Subsidized Food Webs:
Isotopic Tracking of Trophic Interactions and Riparian Songbird Ecology

By

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ABSTRACT

Food webs are networks of trophic interactions that describe ecosystems at a fundamental level. Food web subsidies have emerged as an important motif in the architecture of these webs. Defined as resources consumed in an ecosystem distinct from the one in which they were produced, food web subsidies can link landscape dynamics and play an important role in shaping the ecology of recipient consumers and communities. The question of how consumers behave in landscapes of variable subsidy availability remains open, with important implications for food web function and stability.

Advances in stable isotope ecology have the potential to help resolve food web relationships and are the focus of the first two chapters. Chapter 1 summarizes and critically reviews compound-specific stable isotope analysis of amino acids (CSIA-AA), a method at the forefront of empirical advances in food web ecology. The advantage of CSIA-AA over conventional bulk analysis is that it produces more precise and more voluminous data from a given sample as separate isotope ratios are determined for each amino acid. Our reviews of tens of thousands of amino acid $\delta^{13}C$ and $\delta^{15}N$ values, the largest dataset of its kind assembled, clarified how habitat (aquatic/terrestrial), phylogeny, and trophic level were reflected in amino acid isotopic rations. Carbon and nitrogen have often been studied separately, with carbon used for phylogenetic questions and nitrogen for trophic ones, but both have a broad range of applications. Data supported the concept of characteristic isotope fingerprints at the base of food webs, which may be crucial to the isotopic tracking of resource fate in subsidized food webs.

Despite its utility, a major challenge to broader implementation of CSIA-AA has been that few labs perform the time-consuming and involved procedures. Chapter 2 describes a method developed to facilitate the rapid analysis of amino acids, especially those derived from

biological materials. While established methods often rely on long hydrolysis, multi-step derivatization, and lengthy chromatography, we adapted a method based on high-temperature hydrolysis, rapid methyl-chloroformate based derivatization, and efficient polar chromatography. In tests of standards and biological materials, analytical precision and accuracy were within the range of other studies but were achieved with substantial time savings. A case study of turtles produced $\delta^{15}N$ values that corresponded well with trophic expectations. Developing this method allowed for high throughput of samples to facilitate the study of a riparian food web.

Riparian ecosystems have long been a focus of the food web subsidy literature because of the important and diverse exchanges between streams and uplands. These exchanges are often concentrated at the shoreline, but the mobility of subsidies and their consumers creates the potential for broader landscape impacts. Chapter 3 presents a detailed study of tree swallows (*Tachycineta bicolor*), a generalist songbird that feeds on both terrestrial insects and emergent aquatic insects, an important subsidy. Swallows rely heavily on nestboxes. This allowed us to install "nestbox grids" that created nesting habitat from 25 to 425 meters from a creek, exposing swallows to an order of magnitude difference in aquatic prey availability based on where they settled. Contrary to predictions, swallows showed no discernible preference for nesting near water, and there was no decline in aquatic prey use or reproductive performance with distance from water. CSIA-AA of nestling feathers revealed that birds averaged 66 – 75 % aquatic prey use and were part of an algae-based food web even hundreds of meters from water. This highlights the importance of mobile consumers in increasing the spatial extent of subsidies and the value of using isotopic probes to discern food web relationships that may not be immediately apparent. Overall, the research presented documents the dynamic movement of elements through food webs and attempts to follow some important organismal pathways.

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I want to close by dedicating this dissertation to my grandparents, Bob and Patricia Hawkins. They bought me my first bird book (the Reader’s Digest *Book of North American Birds*), which inspired a lifelong love of birding and nature in seven-year old, second-grade me. My grandma kept me constantly apprised of the birds coming to her backyard bird feeders, and my grandpa and I made countless birding trips all across Southern California. My grandma passed away in January of 2017, but this dissertation was completed in no small part thanks to her love, support, and prayers. She is dearly missed.

CONTENTS

CHAPTER 1

Ecological Inferences from Organismal Amino Acid $\delta^{13}C$ and $\delta^{15}N$

Robert Walsh

Abstract

Compound-specific stable isotope analysis of amino acids (CSIA-AA) has propelled numerous recent advances in organismal ecology. By determining the isotope ratios of many amino acids per sample, this method produces more precise and more voluminous data than does conventional bulk analysis. To address the need for aggregating and analyzing CSIA-AA data from disparate biological fields, we performed a literature search and aggregated some 144 publications that analyzed several hundred species spanning broad spatial and temporal extents, the largest dataset of this kind assembled to date. $\delta^{13}C$ and $\delta^{15}N$ values of AAs and associated metadata were then used to examine correlations with various ecological and evolutionary factors. Most studies were observational and focused on marine eukaryotic organisms. Most of 16 commonly measured AAs were positively correlated with one another, but from principal components analysis, up to three meaningful axes of variation were suggested for both $\delta^{13}C$ and $\delta^{15}N$. For carbon, these related to source material and phylogeny, and for nitrogen, axes related to trophic level and habitat (aquatic or terrestrial). In models, habitat and taxonomic variables were important predictors of AA $\delta^{13}C$ and $\delta^{15}N$ values, and trophic position was important for some AAs. The age of the sample appeared to have an isolated impact, and lab effects were suggested but would require further investigation. For primary producers, the concept of a characteristic AA isotope fingerprint based on phylogeny has largely been supported by the literature. Linear discriminant analysis assigned jackknifed samples to the correct broad taxon (domain to class) in the majority of cases. $\delta^{15}N$ values are rarely used for this task but perform about as well as $\delta^{13}C$ values. Predicting offsets between consumers and their diets is crucial for work at higher trophic levels, but both lab experiments and field studies suggest consume-diet AA $\delta^{13}C$ offsets may be greater than is commonly assumed. Nonetheless, there are many potential and proven ecological applications for CSIA-AA, and we demonstrate how adding more data may improve tasks such as assigning trophic position. More experimental work and better coordination of data may be important to accelerating advances in this field.

