CHAPTER

7

Amino Acid Isotope Analysis: A New Frontier in Studies of Animal Migration and Foraging Ecology

Kelton W. McMahon[1] *and Seth D. Newsome*[2]

[1]University of Rhode Island, Narragansett, RI, United States, [2]University of New Mexico, Albuquerque, NM, United States

7.1 INTRODUCTION

Whether it is diel, meter-scale migrations of zooplankton toward the ocean surface or the annual 70,000 km global migrations of Arctic Terns (*Sterna paradisaea*), animal migration represents one of the most biologically significant redistributions of biomass on Earth (Dingle, 2014). Accordingly, there is a need to develop powerful, precise, and integrative tools to describe and quantify these spectacular migratory movements (Hobson, 1999; Hussey et al., 2015; Kays, Crofoot, Jetz, & Wikelski, 2015; Chapter 1: Animal Migration: A Context for Using New Techniques and Approaches). Given that the acquisition and allocation of dietary resources is a fundamental requirement for all animals and an integral part of animal movement (Fryxell & Sinclair, 1988), movement ecology is intimately linked with foraging ecology in space and time.

Ecogeochemistry is the application of geochemical techniques to fundamental questions in ecology (McMahon, Hamady, & Thorrold, 2013a). This field offers a powerful suite of tools to explore the intricate connections between movement and foraging dynamics. Physical, chemical, and biological processes in nature create geospatial structure in the distribution of stable isotopes; mapping these distributions illustrates the invisible "isoscapes" in which organisms operate (Bowen, 2010). As animals interact with isoscapes, they incorporate the local isotopic signals of the environment into their tissues (Graham, Koch, Newsome, McMahon, & Aurioles, 2010; Hobson, 1999). Herein lies one of the biggest challenges in interpreting isotope data in the context of movement ecology: the isotope composition of a consumer reflects information about both the baseline isoscape and the subsequent biochemical modifications of organic

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matter as it flows through food webs (Chapter 4: Application of Isotopic Methods to Tracking Animal Movements).

The trophic modification of organic matter and its isotopic composition results in a series of complex ecogeochemical "filters" between the baseline isoscape and the corresponding consumer tissue isotope δ-value. To use stable isotopes to track animal movement among isoscapes, we need to understand how these filters modify the isotope value of consumers relative to their baseline isoscapes. Conventional isotopic approaches to examining movement ecology have focused on the isotope analysis of "bulk" tissues, i.e., the weighted average of all components within in a specific tissue. While quite successful (Hobson & Wassenaar, 2008), it can be difficult to determine whether variation in bulk tissue isotope values is due to differences in (1) diet (e.g., trophic level), (2) tissue types analyzed, (3) physiology or metabolism, (4) isotopic composition at the base of the food web, or (5) some combination of these factors (Post, 2002). This can be particularly problematic when studying mobile organisms in which resource and habitat use change across space and time.

Whereas bulk tissue isotope analysis averages all macromolecules in a sample, compound-specific isotope analysis of amino acids (CSIA-AA) takes a *molecular approach* based on well-established biochemical pathways. The power of the molecular approach lies in the differential isotopic (e.g., $\delta^{2}H$, $\delta^{13}C$, $\delta^{15}N$) discrimination of individual AAs during the transfer of organic matter between diet and consumer (i.e., trophic transfer) or biochemical processing within the consumer. In this chapter, we explore the potential of CSIA-AA to study movement and foraging dynamics. We start with a brief primer on the roles of metabolism and physiology on AA stable isotopic discrimination. We highlight the strengths and limitations of the CSIA-AA approach to movement ecology by describing case studies under three major themes: (1) using CSIA-AA to refine isoscapes, (2) disentangling movement and foraging ecology, and (3) examining movement and resource utilization across different ecosystems. We provide a brief overview of the analytical process needed to generate and interpret CSIA-AA data and an outlook toward future research needed to expand and refine the use of CSIA-AA for studying animal movement and foraging ecology.

7.2 PRIMER ON AMINO ACID BIOCHEMISTRY AND ISOTOPE DISCRIMINATION

To track animal movement through space and time with stable isotopes, we must be able to link the isotope δ-value(s) of a consumer tissue to an underlying baseline isoscape. However, the isotope values of the baseline isoscape (e.g., primary producers) are potentially modified through consumer metabolism in a variety of ways depending on the number and isotopic effect of enzymatic reactions, as well as the flux of elements through these metabolic pathways (reviewed in Hayes, 2001; McMahon & McCarthy, 2016; Ohkouchi et al., 2017). The differential isotope fractionation of individual AAs during metabolism provides a mechanism to disentangle signals of the baseline isoscape from subsequent metabolic modifications of consumer isotope values. Therefore in this section we present a brief primer on the basics of how AA metabolism influences nitrogen, carbon, and hydrogen isotopic discrimination.

7.2.1 Nitrogen Metabolism

Transamination (transferring an amine group) and deamination (removing an amine group) are the two dominant enzymatic processes that control the flow of nitrogen, and thus nitrogen

isotope fractionation, in proteinaceous AAs (Braun, Vikari, Windisch, & Auerswald, 2014). The diversity of transaminases and deaminases, each with different N isotope effects, and the variable degree of transamination and deamination among AAs, result in a wide range of nitrogen isotopic discrimination patterns among individual AAs (McMahon & McCarthy, 2016). For δ^{15}N analysis, protein AAs are commonly divided into two groups, the minimally discriminating "source" AAs and the heavily discriminating "trophic" AAs (Popp et al., 2007), based on their nitrogen metabolism and corresponding isotopic discrimination. While this division is often confused with the more familiar essential versus nonessential AA groupings for carbon, these groupings not only represent different AAs, but are also based on fundamentally different biochemical mechanisms related to nitrogen cycling (source and trophic AAs) versus synthesis of carbon sidechains of AAs (essential and nonessential AAs). Below we explore the mechanisms of differential ^{15}N discrimination among AAs, which allow ecologists to disentangle the relative influence of baseline versus trophic isotope variability on consumer δ^{15}N values.

