gly and Ala) had lower $\delta^{13}C$ values in albumen than in muscle. For the other two AA_{NESS} (Glx and Pro), it is notable that Glx $\delta^{13}C$ values were lower in albumen than yolk, consistent with at least some synthesis using lipid-derived carbon. Because emperor penguins had likely been fasting for nearly 6 weeks at the time of albumen formation (Adams, 1992; Ancel et al., 2013; Groscolas, 1990; Groscolas et al., 1986), this de novo synthesis would have used ^{15}N-enriched, recycled endogenous nitrogen. Accordingly, four AA_{NESS} had higher $\delta^{15}N$ values in albumen than in muscle. The two which did not (Asx and Glx) may have had their $\delta^{15}N$ values in yolk buffered from substantial change by their relatively large pool sizes in the body, similar to yolk.

4.3 | $\Delta^{15}N$ offsets as an index of capital breeding

Almost all trophic AAs exhibited greater offsets between egg tissue and muscle in emperor penguins than in herring gulls, more so than source AAs (see Figure 6). This trend suggests that calculations of $\Delta^{15}N_{Trophic\text{-}Source}$ can be sensitive to the mobilization of endogenous tissue stores. However, the trophic-source pair of $\Delta^{15}N_{Glx\text{-}Phe}$, which to date is the most common pairing (McMahon & McCarthy, 2016; Whiteman et al., 2019), did not differ between penguins and gulls. Instead, $\Delta^{15}N_{Pro\text{-}Phe}$ was a better marker: larger values of this offset in egg tissue than in muscle tissue may therefore represent an index of capital breeding. We speculate that $\Delta^{15}N_{Pro\text{-}Phe}$ was more sensitive than $\Delta^{15}N_{Glx\text{-}Phe}$ because the relative pool size of endogenous Glx is much larger than that of Pro. As a result, greater mobilization and redeposition should be required of Glx before an isotopic offset of similar magnitude can be achieved. Using $\Delta^{15}N_{Pro\text{-}Phe}$ as a quantitative index of reproductive strategy, or any other AA trophic-source pair, requires establishing an expected range of values across a spectrum from complete capital to complete income breeding. We hypothesize that this range of values is broad and contains predictable patterns that reflect animal physiology and ecology, similar to the range of trophic discrimination factors that have been observed when using $\Delta^{15}N_{Glx\text{-}Phe}$ to calculate trophic position (McMahon & McCarthy, 2016). Importantly, fractionation during mobilization of endogenous tissue stores is caused by biochemical mechanisms that are likely consistent across a diversity of species, suggesting that the value of $\Delta^{15}N_{Pro\text{-}Phe}$ may be a generalizable predictor of capital breeding.

A potential quantitative index of reproductive strategy is exciting because such an index has proven elusive. While earlier studies of capital breeding provided important advances, they required assumptions about material balance when estimating mass loss during laying (reviewed by Hobson, 2006) or about variation in dietary isotopic values and isotopic discrimination between endogenous stores and eggs. Our AA approach has the advantage of explicitly quantifying isotopic difference between tissues within a consumer, avoiding these assumptions. Using $\Delta^{15}N_{Pro\text{-}Phe}$ values as an index has considerable potential implications for the study of nutrient allocation strategies in breeding birds (Stephens et al., 2009), evolution of reproductive strategies (Drent, 2006), population dynamics in a rapidly changing world (Hupp et al., 2018), and the transfer of nutrients and contaminants among biomes by migrating individuals (Hebert et al., 2016). It is important to note that our inferences were constrained by the limited sample collection that was possible from wild emperor penguins; future studies can provide additional validation by using large sample sizes of eggs and endogenous tissue that are consistently collected from the same individual. We encourage such studies to build on the isotope framework presented here and to evaluate the $\Delta^{15}N_{Pro\text{-}Phe}$ offset, as well as other trophic-source pairings of AAs, in avian species spanning the capital versus income spectrum. This spectrum also occurs in non-avian wildlife (Stephens et al., 2009), and researchers focused on other taxa should consider applying our approach for their study systems.

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AUTHORS' CONTRIBUTIONS

J.P.W., S.D.N. and K.A.H. conceived the ideas and designed methodology; Y.C. and P.B. provided the samples; J.P.W. conducted isotope analysis and analysed the data; all the authors interpreted the data, contributed critically to the writing and gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data available from the Dryad Digital Repository https://doi.org/10.5061/dryad.j3tx95xcb (Whiteman et al., 2020).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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