INTRODUCTION

Much of an organism's ecology is impossible or impractical to observe directly, and stable isotope analysis has played an indispensable role in revealing some of these unseen aspects. From mere milligrams of biomass, isotopic signatures can be measured and used to

elucidate diet, trace movements, reconstruct physiological states, and draw many other ecological inferences (Peterson & Fry 1987; Hobson & Wassenaar 1999; Fry 2014). However, the most common method for organismal ecology, bulk analysis, is far from being the most informative. Bulk stable isotope analysis determines an overall average isotope ratio for whole organisms, organs, or tissues. These values can be extremely useful, but obscure the ecologically meaningful isotopic differences among an organism's constituent compounds. A focus on isotopic heterogeneity at the molecular level, particularly compound-specific stable isotope analysis of amino acids (CSIA-AA), is allowing researchers to surpass the limitations of conventional bulk analysis. Recent advances based on the $\delta^{13}C$ and $\delta^{15}N$ of AAs have been made in areas as varied as paleoceanography (McMahon et al. 2015), microbial food webs (Steffan et al. 2015), commercial fisheries (Madigan et al. 2014), and human ecology (Naito et al. 2015). CSIA-AA is increasingly recognized as a powerful, adaptable tool for ecological inquiry, but the literature is widely scattered. The purpose of this study is to aggregate and synthesize a global CSIA-AA dataset in order to evaluate the relationship between AA isotopes and major aspects of organismal ecology.

The most compelling reasons to use CSIA-AA are that it increases the specificity and volume of data produced relative to bulk stable isotope analysis (Hayes et al. 1990, Meier-Augenstein 1999, Evershed et al. 2007). Specificity is improved because isotope ratios are determined for particular AAs, not an uncharacterized mix of molecules and compounds comprising bulk biomass. This makes it possible to compare multiple organisms on a precise compound to compound basis and to develop mechanistic hypotheses about how ecological factors influence isotope biochemistry. CSIA-AA also increases the volume of data produced. Established methods routinely characterize 9-16 AAs per element per sample (Hofmann et al.

2003, Corr et al. 2007), whereas bulk analysis generates just one isotope ratio per element per sample.

More specific and voluminous data from CSIA-AA would be useless if they simply imparted redundant information. This is not the case. Despite structural similarities and numerous biochemical links between AAs, early research established that several are isotopically distinctive and fractionate independently (Abelson & Hoering 1961, Gaebler et al. 1963). The various AAs are suited to investigating different questions. For example, the $\delta^{13}C$ value of lysine makes a useful axis for separating aquatic from terrestrial consumers (Webb et al. 2015), and the $\delta^{15}N$ value of phenylalanine in marine organisms has been used to track changes in the nitrogen baseline of the Pacific Ocean (Broek et al. 2013, Décima et al. 2013, Vokhshoori & McCarthy 2014). In both cases, results may be more precise and specific than what bulk analysis can achieve. Leveraging multiple AAs may be even more useful for analysis. For example, the difference between the $\delta^{15}N$ values of an organism's glutamic acid and phenylalanine is widely used to estimate trophic position more accurately than is possible with bulk nitrogen (McClelland & Montoya 2002, Chikaraishi et al. 2009), and multivariate methods allow researchers to use the $\delta^{13}C$ values of the proteinaceous AAs to separate organisms phylogenetically (Larsen et al. 2009) or physiologically (Jackson et al. 2015). Clearly, there is sufficient isotopic variation among AAs to warrant compound-specific analysis.

Despite the many potential applications of CSIA-AA in organismal ecology, compound-specific data were generated somewhat sporadically until the 1990s. It wasn't until significant methodological advancements were made (Sano et al. 1976, Matthews & Hayes 1978) and the required instruments became commercially available that CSIA-AA became more practical and widely used (Popp 1991, Meier-Augenstein 1999, Lichtfouse 2000). Indeed, stable isotope

analysis of individual AAs at natural isotope abundance has gone on since the 1930s (Schoenheimer & Rittenberg 1939), but the median publication date of papers used in this study is 2012. It seems that after decades of calls for more mechanistic, compound-focused research in organismal ecology (Gannes et al. 1997, Martínez del Rio et al. 2009), the use of CSIA-AA has grown exponentially to the point that isotope ratios of AAs have now been presented in well over 100 publications (Fig. 1-1). The scope of the work, too, has broadened, including more microbes and organisms with unique life histories to shed light on diverse ecosystem components.

What remains lacking is a synthesis of the collective tens of thousands of available AA $\delta^{13}C$ and $\delta^{15}N$ values. For this paper, we attempted to gather all available natural abundance carbon and nitrogen isotope data from organismal AAs, regardless of the biological discipline (e.g., anthropology, nutrition, physiology). Both carbon and nitrogen were examined because they are by far the most commonly analyzed elements in AAs and because both have demonstrated their usefulness in making ecological inferences.

We had three aims after compiling this global dataset. (1)Quantify the range of organismal AA $\delta^{13}C$ and $\delta^{15}N$ values and their relationships. This basic, descriptive work is essential to evaluating the potential for new ecological information to be gained by analyzing additional AAs. (2)Model AA $\delta^{13}C$ and $\delta^{15}N$ values as functions of ecologically relevant variables. How well do some major aspects of organismal ecology explain isotopic variation in a global dataset? (3)Use experimental and field data to evaluate how an organism's AA $\delta^{13}C$ and $\delta^{15}N$ values differ from those of its source material. For autotrophs and some detritivores, this evaluation focused on the concept of an "isotop(e/ic) fingerprint" (Blair & Leu 1985, Larsen et al. 2009) characteristic of various phylogenetic groups. For consumers, the evaluation focused on the predictability of offsets between AAs in their diet and biomass, as diet is generally believed

to more strongly influence isotopic composition than phylogeny. The existence of discernible fingerprints and predictable consumer-diet offsets is crucial to most all ecological applications of CSIA-AA data. Underlying all of our aims was the intent to highlight areas where more work is needed and to critically examine common assumptions, methods, and interpretations.

METHODS

Literature Search and Data Compilation

Relevant publications were located by querying "compound-specific isotop* 'amino acid*'" in Web of Science (Thomson Reuters). The cut-off for inclusion was December 2015. 179 results were returned and all were evaluated. We then opportunistically searched references and forward citations of the initial results with Web of Science and Google Scholar, which helped to add older papers that did not necessarily use the most common contemporary key words and acronyms. Data from any study presenting organismal $\delta^{13}C$ or $\delta^{15}N$ AA values were compiled directly or, in the case of graphical data presentation, by using WebPlotDigitizer (Rohatgi 2015) to estimate values.

We excluded data from samples of ambiguous or mixed organismal origin (e.g., soil, biofilms, unsorted plankton) and from experiments employing highly-enriched isotopic tracers. Humans, domesticated species, and organisms from experimental studies were included because the distinction between natural and artificial diets and growth conditions was often somewhat arbitrary. We used taxonomic identity as a fixed effect in our models (see below), but samples were not grouped or averaged by species because we had no reason to presume that species identity trumped habitat, diet, and other factors in influencing AA $\delta^{13}C$ and $\delta^{15}N$ values. If there were multiple measurements of the same sample, we treated them as replicates and averaged

them. However, if samples came from different tissues of the same organism, they were not treated as replicates because different tissues often have distinct isotopic profiles and may reflect ecology from different time periods (e.g., McMahon et al. 2012, Ellis et al. 2014, Ohkouchi et al. 2015). Finally, if a sample was measured by multiple methods, we selected the one with data for the most AAs or, in case of a tie, with the most precise data.