7.2.1.1 Minimally Fractionating "Source" Amino Acids

The source AAs (e.g., phenylalanine: Phe; methionine: Met; lysine: Lys) show relatively little N isotope discrimination between diet and consumer, likely because their dominant metabolic pathways typically do not form or break C–N bonds during metabolism (O'Connell, 2017). A metaanalysis of published controlled feeding studies (70 species, 317 individuals, and 88 distinct consumer–diet combinations) found minimal N isotopic discrimination of Phe ($\Delta^{15}N_{C-D} = -0.1 \pm 1.6$‰), Met ($0.4 \pm 0.4$‰), and Lys ($0.8 \pm 1.5$‰) between consumer (C) and diet (D) (McMahon & McCarthy, 2016). The δ^{15}N values of these "source" AAs are therefore thought to directly reflect the δ^{15}N of the baseline isoscape without the confounding issue of trophic isotope discrimination. However, alternative metabolic pathways can impart N isotope fractionation via transamination or deamination for many source AAs. ^{15}N-labeling experiments have shown that the central pool of dietary nitrogen can be incorporated into Phe, albeit at low levels relative to most other AAs (Hoskin, Gavet, Milne, & Lobley, 2001). Identifying under what dietary and physiological conditions source AA ^{15}N discrimination becomes significant is a current area of research in CSIA-AA.

Finally, it should be noted that several other AAs that were originally termed "source" AAs, namely Glycine (Gly), Serine (Ser), and Threonine (Thr) (Popp et al., 2007), were done so based largely on the empirical results reported in McClelland and Montoya (2002). Subsequently, across a much broader range of consumers, the variability in mean ($\pm$SD) $\Delta^{15}N_{C-D}$ values for these AAs has been shown to be extremely large: Gly (3.9 ± 4.9‰,), Ser (2.9 ± 4.6‰), and Thr (-5.8 ± 3.2‰) (McMahon & McCarthy, 2016). As a result, we suggest caution be used when interpreting δ^{15}N values of these particular AAs in the context of animal movement and foraging ecology without further mechanistic studies of their isotopic discrimination patterns.

7.2.1.2 Heavily Fractionating "Trophic" Amino Acids

The trophic AAs (glutamic acid: Glu; aspartic acid: Asp; alanine: Ala; isoleucine: Ile; leucine: Leu; proline: Pro; valine: Val) typically undergo significant N isotopic discrimination during metabolism associated with extensive transamination and deamination (O'Connell, 2017). The canonical trophic AA Glu often has the highest mean $\Delta^{15}N_{C-D}$ of all AAs (6.4 ± 2.5‰; McMahon & McCarthy, 2016). The other trophic AAs typically exhibit discrimination patterns that closely resemble Glu because

they exchange nitrogen with the central nitrogen pool in an organism via transamination chains linked to Glu (McCarthy, Lehman, & Kudela, 2013; O'Connell 2017).

Together, the δ^{15}N values of a consumer's trophic and source AAs provide ecologists with a potential tool to calculate consumer trophic position ($TP_{CSIA\text{-}AA}$) that is internally indexed to the δ^{15}N of the base of the food web. However, several controlled feeding studies have noted that the degree of N isotope discrimination of trophic AAs between diet and consumer is not constant (Chikaraishi, Steffan, Takano, & Ohkouchi, 2015; McMahon, Polito, Abel, McCarthy, & Thorrold, 2015; McMahon, Thorrold, Elsdon, & McCarthy, 2015; Yamaguchi et al., 2017). As with trophic patterns observed in bulk tissues (Vanderklift & Ponsard, 2003), diet quality and consumer mode of nitrogen excretion (e.g., ammonia vs urea) are potentially key variables influencing the degree of trophic AA isotopic discrimination (Germain, Koch, Harvey, & McCarthy, 2013; McMahon, Thorrold, et al., 2015; O'Connell, 2017). This variability in nitrogen isotopic discrimination among trophic AAs can significantly impact the accuracy of trophic position estimates (e.g., Dale, Wallsgrove, Popp, & Holland, 2011; Lorrain et al., 2009). Therefore we need to increase the number of controlled feeding studies examining AA isotope fractionation to better understand and account for the underlying mechanisms controlling variability in trophic AA ^{15}N discrimination.

7.2.2 Carbon Metabolism

For carbon, AAs have conventionally been classified into two categories, essential and nonessential AAs, which relate to an organism's ability for de novo synthesis of AA side chains. Animals cannot synthesize essential AAs at a rate adequate for normal growth (Wu, 2009). Typically, δ^{13}C data are reported for seven essential AAs (Ile, Leu, Lys, Met, Phe, Thr, and Val), which contribute ~25%–40% of the AA budget of animal tissues commonly analyzed by ecologists (Wolf, Newsome, Peters, & Fogel, 2015). Conversely, nonessential AAs are those that organisms can de novo synthesize from a common carbon pool (Wu, 2009). δ^{13}C data are routinely reported for eight nonessential AAs (Gly, Ser, Ala, Glu, Asp, Pro, arginine: Arg, and tyrosine: Tyr), which contribute ~60%–75% to the total AAs budget of animal tissues (Wolf et al., 2015). Some of these nonessential AAs are considered conditionally essential (e.g., Pro, Arg, and Tyr), meaning their de novo synthesis can be limited under certain physiological conditions. In contrast to the general classification scheme used for δ^{15}N (source vs trophic) that is empirically derived, these essential and nonessential designations for δ^{13}C are based on well-established biochemical pathways.