The publications aggregated provided sufficient data for $\delta^{13}C$ and $\delta^{15}N$ analysis of 16 proteinaceous AAs: alanine (Ala), arginine (Arg), aspartic acid (Asp), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tyrosine (Tyr), and valine (Val). In both the carbon and nitrogen datasets, all of these AAs were analyzed in ≥102 distinct samples pooled from ≥10 different publications, usually many times more. Indicators of measurement precision (e.g., standard deviation of replicates) were recorded when available. Four of the 20 common proteinaceous AAs could not be analyzed. Cysteine and tryptophan were excluded because they are degraded during acid hydrolysis, a nearly universal preparatory step for CSIA-AA. Acid hydrolysis also converts asparagine and glutamine to their acidic forms, so they became indistinguishable parts of the aspartic acid and glutamic acid fractions, respectively.

Methodological, provenance, and organismal metadata were added to each sample in the global $\delta^{13}C$ and $\delta^{15}N$ datasets and consumer-diet offsets from controlled, known-diet studies were also assembled for subsequent analysis. These comprise data from studies in which both the organism and its diet underwent CSIA-AA, allowing for the calculation of consumer-diet offset, $\Delta^{13}C$ and $\Delta^{15}N$. The vast majority of known-diet studies were controlled experiments, though selected field studies were included when both consumer and diet were known with an extremely high degree of confidence, such as in host-specific parasitoid systems (e.g., Steffan et al. 2013).

For all statistical analyses of the datasets, R Version 3.2.3 (R Core Team 2015) was used.

AA Relationships

A Spearman's rank correlation matrix was used to gauge the correlation between pairs of AAs to minimize influence from the dataset's outliers. To explore relationships among all AAs, we performed a principal components analysis (PCA) with imputation of missing values based on a variational Bayesian framework using the R package pcaMethods (Stacklies et al. 2007). VBPCA has been found to produce reasonable results even when datasets are sparse (Ilin & Raiko 2010). To be somewhat conservative, we restricted analysis to those samples where isotope ratios of at least half of the 16 possible AAs had been determined. Samples were scaled to have unit variance prior to analysis. Use of a non-imputed dataset would have been preferable, but only 19 samples for carbon and 16 samples for nitrogen reported values for all 16 AAs of interest, which was inadequate for a broad, heuristic characterization of AA relationships.

CSIA-AA Ecological Models

The next stage of analysis was examining which ecological factors best explained variance in AA $\delta^{13}C$ and $\delta^{15}N$ values. A linear mixed model was chosen for blanket application because AA $\delta^{13}C$ and $\delta^{15}N$ values were effectively continuous and distributions often met or approached normality. We used the R package 'lme4' (Bates et al. 2014). Trophic position from the literature, habitat, taxon, and sample age were treated as fixed effects, and derivatization method was added as a random effect (operational definitions in Appendix 1-1). These variables were selected *a priori* because they could be determined for all organisms and represented several major axes of variation. During initial model exploration and comparisons, taxon proved to be a particularly important variable. Using fishes for exploratory analysis, the narrower the taxonomic assignment, the more that model AICc values improved until ultimately reaching the

species level. We did not narrow taxon beyond the rank of class, however, because this allowed for important phylogenetic divisions to be made while maintaining a reasonable variety and number of samples per taxon. Interactions between at least some of the variables are suspected to be important—for example, the effect of trophic level on the $\delta^{15}N$ value of Glu may depend on taxon (Germain et al. 2013). However, interactions were not included in order to avoid overfitting and loss of power, a concern for the less frequently measured AAs. More importantly, many potential levels for interactions were poorly covered by the data, if at all. Conditional R^2 values were generated for the mixed models (Nakagawa & Schielzeth 2013).

Isotope Fingerprints

We define an isotope fingerprint as a set of AA $\delta^{13}C$ or $\delta^{15}N$ values characteristic of some phylogenetic group. This general concept has been discussed under many headings including "isotopic molecular mapping" (Macko et al. 1987), "taxon-specific isotope fractionation" (Abraham & Hesse 2003), "biosignature" (Scott et al. 2006), and "isotop(ic) fingerprint" (Blair et al. 1985, Larsen et al. 2009), the term we have used.

To more concretely illustrate the concept of isotope fingerprints, we selected species that had been studied under broadly different growth conditions and plotted the full range of their Ala-normalized AA $\delta^{13}C$ values and Glu-normalized AA $\delta^{15}N$ values. Normalization makes more coherent fingerprints by correcting for differences in isotopic baselines. Others have performed normalization based on multiple AAs (e.g., Larsen et al. 2009, McCarthy et al. 2013), but we normalized to Ala and Glu because both molecules are commonly measured and have central metabolic positions for carbon and nitrogen biochemistry, respectively. For carbon analysis, the fingerprint of bread wheat (*Triticum aestivum*) was based on 18 different samples (Paolini et al. 2015) and that of a diatom (*Thalassiosira weissflogii*) on 28 different samples

(Larsen et al. 2015). For nitrogen analysis, the fingerprint of bread wheat was based on 28 different samples (Ostle et al. 1999, Styring et al. 2014, Paolini et al. 2015) and that of a cyanobacterium (*Synechococcus* sp.) on five different samples (Chikaraishi et al. 2009, McCarthy et al. 2013).

To more broadly determine the discernibility of taxon-specific isotope fingerprints, we assembled CSIA-AA data from organisms capable of synthesizing all of the proteinaceous AAs, a mix of autotrophs and detritivores (taxonomic kingdoms Archaea, Bacteria, Chromista, Fungi, Plantae). Conventionally, discernment has been based on linear discriminant analysis (LDA) using $\delta^{13}C$ values of AAs, especially the essential AAs (Scott et al. 2006, Larsen et al. 2009). Because of missing data, we could not exactly replicate this approach. Imputation was an inappropriate solution because it would muddle taxon-specific differences by using multiple taxa to impute AA isotope ratios. Thus, we limited the dataset to organisms that had been analyzed for all of the most common AAs, eight for carbon (Ala, Glu, Gly, Ile, Leu, Phe, Thr, Val; n = 226) and seven for nitrogen (Ala, Glu, Gly, Ile, Leu, Phe, Val; n = 180). Then, we performed LDA using R package 'MASS', based on Venables & Ripley (2002). The rate of correct taxonomic classification was determined based on assignments of jackknifed samples at the levels of domain, kingdom, phylum, and class. For each stepped increase in taxonomic specificity, we dropped any taxa for which there were fewer than three samples. This usually led to a slight reduction in the taxonomic breadth and number of total samples with each increase in specificity, but there were never fewer than 160 samples.

Consumer-Diet Offsets

The difference between the AA $\delta^{13}C$ or $\delta^{15}N$ values of a consumer's biomass and its diet, the consumer-diet offset (also known as discrimination factor or enrichment factor), was

calculated in two ways. First, we simply took the mean of offsets from the known-diet dataset. A meta-analysis approach would have been preferable, but in many cases, offsets were reported for only a single individual or without fully accounting for methodological propagated error (Docherty et al. 2002), both of which made estimates of variance, and thus meta-analysis, problematic. If there were replicates of a consumer species on a particular diet in a particular study, these were averaged. The second method of calculating consumer-diet offsets was by using the regression of an organism's AA $\delta^{13}C$ or $\delta^{15}N$ values against literature-based trophic position. Linear regression was used to make offset values comparable to those calculated from known-diet studies (i.e., without different offsets for different consumer trophic positions). We also modeled how well various Phe-normalized AA $\delta^{15}N$ values predicted empirical trophic position. Models were based on all samples for which $\delta^{15}N$ values of Glu, Phe, Pro, and Thr had been obtained (n = 606) as these AAs are of particular interest for diet studies. Linear models were compared using AICc values (Burnham & Anderson 2004).

RESULTS

Sampling Scope and Analytical Patterns

A total of 26,945 AA $\delta^{13}C$ and $\delta^{15}N$ values were gathered from 144 publications (Appendix 1-2). Datasets for both AA carbon and nitrogen spanned a wide range of taxa, biomes, geographical locations, and time periods. Chordates account for 60.5% of carbon samples and 60.9% of nitrogen samples. Aquatic organisms comprise 68.3% of carbon samples and 68.0% of nitrogen samples. 24.1% of carbon and 11.4% of nitrogen samples are ancient materials (preserved 100 – 175,000 years B.P.), and 19.0% of carbon samples and 19.4% of nitrogen samples come from organisms raised in controlled laboratory conditions.

The overwhelming majority of samples (96.6% for carbon, 99.2% for nitrogen) were analyzed with variations on a three-part procedure: (1)Acid hydrolysis of a protein sample, (2)Derivatization of AAs and separation by gas or liquid chromatography, and (3)Combustion and isotope-ratio mass spectrometry. The balance of studies omitted hydrolysis and analyzed free AAs (e.g., Petzke & Lemke 2007) or did not employ derivatization (e.g., Abelson & Hoering 1961). Within the conventional three-part procedure, at least seven variations on acid hydrolysis and nine different derivatization methods were employed. Nearly all samples were measured in duplicate or triplicate—less than 1% of the data was not the average of technical/analytical replicates of the same sample. This made it possible to assess which AAs had higher or lower precision than average compared to other AAs from the same sample (Appendix 1-3). For carbon, AAs without heteroatom-containing side-chains (e.g., Val, Phe, Leu) can be analyzed most precisely, but for nitrogen, patterns cannot be as explicitly tied to molecular structure. Precise measurements of Arg, His, and Thr are a challenge for both elements.

AA Relationships

The $\delta^{13}C$ values of all AAs are significantly positively correlated (Fig. 1-2, $R_s = 0.30 – 0.90$). From VBPCA, PC1 explains 75.7% of the total variance (Fig. 1-3) and relates to source carbon or the organism's environment. PC2 provides some phylogenetic separation of organisms, particularly fungi, based primarily on $\delta^{13}C$ values of AAs with aromatic and heteroatom-containing side-chains. The first three PCs have eigenvalues >1, a commonly used cutoff for non-trivial components (Jackson 1993). Based on samples in which at least two different AAs had been measured, the average range of AA $\delta^{13}C$ values within a single organism is 19.59‰ (σ = 7.03‰)

Correlations of $\delta^{15}N$ values are more variable (Fig. 1-2, R_s = -0.75 – 0.95). Most pairs are significantly positively correlated, but Thr $\delta^{15}N$ values are significantly negatively correlated with the $\delta^{15}N$ values of most other AAs. Ten pairs of AAs have no significant correlation; most of these pairs include Arg, Met, and/or Phe. From VBPCA, PC1 explains 66.1% of the total variance and relates strongly to trophic position, and all AAs but threonine load on this component in the same direction (Fig. 1-3). The first three PCs have eigenvalues >1. Based on samples in which at least two different AAs had been measured, the average range of AA $\delta^{15}N$ values within a single organism is 21.20‰ (σ = 13.5‰).

CSIA-AA Ecological Models

Organismal AA $\delta^{13}C$ and $\delta^{15}N$ values are strongly predicted by habitat and taxonomic class. Type III F-tests show these factors explain a significant amount of variation in the $\delta^{13}C$ and $\delta^{15}N$ values of nearly all AAs, warranting their inclusion in models (Table 1-1). The direction of their effect varies by AA, but overall, marine organisms and vertebrates had relatively higher $\delta^{13}C$ and $\delta^{15}N$ values. Trophic position is another significant explanatory variable of the $\delta^{15}N$ values of 12 AAs and of the $\delta^{13}C$ values of nine AAs. Generally, values of both $\delta^{13}C$ and $\delta^{15}N$ increase with trophic position. Sample age, the final fixed effect in the models, generally does not account for a significant amount of variation in AA $\delta^{13}C$ or $\delta^{15}N$ values. A few exceptions include Ser for both elements. R^2 estimates of carbon models average 0.531, lower than R^2 estimates of nitrogen models, which average 0.663.

Derivatization method was included as a random effect, and its inclusion is generally supported by likelihood ratio analysis ($p < 0.05$, Table 1-1). The AAs for which derivatization method was not found to be statistically significant were among the most infrequently measured AAs (e.g., Arg, His, Met). Inspection of the models for these AAs revealed they had usually been

analyzed by just two or three derivatization methods, and a fixed effect with fewer than five levels produces unreliably low estimates of variance for the fixed effect (Gelman & Hill 2007).

Isotope Fingerprints

The concept of isotope fingerprints based on phylogeny has empirical support in the literature. Several such fingerprints were plotted for illustrative purposes (Fig. 1-4). The specificity of the fingerprint can be thought of in comparative terms; what is the isotopic range of an AA for the species compared to the range of that AA in the global dataset? On average, wheat AA $\delta^{13}C$ values span 21.2% of the global range, diatom AA $\delta^{13}C$ values span 18.6% of the global range, wheat AA $\delta^{15}N$ values span 32.8% of the global range, and cyanobacteria AA $\delta^{13}C$ values span 25.6% of the global range. These comparative ranges are slight overestimates as the global range to which they are compared was trimmed of the most extreme 5% of values.