7.2.2.1 *Essential Amino Acids*

Animals have lost the enzymatic pathways required to synthesize sufficient quantities of essential AAs and hence must acquire the intact carbon skeletons of essential AAs directly from their dietary proteins (Howland et al., 2003; McMahon, Fogel, Elsdon, & Thorrold, 2010; O'Brien, Fogel, & Boggs, 2002) or from their prokaryotic gut microbiome (Ayayee et al. 2015; Newsome, Fogel, Kelly, & Martinez del Rio, 2011). As a result, essential AAs typically show minimal ^{13}C isotopic discrimination between diet (or gut microbe essential AAs) and consumer tissue (Howland et al., 2003; McMahon et al., 2010). Consumer essential AA δ^{13}C values therefore represent the isotopic signature of the producers of those AAs at the base of the food web, without the confounding variable of significant trophic discrimination.

On the other hand, plants, algae (protists), bacteria, and fungi can synthesize essential

AAs de novo from a variety of inorganic and organic carbon sources. These organisms use a variety of pathways and associated isotope effects to synthesize common essential AAs (Hayes, 2001). This metabolic diversity in synthesis pathways imprints on the relative $\delta^{13}C$ values of essential AAs synthesized by different groups of producers. Multivariate analyses using carbon isotope data from a suite of essential AAs results in unique essential AA $\delta^{13}C$ "fingerprints" among plants, algae, bacteria, and fungi (Larsen, Taylor, Leigh, & O'Brien, 2009; Larsen et al., 2013; Scott et al. 2006). Assuming the contribution of essential AAs synthesized de novo by gut microbiota is minimal (but see Ayayee, Jones, & Sabree, 2015 and Newsome et al. 2011 for counterarguments), then direct assimilation (routing) of dietary essential AAs results in little C isotopic alteration of these molecules as they are passed from diet to consumer (Howland et al., 2003; McMahon et al., 2010). By extension, the essential AA $\delta^{13}C$ fingerprints of consumers at any trophic level could be used to evaluate the relative importance of different sources of primary production at the base of the food chain (Arthur, Kelez, Larsen, Choy, & Popp, 2014; McMahon, McCarthy, Sherwood, Larsen, & Guilderson, 2015; McMahon, Thorrold, Houghton & Berumen, 2016). As discussed in Section 7.4.2, when these distinct primary producers occupy spatial gradients or discrete habitats, the AA C isotope fingerprinting approach can be used to identify foraging across space and time.

7.2.2.2 Nonessential Amino Acids

In contrast to essential AAs, animals can acquire nonessential AAs in several ways: (1) synthesize them de novo from a bulk carbon pool of protein and nonprotein dietary macromolecules (Newsome et al., 2011; O'Brien et al., 2002; Wu et al., 2014), (2) route them directly or indirectly (via the gut microbiome) from dietary protein (McMahon et al., 2010), and (3) route them directly from nonessential AAs produced by the gut microbiome from dietary carbohydrate (Ayayee et al., 2015; Newsome et al., 2011) and lipid precursors (Newsome et al. 2014). Nonessential AAs can be further grouped into two general types: (1) those synthesized from intermediaries in glycolysis (Gly/Ser/Ala) and (2) those synthesized from intermediaries in the tricarboxylic acid (TCA) cycle (Glu/Asp/Pro/Arg), both of which have distinct sources of carbon and synthesis pathways. However, routing of intact nonessential AAs directly from dietary protein into consumer tissues (just like essential AAs) is energetically most favorable (Schwarcz, 1991), as the use of premanufactured carbon skeletons reduces the metabolic costs of synthesis (Wu et al., 2014). Isotopic discrimination of C between nonessential AAs and bulk diet is highly variable and is likely dependent on the degree of de novo synthesis, the nonprotein substrates utilized during synthesis (e.g., carbohydrates vs lipids), and the degree of direct routing of nonessential AAs sourced from dietary protein (Howland et al., 2003; McMahon et al., 2010; McMahon, Polito, et al., 2015; Newsome et al., 2011). Empirical studies have demonstrated that the relative degree of de novo synthesis versus direct routing of nonessential AAs is primarily driven by dietary protein content (Newsome et al., 2011; Newsome, Wolf, Peters, & Fogel, 2014; O'Brien et al., 2002). Understanding the patterns in direction and magnitude of nonessential AA carbon isotope fractionation, as well as the underlying drivers, is an area of active research in ecogeochemistry.

7.2.3 Hydrogen Metabolism

Only one study has reported $\delta^{2}H$ values of individual AAs (Fogel, Griffin, & Newsome, 2016). However, given the ~200‰ δ-range in global $\delta^{2}H$ isoscapes (Bowen, 2010; McMahon

et al., 2013a), AA δ^2H analysis, just like hydrogen isotope analysis of bulk tissues, may be the most promising system for studying terrestrial animal movement and migration patterns. While food is the only source of carbon and nitrogen available for proteinaceous tissue synthesis in animals, hydrogen from both food and water is used to construct tissues (Hobson, 1999; Wolf, Newsome, Fogel, & Martinez del Rio, 2013). The experiment reported in Fogel et al. (2016) focused on bacteria (*Escherichia coli*) grown in water of varying δ^2H values and on medium that did or did not contain protein. Interestingly, the essential and nonessential classification scheme appears to work well for AA δ^2H, likely because most of the hydrogen in AAs is bonded to carbon and does not exchange with body water. This experiment showed that most ($>80\%$) of the hydrogen in *E. coli* cells was routed directly from a protein-rich substrate (tryptone). The only exception to this pattern was in the glycolytic nonessential AA alanine, which had $\sim 40\%-50\%$ of its hydrogen derived from environmental water (i.e., cellular water) even when exogenous protein was available for cellular synthesis.