LDA assigns most species to the correct taxon using CSIA-AA data. Over 80% of jackknifed samples can be assigned to the correct taxonomic domain based on LDA of $\delta^{13}C$, normalized $\delta^{13}C$, $\delta^{15}N$, or normalized $\delta^{15}N$ values (Fig. 1-5). As taxonomic ranks narrow, correct assignments using $\delta^{13}C$ and normalized $\delta^{13}C$ values decline monotonically to 61.1% and 56.6% of samples classified to the correct class, respectively. $\delta^{15}N$ and normalized $\delta^{15}N$ values are both least successful at correctly assigning samples to kingdom, but assignment within the narrower taxa of phylum and class improves. For all taxonomic ranks and both elements, LDA based on normalized values was always equally or less successful at taxon-assignment than analysis based on non-normalized values.

Consumer-Diet Offsets

Significant consumer-diet offsets in $\delta^{13}C$ values are the norm but vary by the method used to estimate the offset (Fig. 1-6). In known-diet studies, the offset between a consumer's AA

$\delta^{13}C$ values and those of its diet average 2.18‰ ($\sigma^2 = 0.83$‰). One-sample t-tests show that the mean offsets of Arg, Gly, His, Ile, Lys, Met, and Tyr are not significantly different than 0 ($p > 0.05$). Offsets based on the regression of AA $\delta^{13}C$ values against empirically-determined trophic position average 2.29‰ ($\sigma^2 = 0.28$‰), and Arg, Lys, Met, and Tyr have slopes not significantly different than 0 ($p > 0.05$). For individual AAs, offsets calculated by the two methods are weakly positively correlated ($R^2 = 0.229$ $p < 0.001$).

Calculations of consumer-diet offsets in $\delta^{15}N$ values also vary by the method used to estimate the offset (Fig. 1-6). In known-diet studies, the offset between a consumer's AA $\delta^{15}N$ values and those of its diet average 3.36‰ ($\sigma^2 = 0.64$‰). One-sample t-tests show that the mean offsets of His, Met, Phe, and Tyr are not significantly different than 0 ($p > 0.05$). Offsets based on the regression of AA $\delta^{15}N$ values against empirically-determined trophic position average 3.41‰ ($\sigma^2 = 0.22$‰), and Arg has a slope not significantly different than 0 ($p > 0.05$). For individual AAs, offsets calculated by the two methods are positively correlated ($R^2 = 0.373$, $p < 0.001$). Arg was an outlier with a major discrepancy between the consumer-diet $\delta^{15}N$ offsets calculated from known-diet studies versus regression with empirical trophic position. If Arg is excluded from analysis, the correlation between the two methods is much stronger ($R^2 = 0.850$, $p < 0.001$).

Models using Phe-normalized AAs to characterize offsets and predict empirical trophic position are generally more successful as more AAs are included (Table 1-2). Including Phe-normalized Glu, Pro, and Thr $\delta^{15}N$ values produces the lowest AICc value and the model has considerable predictive power ($R^2 = 0.832$). It is only marginally better than a model that does not include Phe-normalized Pro.

DISCUSSION

The largest CSIA-AA dataset to date has been assembled to provide deeper isotopic insights into ecology. By taking an organismal perspective, a unified picture sequentially emerges. First, there are multiple important axes of variation for an organism's AA $\delta^{13}C$ and $\delta^{15}N$ values. A handful of ecological variables—habitat, phylogeny and trophic position—can explain most of the variation observed in most AAs. Characterizing how isotopic differences arise is the next step. For autotrophs and some detritivores, the concept of an isotope fingerprint explains differences between an organism's source material and biomass based on phylogeny and has reasonable predictive power. For heterotrophs, the related concept of predictable consumer-diet offsets is also important, but offsets are neither as simple nor as uniform as suggested in some prior studies. The ultimate goal of applying CSIA-AA to answer ecological questions fundamentally relies on the isotope fingerprint and/or offset concepts. Explicitly molecular-ecological mechanisms are not required to apply CSIA-AA, but proposing such mechanisms helps focus the development and interpretation of ecological applications.

Sampling Scope and Analytical Patterns

For both carbon and nitrogen, the most well-represented organisms in the dataset are contemporary, free-living, aquatic chordates, particularly bony fishes (Actinopterygii). This bias is not inherently problematic, but it should be kept in mind when interpreting metrics and patterns that summarize the dataset as a whole. The focus on animal field studies suggests a greater eagerness for applying CSIA-AA in natural settings than for lab studies. There is four times as much field data as lab data, but the important grounding provided by lab studies has led to calls for more (Martínez del Rio et al. 2009, Boecklen et al. 2011).

The particulars of CSIA-AA methods vary, but the prioritization of precision (i.e., duplicate or triplicate analyses of the same sample) over breadth of analysis is widespread and has important implications. A single analytical run may monopolize about an hour of instrument time for gas chromatographic techniques or two to three hours for liquid chromatographic techniques (Hofmann et al. 2003, Corr et al. 2007, Dunn et al. 2011), and these figures do not include the need for reference and quality control runs. The rate of output many be just a few samples per instrument per day when replication is particularly extensive. The optimal balance between precision and extent of analysis must be determined on a case-by-case basis, but single runs may produce results with precision not far from the level achieved with replicated runs (Walsh et al. 2014). With only a few thousand samples analyzed by CSIA-AA, it is worth considering whether marginally less precise but more broadly exploratory work could be useful.

AA Relationships

AAs do not converge on a common $\delta^{13}C$ value but span an average of 19.59‰ within a single organism. That is nearly as expansive as the range of bulk $\delta^{13}C$ values of the world's major ecosystem components (Peterson & Fry 1987). Yet even this substantial inter-organismal range is almost certainly an underestimate as most studies did not analyze all AAs, precluding the true range from being known. Despite such heterogeneity at the molecular level, all AAs load strongly and unidirectionally on the PC1 axis apparently reflecting source, with phylogenetic separation apparent secondarily. No clear categories of AAs, such as essential/non-essential or glycolytic/ketolytic, appear to be exceptionally clustered in VBPCA, though the overall tightness of clustering makes such patterns difficult to discern (Fig. 1-3). AAs separated by a single biosynthetic step (e.g., Gly/Ser, Phe/Tyr) did not necessarily have the most strongly correlated

$\delta^{13}C$ values (Fig. 1-2), which could be attributed to problems with analytical accuracy or to the complexity of fractionation along even the simplest metabolic pathways.

Despite obtaining nitrogen from a common Glu pool, AA $\delta^{15}N$ values are even more heterogeneous than $\delta^{13}C$ values. The range of AA $\delta^{15}N$ values within a single organism averages 21.20‰. Many aquatic predators had much broader ranges; Choy et al. (2015), for example, report a 67.5‰ difference between the $\delta^{15}N$ values of Ala and Thr in a broadbill swordfish (*Xiphias gladius*). That span is many times greater than the range of bulk $\delta^{15}N$ values across the world's major ecosystem components (Peterson & Fry 1987). Such remarkable observations are driven by the tendency for Thr to become more depleted as most other AAs become more enriched in ^{15}N at higher trophic positions. This pattern is evident in VBPCA (Fig. 1-3), an indication that the major axis is a trophic one. The unique dynamics of Thr have long been noted but not yet satisfactorily explained. Hypotheses include preferential incorporation of Thr with ^{14}N into proteins (Gaebler et al. 1966); unusual enzymatic dynamics with an "inverse isotope effect" during Thr catabolism (Hare et al. 1991) which is amplified by successive trophic transfers (Styring et al. 2010); and high demand for Thr in blubber-producing organisms (Germain et al. 2013). Several other AAs, particularly Met and Phe, do not load strongly in either direction on the major axis in VBPCA but do along the axis for PC2, which appears to separate organisms by aquatic versus terrestrial habitat.

Based on correlations, the most unexamined AAs may also be the most biochemically interesting. Pairwise correlations should not be over-interpreted because the dataset is plagued by missing data. Still, it is interesting to note that the set of "typical" AAs (i.e., those with the highest average correlation with all other AAs) is identical for both carbon and nitrogen: Ala, Asp, Glu, Ile, Leu, Lys, Pro, and Val. By this convention, the "atypical" AAs are Arg, Gly, His,

Met, Phe, Ser, Thr, and Tyr. The list of typical AAs includes all of the pyruvate and α-ketoglutarate derived AAs, while list of atypical AAs is expectedly heterogeneous, with the largest and smallest AAs, the AAs with the fewest and most heteroatoms, and AAs synthesized along a variety of different pathways. Unfortunately, the atypical AAs include all of the least frequently measured AAs. Their lack of adherence to an organism's overall isotopic patterns suggests they would be valuable targets for future study. More analysis would make it possible to determine whether they are subject to high analytical error, only appear unusual because they are represented by a few unrepresentative datasets, or indeed have unique isotopic dynamics reflecting interesting aspects of organismal ecology.

CSIA-AA Ecological Models

Ecological models of AA $\delta^{13}C$ and $\delta^{15}N$ values were generally successful in that, for nearly all AAs, most of the parameters included had significant explanatory power and the majority of variance was explained. The key ecological variables of habitat, taxon, and trophic level are often related, but a hierarchy of their effects on organismal AA $\delta^{13}C$ and $\delta^{15}N$ values is not clear-cut.

Habitat must have some fundamental importance because the pools of carbon and nitrogen from which biomass is built differ isotopically at the landscape scale (Peterson & Fry 1987, Hobson et al. 1999). In general, the AAs of aquatic organisms have higher $\delta^{13}C$ and $\delta^{15}N$ values than those of their terrestrial counterparts, a pattern in agreement with the bulk stable isotope literature. The aquatic/terrestrial habitat divide is particularly apparent in the relatively low $\delta^{13}C$ and $\delta^{15}N$ values of Phe in terrestrial organisms. Some hypothesize that this is related to the differing fates of Phe in tracheophytes versus algae (Larsen et al. 2013, Ohkouchi & Takano 2014). Tracheophytes are unique in that they convert much Phe to lignin and other secondary

compounds important to a terrestrial lifestyle (Douglas 1996), which can result in fractionation of ^{13}C and ^{15}N of Phe. Studies that include aquatic tracheophytes (e.g., marine grasses), however, suggest phylogeny may matter more than habitat for both carbon (McMahon et al. 2011, Larsen et al. 2012, Larsen et al. 2013) and nitrogen (Vander Zanden et al. 2013). Contrastingly, Larsen et al. (2013) found that a phylogenetically diverse assemblage of microalgae (cyanobacteria, diatoms, haptophytes, and others) converged on a similar AA $\delta^{13}C$ profile in their aquatic environment. This was true even for chlorophytes, which are more closely related to tracheophytes than to other taxa in the polyphyletic microalgae group. Put simply, habitat has explanatory power for AA isotope ratios in autotrophs, but there is no one universal explanation for its importance.

The aquatic/terrestrial habitat divide may influence animal AA isotope biochemistry via excretory pathways. Nitrogen may be excreted as ammonia, urea, or uric acid. There is variation in the predominant form of excreted nitrogen within amphibians, reptiles, arthropods, and fishes that is related to whether an organism inhabits marine, freshwater, or terrestrial environments (Walsh & Wright 1995, Wright 1995). Evidence about the importance of the form of nitrogen excretion to particular AA $\delta^{15}N$ values is conflicting (Germain et al. 2013, Hoen et al. 2014, Nielsen et al. 2015). Still, the potential impacts of such different excretory strategies may at least partly explain why habitat was an important explanatory variable for modelling the $\delta^{15}N$ values of AAs associated with excretory pathways such as Ala, Arg, and Glu.

Taxon is another key explanatory factor in models of AA $\delta^{13}C$ and $\delta^{15}N$ values, even for heterotrophs. Evolutionary constraints are fundamental to an understanding of metabolic biochemistry of AAs and other compounds (Lewis et al. 2012). For example, bacteria synthesize Val somewhat differently than plants, which may lead to detectable isotopic differences by taxon

(Keil & Fogel 2001). The concept loses explanatory power for heterotrophs. Generalists like humans can subsist on strikingly different diets, and there is no evidence that the body brings dietary AA $\delta^{13}C$ and $\delta^{15}N$ values to some characteristic human norm. As codified by DeNiro & Epstein (1976), "you are what you eat--plus a few permil." Thus, humans and their pet dogs (*Canis lupus*) from the same village may appear isotopically similar despite being phylogenetically distant (Choy et al. 2010, Honch et al. 2012), whereas humans living under different food cultures may appear isotopically dissimilar despite being phylogenetically close (Corr et al. 2005, Honch et al. 2012). If the global dataset is primarily based on heterotrophs that more or less reflect their diet, then why is phylogeny so important to explaining AA $\delta^{13}C$ and $\delta^{15}N$ values? The variable "taxon" may act as a catchall that captures physiological, ecological, and other related characteristics and which may collectively shape AA $\delta^{13}C$ and $\delta^{15}N$ values in distinctive ways.