By contrast, a relatively high proportion of hydrogen ($\sim 35\%-75\%$) in both nonessential and essential AAs was sourced from environmental water when *E. coli* were grown on a glucose medium containing no protein. Amino acid δ^2H data from controlled feeding experiments on other organisms have yet to be reported, however, the biochemical pathways (glycolysis and TCA cycle) used by *E. coli* to synthesize nonessential AAs are like those used by heterotrophic eukaryotes (Kanehisa, Furumichi, Tanabe, Sato, & Morishima, 2017). Overall, these patterns suggest that the majority ($\sim 70\%-80\%$) of hydrogen in the proteinaceous tissues of omnivorous and carnivorous animals is obtained from diet, which may obscure the relationships between tissue δ^2H and that of precipitation that are commonly used to trace animal movement. But for herbivorous or frugivorous species that consume relatively low protein diets, AA δ^2H may be a more faithful recorder of local environmental water, and thus may be a strong proxy for tracking latitudinal or altitudinal movements.

7.3 ACCOUNTING FOR CONSUMER PHYSIOLOGY

Animals that migrate use a diverse array of life history strategies to maximize fitness when engaging in long-distance movements and extended residence in different ecosystems: aquatic versus terrestrial, freshwater versus marine, and tropics versus high latitudes (Johnson & Gaines, 1990). These migration strategies are associated with major shifts in organismal physiology (Gwinner, 2012). For example, animal migration is often accompanied by major shifts in energy output associated with the physical act of migrating, alterations to metabolic rate associated with environmental temperature shifts and/or physical activity, and changes in body condition associated with fasting and/or shifts to endogenous energy reserves (e.g., body fat or muscle). These changes are often concurrent with additional changes in physiology associated with the drivers of migration, such as shifts in hormones and body condition associated with reproduction.

There is a wealth of bulk tissue isotope data on how major physiological stressors associated with migration, reproduction, and disease impact consumer C isotopic discrimination (e.g., Gannes, Martinez del Rio, & Koch, 1998), but comparatively little information for individual AAs. For example, McMahon, Polito, et al. (2015) found that the $\Delta^{13}C_{C-D}$ of nonessential AAs in captive penguins varied significantly depending on the duration of fasting prior to feather molt. The strong ^{13}C-depletion of AAs associated with the TCA cycle

(Glu, Asp, and Pro) appeared to be related to increased reliance on endogenous lipid stores, which are catabolized and readily converted to TCA precursors during nutritional stress from molting and migration. Similar patterns have been observed in captive mice fed lipid-rich diets (Newsome et al., 2014). These variable AA isotopic discrimination patterns associated with changes in physiology, disease, and resource utilization make it challenging to accurately interpret migration and residence patterns of mobile consumers. There is a critical need for more controlled feeding experiments in the lab and comparative field studies examining the impacts of these major changes in physiology on the isotopic discrimination of individual AAs, which can create additional mismatches between consumer isotope values and the underlying isoscape.

With proper calibration, these AA isotopic discrimination patterns that currently pose challenges to tracking animal migration could instead provide powerful opportunities to study organismal physiology of migrating animals. Animals that roam over vast distances are inherently difficult to capture and sample, often making it impossible to reliably capture the same individual multiple times to provide a longitudinal ecological or ecophysiological record. CSIA-AA could enable tracing of carbon as it is shuttled among tissue types, helping to illuminate temporal variation in the mobilization of different reservoirs of endogenous versus exogenous resources. Empirical data collected from a wide variety of organisms including bacteria, fungi, plants, and animals show that endogenous lipids and protein, which are the two largest endogenous reservoirs that animals use during times of nutritional stress, have very different $\delta^{13}C$ and $\delta^{2}H$ values (DeNiro & Epstein, 1977; Estep & Hoering, 1980; Hayes, 2001). Bulk adipose tissue, or lipids extracted from proteinaceous tissues, have $\delta^{13}C$ and $\delta^{2}H$ values 6‰–8‰ and ~100‰–150‰ lower than tissue proteins, respectively (e.g., Newsome et al., 2010; Newsome, Wolf, Bradley, & Fogel, 2017). Conversion of one reservoir into another should be traceable at the molecular level, especially for compounds (e.g., nonessential AAs) that consumers can synthesize de novo while minimally fractionating essential AAs provide concurrent information about the sources of primary production at the base of the food web supporting those consumers (e.g., McMahon, Polito, et al., 2015). Note that isotopic effects of such changes in movement, foraging, and physiology will only be observable in tissues with isotopic incorporation rates that can record shifts on the same time scales as the changes in resource allocation and use.

Another topic linked to exploring migration and physiology with CSIA-AA that represents both a current complication and a potential opportunity is the role that the gut microbiome plays in consumer protein metabolism. Gut microflora likely play an important role in the protein metabolism of many animals, particularly herbivorous and omnivorous hosts consuming diets deficient in dietary protein quantity or quality. In such cases, the gut microbiome often supplements the essential AA budget of its host organism by converting nonprotein dietary macromolecules, such as carbohydrates or even lipids, into the carbon skeletons needed for de novo synthesis of essential AAs. Only a few studies focused on a small suite of essential AAs (Lys and Thr) have examined this topic in humans (Metges, 2000), rats (Torrallardona, Harris, Coates, & Fuller, 1996), fish (Newsome et al., 2011), and insects (Ayayee et al., 2015) in a laboratory setting. This topic merits continued study as the gut microbiome adds an important filter between consumers and the baseline isoscape, as well as a potential opportunity to explore patterns of resource utilization, metabolism, and physiology in migratory species.

7.4 CASE STUDIES IN MOVEMENT AND FORAGING ECOLOGY USING CSIA-AA

Perhaps the most striking advantage of CSIA-AA is the ability to isolate information about both the baseline isoscape and food web structure from a single sample. In this section, we explore case studies under three important themes to the development and application of CSIA-AA in movement and foraging ecology: (1) using CSIA-AA to refine isoscapes, (2) disentangling movement and trophic dynamics in space and time, and (3) examining movement and resource utilization across different ecosystem types.