Increased trophic position was often accompanied by an increase in AA $\delta^{13}C$ and especially $\delta^{15}N$ values. This pattern is explained by kinetic isotope effects during nitrogen excretion that are compounded with each trophic transfer, the principal which underlies the widespread use of bulk $\delta^{15}N$ values to estimate trophic position (DeNiro & Epstein 1976, Boecklen et al. 2011). The set of AAs for which trophic position is not important in predicting $\delta^{15}N$ values include those that are not transaminated as an initial step of catabolism, such as Phe (Gaebler et al. 1966, Chikaraishi et al. 2007, Ohkouchi & Ogawa 2014). Since nitrogen-bonds are not broken during their catabolism, fractionation of nitrogen is hypothesized to be less pronounced than for the other AAs. Trophic position is not as important to modelling AA $\delta^{13}C$ values. With 1-8 other carbon atoms per AA, fractionation during catabolism may be present but muted by all the other atoms not involved in the reaction. AA $\delta^{13}C$ values may also respond to

whether consumers obtain AAs directly from dietary protein ("routing") rather than by synthesizing AAs from lipids, which are ^{13}C-poor (DeNiro & Epstein 1978). More generally, the ratio of carbohydrate, fat, and protein should vary by trophic position and make trophic position an important predictor for both carbon and nitrogen (Hoen et al. 2014, Nielsen et al. 2015).

The final fixed effect to be included in the models was sample age on the grounds that proteins and their AAs change markedly during decay and diagenesis. However, sample age was not a significant explanatory factor for most AA $\delta^{13}C$ and $\delta^{15}N$ values. This is in line with the finding that there is no marked fractionation during some time-related changes to proteins, such as the racemization of AAs (Schroeder & Bada 1976, Silfer et al. 1994). As biomass breaks down, some fractions will maintain a more or less faithful record of the AA isotopic profile from life (Hare et al. 1991, Fogel & Tuross 1999, Keil & Fogel 2001). It is worth noting that the most frequently analyzed ancient material in the global dataset was collagen, which has been labeled as a "survivor protein" particularly resistant to degradation over time (Wadsworth & Buckley, 2014). Including sample age was important for modelling Ser $\delta^{13}C$ and $\delta^{15}N$ values; ancient samples had relatively higher $\delta^{13}C$ and lower $\delta^{15}N$ values compared to modern ones. Ser has been singled out as an important modern contaminant of ancient materials, which may explain this finding (Hare et al. 1991). This is based on the pronounced lability of Ser. Over time it is converted to other forms, so any Ser that is detected after a long period may in fact more likely come from contemporary contaminating proteins. Otherwise, there are few indications that sample age plays a major role in shaping $\delta^{13}C$ and $\delta^{15}N$ values.

The choice of derivatization method was included as a random effect to control for its direct influence on CSIA-AA results (Dunn et al. 2011) and as a reasonably good proxy for overall "lab effects," and these should not be overlooked. The handful of labs that perform most

CSIA-AA tend to repeatedly use their own characteristic set of hydrolysis, derivatization, and chromatographic methods. Likelihood ratio tests supported the inclusion of derivatization method for models of nearly all AAs of both elements. This may not be a concern for interpretation of data analyzed by the same lab and based on relative differences, but it is of concern for those interested in obtaining true AA $\delta^{13}C$ and low $\delta^{15}N$ values and for those comparing data between labs. For example, inter-lab calibration between the University of Kiel and the University of California, Davis, found trivial inter-lab differences in the $\delta^{13}C$ values of Ile, Leu, Phe, and Val (<1‰) but a pronounced difference in the inter-lab $\delta^{13}C$ values of Lys (5.5‰; Ayayee et al. 2015). An inter-lab calibration between the University of Kiel and the University of Hawaii found trivial inter-lab differences in $\delta^{13}C$ values of Lys, Thr, and Val (<1‰), but inter-lab differences in the $\delta^{13}C$ values of Ile, Leu, and Phe exceeded 1‰ (Arthur et al. 2013). At present, there are no international standards for CSIA-AA (i.e., reference proteins with known AA values), so lab effects remain a critical but often overlooked consideration.

Isotope Fingerprints

An important question about isotope fingerprints is whether they are best understood on phylogenetic grounds. Qualitatively, a characteristic pattern for each species emerges based on both absolute and relational AA $\delta^{13}C$ and $\delta^{15}N$ values (Fig. 1-4). The considerable breadth of values spanned by single AAs, however, indicates that the isotope fingerprint does not have a strictly phylogenetic basis, in which case we would expect source-normalized values to have a narrower span reflecting analytical error alone (~1‰). Indeed, some environmental or environmentally-induced factors cause an organism's isotope fingerprint to "smudge." For example, the $\delta^{13}C$ values of Ala-normalized Phe in diatoms span 10‰ under different growth conditions (Larsen et al. 2015), and Paolini and Ziller (2015) were able to use the wide range of

Glu $\delta^{15}N$ values to distinguish between organic and non-organic origins of samples from a single wheat species (Fig. 1-4). Further smudging may be caused by physiological considerations, including the compartmentalization of different chemical reactions. This is suspected to give rise to the slightly different fingerprints for seeds and leaves of the same plant (Lynch et al. 2011). Further progress in separating the phylogenetic from environmental basis of isotope fingerprints will be made by examining more species grown under a wide range of conditions and looking for uniquely stable components of the fingerprint.

Looking to $\delta^{15}N$ AA values for fingerprinting, while uncommon, may be as valid as using $\delta^{13}C$ AA values. In the global dataset, most recent work on isotope fingerprints has heavily emphasized separating taxa on the basis of AA $\delta^{13}C$ values. These can correctly categorize the vast majority of jackknifed samples to domain, kingdom, or phylum, even with the constraints imposed by our dataset (i.e., relatively few AAs per sample and measurements not standardized to account for different labs). LDA with AA $\delta^{15}N$ values relied on a different pool of samples, so direct comparisons between the two elements are invalid. Yet it is clear that nitrogen isotope fingerprints deserve more attention, as others have also suggested (McCarthy et al. 2013). LDA informed by just six different, Glu-normalized AA $\delta^{15}N$ values successfully identified most biological samples to taxonomic class. Though not presented here, we also explored LDA with categories other than phylogenetic taxa. This is because many authors have looked for fingerprints characteristic of such eco-genetic groupings as prokaryotes (Larsen et al. 2009, McCarthy et al. 2013, McMahon et al. 2015), detritivores (McMahon et al. 2016), graminoid plants (Lynch et al. 2016), and microbes with particular metabolic strategies (Scott et al. 2006). Adding isotopic data from more AAs, additional non-AA molecules, or position-specific values would make the potential to resolve distinct fingerprints even greater. These fingerprints needn't

be drawn on a purely phylogenetic basis, but phylogeny may be the single-most useful categorizer.