7.4.1 Developing CSIA-Based Isoscapes

Isoscapes form a valuable framework for tracking the movement of animals in space and time (Graham et al., 2010; Hobson, 1999; McMahon, Hamady, & Thorrold, 2013b; Wunder & Norris, 2008). Primary producers integrate the physical, chemical, and biological cycling of elements in the environment and form a link to upper trophic level consumers. The tissues (e.g., leaves) of most primary producers in terrestrial environments integrate environmental information over long time periods and thus may be suitable for the construction of isoscapes at the primary producer level. In contrast, the typically short life spans and fast isotopic incorporation rates of primary producers in marine environments (e.g., phytoplankton) relative to upper trophic level consumers can create a mismatch between the highly dynamic baseline isoscape and consumer isotope values. To circumvent this issue, marine isoscapes are often empirically constructed using data from primary consumers with longer integration times that more closely match those of the target organism. The biochemical and physiological processing of that baseline isoscape signal as it moves up the food chain imparts additional isotopic discrimination and uncertainty that can obscure the links between consumer isotope values and the baseline isoscape. CSIA-AA has the potential to expand and refine the use of isotopes for studying animal movement by constraining (1) the development of baseline isoscapes (this section) and (2) the offsets between baseline isoscapes and consumer stable isotope values (Section 7.4.2).

Generating isoscapes using minimally isotope fractionating AAs of primary or secondary consumers provides a temporal integration of the baseline signal without the confounding issue of significant trophic discrimination. For example, Vokhshoori and McCarthy (2014) used δ^{15}N values of source AAs from sessile, filter-feeding mussel tissue (*Mytilus californianus*) to generate CSIA-AA isoscapes across 10° latitude in the dynamic coastal zone of the California Current System. They found strong latitudinal gradients in mussel Phe δ^{15}N values, reflecting the mixing of northern ^{15}N-depleted water brought south by surface flow of the California Current and southern ^{15}N-enriched water brought north via the California Undercurrent from the denitrification zone of the Eastern Tropical Pacific. In systems that are spatially and temporally constrained, the empirical generation of AA-based isoscapes may provide a robust geospatial framework for examining animal movement.

The primary reason why CSIA-AA has not been routinely used to construct isoscapes is analytical. The requirement of significant specialized isotope mass spectrometer instrumentation, time and expense of analyses, and analyst expertise required for proper interpretation of CSIA-AA data mean it is not feasible to analyze hundreds or thousands of samples to generate large-scale isoscapes from AA isotope data. In all likelihood, the true value of CSIA-AA to the development of isoscapes will lie in the ability to help constrain the underlying drivers of

observed geospatial structure in isoscapes that are constructed using bulk tissue data. For example, MacKenzie, Longmore, Preece, Lucas, and Trueman (2014) used lion's mane jellyfish (*Cyanea capillata*) to construct a robust pelagic isoscape for the North Sea. However, jellyfish are opportunistic feeders, preying on a wide range of pelagic organisms. To address this potential trophic variability issue, the authors compared their isoscapes to similar isoscapes generated from benthic, sessile scallops in the North Sea from Jennings and Warr (2003). They concluded that the remarkably similar gradients in isotope value between these two consumers, despite their differences in diet (trophic level) and habitat, suggested that the observed North Sea isoscape was a function of regional hydrography and biogeochemical cycling rather than trophic dynamics. A further test of this hypothesis could apply CSIA-AA of select samples along the major isotopic gradients. Thus bulk SIA facilitates the sample sizes necessary to generate robust isoscapes in space and time, while CSIA-AA provides a tool to further validate the structure of the observed isoscapes through insights into the underlying mechanisms generating such geospatial patterns.

7.4.2 Disentangling Movement and Foraging Ecology

Movement and foraging ecology are fundamentally linked. Not only is foraging ecology a major driver of animal migrations (Fryxell & Sinclair, 1988), but food is often a primary vector for transferring the biogeochemical signals of baseline $\delta^{13}C$, $\delta^{15}N$, and $\delta^{2}H$ isoscapes into consumer tissues (Graham et al., 2010; Wunder & Norris, 2008). Perhaps the most common applications of CSIA-AA in ecogeochemistry have been to tease apart the relative influence of baseline and trophic isotope variability on consumer isotope values (see Fig. 1 of McMahon & McCarthy, 2016). These studies often (1) use source AAs to examine local biogeochemical cycling without the confounding issue of consumer trophic discrimination or (2) compare source and trophic AAs to calculate consumer trophic level while controlling for variation in baseline isotope values.

The logical extension of these CSIA-AA approaches is to use the isotope values of minimally isotope fractionating AAs in consumers as proxies for baseline isotope values to track movement across known geochemical gradients, while controlling for potential filters between baseline and consumer imposed by trophic discrimination. One of the first studies to use CSIA-AA to infer animal movement was by Popp et al. (2007), who found strong latitudinal gradients in the source AA $\delta^{15}N$ values of Pacific yellowfin tuna (*Thunnus albacares*) in the eastern tropical Pacific that mirrored patterns in the local baseline isotope values of particulate organic matter. They concluded that these tuna were not undertaking significant ocean basin scale migrations, at least on the monthly time scale of tissue turnover (Bradley, Madigan, Block, & Popp, 2014). Dale et al. (2011) took this approach one step further, using CSIA-AA to identify ontogenetic shifts in nursery habitat use of brown stingray (*Dasyatis lata*) across an isoscape in coastal Hawai'i. They were able to resolve shifts in both movement (migration from nursery habitats within the bay to adult habitats offshore) and foraging (diet shifts with concurrent increases in trophic level) of this benthic predator across a complex tropical isoscape.

A number of other studies have employed CSIA-AA to examine movement associated with foraging (e.g., Lorrain et al., 2009; Ruiz-Cooley, Koch, Fiedler, & McCarthy, 2014), breeding (e.g., Seminoff et al., 2012; Vander Zanden et al., 2013), and range expansion (e.g., Ruiz-Cooley, Ballance, & McCarthy, 2013) across a variety of spatial and temporal scales. Several of these studies report substantial underestimates of top consumer trophic level

based on CSIA-AA in both field and laboratory settings (e.g., Dale et al., 2011; Germain et al., 2013; Lorrain et al., 2009; Ruiz-Cooley et al., 2013, 2014), which likely reflects challenges with our current understanding of the underlying mechanisms of trophic AA isotopic discrimination (O'Connell, 2017). Characterizing the sources and magnitudes of these AA trophic discrimination factors is an area of active research (e.g., Chikaraishi et al., 2015; Germain et al., 2013; McMahon, Polito, et al., 2015; McMahon, Thorrold, et al., 2015; Steffan et al., 2013), as are new approaches to incorporating this variability into equations that estimate trophic level (e.g., McMahon, Polito, et al., 2015). As our understanding of AA isotopic discrimination improves, so will our ability to calculate more accurate and precise trophic levels using CSIA-AA.

δ^2H analysis of individual AAs could provide better spatial resolution than bulk tissue approaches to assigning origins in animals that undertake continental-scale migrations. Specifically, there are two potentially fruitful approaches that could be used to construct compound-specific hydrogen isoscapes. First, nonessential AAs like Ala appear to be synthesized from a high proportion (40%–50%) of water-derived hydrogen. Thus δ^2H analysis of Ala may provide a more direct link between the hydrogen isotopic composition of local precipitation and that of tissues, of which only 20%–30% is sourced from body water. A second approach would be to focus on the δ^2H of essential AAs, which are overwhelmingly derived from food (≥90%), and thus may more faithfully record regional or even landscape level variation in the δ^2H of resources than bulk tissues that are synthesized from a combination of hydrogen from water and food. However, AA δ^2H compound-specific approaches need to be tested with feeding experiments in the laboratory followed by analysis of tissues from known origin individuals (e.g., Langin et al., 2007).

To date, most studies using CSIA-AA to examine migration have focused on large-scale (>100s km) movements of organisms relative to known geochemical gradients. The use of isotopes to geolocate organisms to specific habitats distributed across smaller scales (<10 km) is a potentially powerful but challenging goal. McMahon, Berumen, and Thorrold (2012) used an essential AA δ^{13}C fingerprinting approach to assess ontogenetic migration patterns of Ehrenberg snapper (*Lutjanus ehrenbergii*) among discrete juvenile nursery habitats on the scale of 10s km. To do so, they characterized spatially separated habitat signatures using resident fishes (reflecting local food web structure; McMahon, Berumen, Mateo, Elsdon, & Thorrold, 2011) and then compared those signatures to the core of adult fish otoliths (representing the period of time when those fish were juveniles; McMahon, Fogel, Johnson, Houghton, & Thorrold, 2011). In doing so, they were able to identify important nursery habitats and migration corridors for an ecologically and economically important coral reef fish in the Red Sea. In situations like this with spatially and isotopically distinct habitats, CSIA-AA can provide a powerful tool for identifying habitat use and residence patterns in mobile consumers.

As with all indirect tools for tracking animal movement, we need to validate the patterns of movement determined by CSIA-AA with known locations. For instance, Seminoff et al. (2012) used satellite telemetry and source AA δ^{15}N analysis to show that patterns in bulk tissue isotope values of Pacific leatherback sea turtles (*Dermochelys coriacea*) reflected divergent turtle migratory strategies to distinct foraging groups rather than spatial variation in trophic dynamics. Similarly, Polito et al. (2017) used archival geolocation tags to show that the strong segregation of essential AA δ^{13}C values in Antarctic penguin tail feathers was indicative of distinct migration strategies from the Antarctic Peninsula east into the ice-covered

Weddell Sea versus west into the ice-free Scotia Sea. There is an obvious need to expand upon these efforts across a variety of stable isotopes, ecosystems, and spatiotemporal scales. While no isotopic approach will ever provide the geolocation resolution of satellite telemetry, isotopes have the advantage of recording dietary inputs over a variety of timescales depending on tissue type. Thus coupling isotopes with telemetry could potentially discriminate between movement through and actual use of resources in particular habitats.

7.4.3 Constraining Movement Across Ecosystem

Most studies utilizing bulk and CSIA-AA isotope methods to characterize animal movement have focused on movement within a single ecosystem type (e.g., an ocean basin or a terrestrial grassland). Far fewer studies have used CSIA-AA to rigorously examine movement across ecosystems types (e.g., between marine and terrestrial systems, or from C_3 forests to C_4 grasslands). Some early work in this area assessed use of marine versus terrestrial resources by coastal consumers (including humans) (e.g., Hare, Fogel, Stafford, Mitchell, & Hoering, 1991; Honch, McCullagh, & Hedges, 2012; Naito, Honch, Chikaraishi, Ohkouchi, & Yoneda, 2010). However, interpretation of source or essential AA isotope data can be challenging when adjacent ecosystems have different primary producer sources (Jarman et al., 2017). In comparison to marine producers, very little is known about $\delta^{15}N$ discrimination patterns among source AAs in terrestrial plants (e.g., Chikaraishi et al., 2009). Preliminary data suggest that C_3 and C_4 terrestrial plants have distinct β values (difference between trophic and source AAs in primary producers), and these differences appear to be larger in magnitude than observed trophic discrimination of the most heavily fractionating trophic AA Glu. Assessing trophic dynamics of consumers that move and forage between these distinct ecosystem types (e.g., with different primary producer β values) will require new trophic position models that take this variability into account. For instance, to examine the trophic positions of prehistoric human populations in Rapa Nui, South Pacific, Jarman et al. (2017) used an amino acid $\delta^{13}C$ fingerprinting approach to identify the relative contribution of marine and terrestrial primary production supporting humans, and then used an amino acid nitrogen isotope mass balance model with a β_{mixed} value that multiplies the estimated fraction of marine and terrestrial foods with their corresponding known marine or terrestrial β values. This analytically intensive, but innovative approach, likely reflects the next generation in trophic dynamics models using CSIA-AA. But the success of this approach necessitates a better understanding of the parameters (e.g., B) that go into the model. Furthermore, there have been very few controlled feeding studies to examine trophic discrimination of individual AAs among terrestrial consumers, particularly vertebrates and upper trophic level consumers (but see Nakashita et al., 2011; Steffan et al., 2015). Uric-acid producing insects, for instance, appear to have very different isotopic discrimination patterns of Glu and Phe than many urea and uric-acid producing aquatic consumers (see discussion in McMahon & McCarthy, 2016). In agreement with other recent comments (O'Connell & Collins, 2017), we caution that more data are needed on primary producer β values and AA isotopic discrimination factors between diet and consumer from a wide range of natural marine and terrestrial ecosystems before we can confidently use AA-based equations to quantify trophic level, especially for consumers that forage across multiple primary producer types (e.g., marine algae, terrestrial C_3 plants, and terrestrial C_4 plants). Similar datasets are needed to

further refine the capabilities and limitations of using essential AA $\delta^{13}C$ fingerprints to address questions in both aquatic and terrestrial ecology.

7.5 CSIA-AA METHODOLOGY

Successful application of CSIA-AA to questions in movement and foraging ecology requires significant analytical capital and biochemical expertise to properly analyze, interpret, and evaluate molecular isotope data. Most notably, the isotope analysis of individual compounds requires significantly more time for sample preparation and analysis compared to conventional analysis of bulk tissues via elemental analyzer-isotope ratio mass spectrometry (EA-IRMS). A telling proxy for the intense analytical nature of CSIA-AA is the average sample size in published datasets, often in the dozens rather than the hundreds or thousands. While CSIA-AA is a potentially powerful methodology in animal ecology and ecophysiology, it is currently best suited for a well-defined question that can be answered with a relatively small dataset.

Contemporary analytical methods for determining $\delta^{13}C$, $\delta^{15}N$, or $\delta^{2}H$ values of individual AAs consists of two phases: a wet chemistry phase to purify AAs and an analytical phase to separate the AAs and measure their stable isotope values. In the wet chemistry phase, samples are typically lipid extracted, acid hydrolyzed, purified, and then derivatized (if analyzing via gas chromatograph-combustion (GC-C-)-IRMS, see below). Lipid extraction with polar solvents (e.g., chloroform:methanol or petroleum ether) is necessary when working with samples that contain >2% lipids, as excess lipids can interfere with the derivatization process and degrade chromatography. Hydrolysis with strong (~6 N) hydrochloric acid liberates individual AAs by breaking the peptide bonds that bind the carboxyl and amino terminus of sequential AAs in a polypeptide chain. AA purification with a cation exchange resin (e.g., Dowex) is often necessary when dealing with heterogeneous materials that contain a mixture of protein, lipids, and carbohydrates (e.g., blood plasma, plants, algae, and biofilms) or when working with biomineral tissues that have interfering compounds (e.g., otoliths) (Amelung & Zhang, 2001). However, significant isotopic fractionation can occur with some types of column resins or procedures (e.g., Hare et al., 1991), so consistent analytical procedures with careful evaluation of isotopic fractionation should always be employed.

The vast majority of papers reporting AA isotope data use GC-C-IRMS, which requires the derivatization of individual AAs to reduce AA polarity and increase AA volatility prior to separation and isotopic analysis (Silfer, Engel, Macko, & Jumeau, 1991). Derivatization neutralizes polar carboxyl (−COOH), amino ($-NH_2$), and hydroxyl (−OH) groups in AAs by replacing active hydrogen atoms with nonpolar moieties. The three most common derivatization reagents used in ecological and geochemical studies are: trifluoroacyl-isopropyl ester (TFA/AA/iPr, e.g., Silfer et al., 1991), pivaloyl-isopropyl ester (Pv/AA/iPr, e.g., Chikaraishi, Kashiyama, Ogawa, Kitazato, & Ohkouchi, 2007), and methoxycarbonyl AA ester (MOC/AA, e.g., Walsh, He, & Yarnes, 2014). As is the case with all wet chemistry, great care should be taken to choose the appropriate derivatization protocol for the application at hand. Each of these procedures has trade-offs, e.g., in the amount of time required to derivatize, the stability of the resulting derivatives, the safety risks of exposure (both to humans and instruments), and the ability to maximize the number of AAs measured (Ohkouchi et al., 2017). A smaller number of studies have successfully used high-pressure liquid chromatography-(HPLC)-C-IRMS, which does not require derivatization, to analyze the stable isotope values of

individual AAs (Broek & McCarthy, 2014; McCullagh, Juchelka, & Hedges, 2006). However, GC-C-IRMS continues to be the preferred analytical approach for CSIA-AA.

While the wet chemistry phase is time intensive, the major bottleneck in sample throughput is instrument analytical time. Methods vary, but separation of AAs via GC typically uses a 50–60 m nonpolar GC column and a temperature ramping procedure lasting 40–75 minutes for an individual sample injection. Samples are typically analyzed in triplicate and bracketed by an AA reference material that is derivatized along with the target samples. Reference materials are mixtures of commercially available AAs (e.g., Sigma Aldrich) with known isotope values independently measured via EA-IRMS prior to derivatization. Under ideal analytical conditions when instrumentation is performing well, this run sequence will yield AA isotope data for ~3–4 unique samples (run in triplicate with standards) in a ~24-hour time period. But note that GC-C-IRMSs are complex instruments that require constant attention and expert knowledge to operate. Generating and maintaining good chromatography on any GC system often transcends the boundary between science and art and takes time to learn.

High-quality peak separation in the chromatography is critical for generating high-quality AA isotope data. Peak coelution prevents accurate integration and isotopic measurement. Ensuring that the trace returns as close as possible to baseline between peaks requires the purification steps discussed earlier, proper selection of column type, modifications to the temperature ramps during analysis, and adjustments to peak and baseline integration on the chromatograph postanalysis. Once optimal chromatography is achieved, derivatized AA isotope data must be corrected post hoc for the extrinsic carbon or hydrogen added to the carboxyl and amino terminus of each AA as well as the associated kinetic isotope fractionation of the reaction (for sample equations see Fogel et al., 2016; O'Brien et al., 2002). Corrections accounting for the proportion of extrinsic versus intrinsic carbon and hydrogen vary depending on individual AA structure. No extrinsic nitrogen is added during derivatization so post hoc corrections of $\delta^{15}N$ data are similar to those used for correcting bulk tissue isotope data produced by EA-IRMS.

7.6 SUMMARY AND FUTURE WORK

CSIA-AA has great potential to enhance the study of animal migration, habitat use, and foraging ecology. However, like all indirect tracer-based proxies, there are a number of assumptions and inherent limitations that must be considered when interpreting AA isotope data. The success of any isotope-based application to track resource utilization, be it diet or habitat use, is dictated by our understanding of two fundamental principles of how the isotopic composition of consumer diet and tissues are related: (1) isotopic discrimination factors that control the offset between consumer and baseline isoscapes and (2) tissue incorporation rates that influence the time scale of integration.

First, we must consider the accuracy of the AA discrimination factors used to link the isotopic composition of the consumer to the underlying baseline isoscape. The analysis of source AAs ($\delta^{15}N$) and essential AAs ($\delta^{13}C$ and $\delta^{2}H$) offers a major advantage on this front. However, even these AAs with generally minimal isotopic discrimination exhibit some degree of change in isotope value during trophic transfer, which when propagated through several trophic levels, can impart a significant shift in consumer AA isotope values relative to the baseline isoscape. There is an obvious need for more data on the mechanisms that

influence carbon, nitrogen, and hydrogen isotopic discrimination in individual AAs. This requires controlled laboratory experiments cross-validated with natural experiments across an increasingly wide range of consumer taxa, consumer–resource relationships (e.g., diet composition and quality, and gut microbiota contribution), and consumer physiological conditions (e.g., fasting and disease). Recent developments in position-specific stable isotope analysis may open new doors to mechanistically study biochemical pathways along the synthesis and transformation of compounds (Gauchotte-Lindsay & Turnbull, 2016). Our improved understanding of isotopic discrimination factors will also necessitate better universal practices for accounting for uncertainty in AA isotopic measurements and associated parameters (e.g., β and Δ in trophic level calculations) used in CSIA-AA applications (e.g., Ohkouchi et al., 2017).

Second, the accuracy of an isotope approach to tracking animal movement and habitat use is dependent upon our understanding of the integration time of the sampled tissue relative to the organism's residence time in a habitat. The rate of isotope incorporation into the AAs of a consumer can vary significantly as a function of the degree of transamination/deamination (nitrogen) or synthesis (carbon and hydrogen), the kinetic isotope effects associated with AA transport, and the biochemical reactions controlling metabolic breakdown (Chikaraishi et al., 2007; McCarthy et al., 2013). While isotope turnover rates of individual AAs is still relatively unknown, several studies have found that half-lives of individual AAs in Pacific bluefin tuna (*Thunnus orientalis*) (Bradley et al., 2014) and Pacific white shrimp (*Litopenaeus vannamei*) (Downs et al., 2014) varied from weeks (e.g., Pro) to >1 year (e.g., Ser). Understanding AA isotope turnover rates is critical to constraining the temporal and spatial integrations of the local baseline isotope signals into consumer tissues. Calculation of accurate consumer trophic positions using CSIA-AA will require choosing trophic and sources AAs with comparable turnover rates. However, with proper calibration, variability in individual AA incorporation rates may also provide a framework to create an isotopic clock, as has been done previously by comparing bulk isotope values of multiple tissues with different incorporation rates, to determine the length of time an individual has spent on a new resource (e.g., habitat or food) (Phillips & Eldridge, 2006).

Finally, the accuracy of geolocation assignments using CSIA-AA or any isotope approach is only as good as the accuracy of the underlying isoscapes. Most current regional or continental scale isoscapes used to track large-scale migrations are hampered by the undersampling and uneven distribution of data across space and time (e.g., Jennings & Warr, 2003; McMahon et al., 2013b; Schell, Barnett, & Vinette, 1998), as well as the paucity of calibration samples of known origin (e.g., Langin et al., 2007). With advances in our understanding of isotope systematics (e.g., Hayes, 2001; Macko, Estep, Engel, & Hare, 1986), biogeochemical and ecosystem models (e.g., Magozzi, Yool, Vander Zanden, Wunder, & Trueman, 2017), and statistical approaches (e.g., Wunder & Norris, 2008), significant opportunities exist to expand and refine the development of spatially and temporally robust isoscapes through the development of more mechanistic and comprehensive isoscape models (Trueman, MacKenzie, & Palmer, 2012). When coupled with the improved understanding of individual AA isotopic discrimination and turnover rates, the CSIA-AA approach holds great promise for improving our understanding of animal movement, habitat use, and foraging ecology.

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