Offsets

Isotopic offsets between consumers and their diet can be summarized simply: variable but generally positive. This holds true for most AAs for both carbon and nitrogen. It is a pattern that calls into question the use of binary frameworks for analyzing consumer-diet offsets in AA $\delta^{13}C$ and AA $\delta^{15}N$, namely essential/non-essential and source/trophic.

Consumer-diet offsets are often regarded as insignificant for EAAs, but we found significant deviations from this assumption. Non-essential AAs can be incorporated directly from the diet but can also be synthesized from carbon scavenged from a host of other molecules. Many experiments have been designed to highlight the potential for non-AA carbon sources to become integrated in an organism's non-essential AAs (O'Brien et al. 2003, Jim et al. 2006, Newsome et al. 2014). In theory, essential AAs provide a contrast because they come strictly from dietary protein and cannot be synthesized by most consumers, thereby simplifying interpretation of AA $\delta^{13}C$ values. Experimental support for this concept is based on negligible consumer-diet offsets for some essential AA $\delta^{13}C$ values (Howland et al. 2003, McMahon et al. 2010). Yet the $\delta^{13}C$ values of the essential AAs Leu, Phe, Thr and Val not only have significant consumer-diet offsets in isolated known-diet studies but average a significant, positive offset across all studies. Field data support the experimental findings, as these essential AAs and others had non-zero slopes in regressions of AA $\delta^{13}C$ values against empirical trophic position. Offsets may be caused by kinetic isotope effects during catabolism or reflect the importance of endosymbiotic microbial contributions of essential AAs to host consumers (Newsome et al. 2011, Ayayee et al. 2015). Though the magnitude of the offset is small at about 1-2‰, it is clear

that essential AA $\delta^{13}C$ values should not be assumed to be fixed. The essential/non-essential AA framework could be further improved by considering variable demand for nutritionally non-essential AAs (Wu et al. 2014).

As expected, nitrogen offsets are more pronounced than those of carbon, averaging 3.41‰ across all AAs. This value corresponds well with the 3-4‰ offset observed between consumers and their diet in bulk analysis (DeNiro & Epstein 1981, Minagawa & Wada 1984), most likely because AAs are the major reservoir of nitrogen in animal biomass. Their collective individual trends underlie the bulk trend. The essential/non-essential divide is generally disregarded for nitrogen. Instead, the concept of source AAs (those with minimal offsets) and trophic AAs (those with large, consistent offsets) is paradigmatic (Popp et al. 2007). The distinction between source and trophic AAs has an entirely empirical basis that relies on a few highly influential studies of a limited number of samples (McClelland & Montoya 2002, Popp et al. 2007, Chikaraishi et al. 2007).

Though many of the source AAs do indeed show no or limited change with trophic positions (Table 1-1), results from known-diet studies must be considered the gold standard, and these suggest that offsets fall along a gradient rather than discrete categories. Indeed, Phe appears to be the only good candidate for the title of source AA. The $\delta^{15}N$ values of Phe from 69 known-diet analyses had an average offset of just -0.21‰ ($\sigma^2 = 0.25$), and empirically-based regression based on 1,667 samples suggested an offset of -0.39‰ ($\sigma^2 = 0.12$). His, Met, and Tyr show some potential to function as source AAs, but none are remotely as well studied as Phe, so more work is needed. The conventional "trophic" AAs, exhibited a considerable range of average offsets, and offsets for the same AA could vary markedly between and within studies. For example, the $\delta^{15}N$ values of Pro and Val showed anomalously low consumer-diet offsets for lemon sharks

(*Negaprion brevirostris*), but this was not the case for other shark species from the same well-controlled study (Hoen et al. 2014). At the microbial level, Gutierrez-Rodriguez et al. (2014) suggested that consumer-diet offsets observed in protozoa are so slight as to render them trophically "invisible", yet Steffan et al. (2015) declared microbes to be trophic "analogues" of the offset dynamics observed in animals—though their microbial focus was bacteria and fungi in place of protozoa. Additional, well-controlled experiments will be required to better constrain and characterize consumer-diet offsets in AA $\delta^{15}N$ values.

For both AA $\delta^{13}C$ and $\delta^{15}N$ values, there was significant but imperfect agreement of offsets estimated from known-diet studies with those estimated from the regression of field data against literature-determined trophic positions. Agreement between the two methods is stronger for nitrogen, perhaps because there is more data to make estimates more broadly representative. Major discrepancies in agreement of offsets based on estimation method were noted for for $\delta^{13}C$ values of Ile and for $\delta^{15}N$ values of Arg, both likely due to limited sampling in the known-diet dataset.

The final component of our consumer-diet offset analysis illustrates the value of using multiple, distinctive AAs to study offsets related to trophic position. We modeled TP as a function involving various combinations of Glu, Pro, and Thr normalized to Phe. Glu is the usual focus (Chikaraishi et al. 2007), but Bradley et al. (2014) and Nielsen et al. (2015) have suggested the utility of Thr, and McMahon et al. (2015) have suggested Pro is worth further consideration on the basis of their offset dynamics. The strongest model relating AA $\delta^{15}N$ values to trophic position included Glu, Pro, and Thr, and a marginally weaker model included all but Glu and Thr only. Given that our understanding of consumer-diet offsets requires further refinement, the use of multiple AAs may better resist erroneous interpretations than reliance on any single AA.

Applications

The appeal of CSIA-AA as a general tool is built upon the fact that any ecological factor that affects AA biochemistry has the potential to lead to detectable isotopic fractionation. There are often more precise tools for providing various ecological insights, but AA $\delta^{13}C$ and $\delta^{15}N$ values effectively record information "automatically" without the need for prior intervention, and this information is accessible from a miniscule sample of biomass. Table 1-3 summarizes some of the main thrusts of CSIA-AA in ecology, several of which have already been discussed.

It is clear that most applications are variations on the same empirically-based general approach. An ecological difference of interest is identified and exploratory CSIA-AA finds the AAs, normalized AAs, or sets of AAs that best reflect that difference. There is much overlap between applications, so a rule of thumb is that if some factors are known (e.g., trophic level, origin), it becomes possible to assign remaining variability to other factors, improving inferential power. Much as with stable isotope ecology in general, CSIA-AA ecology has tended to make *a posteriori* explanations of patterns rather than confirm *a priori* predictions. Given the immense complexity of organismal biochemistry and its ecological context, this is entirely reasonable. Yet without theoretical grounding, there is no reason to presume that the best candidate AAs for a particular task have always been found. Thus, the table of methods is being actively developed, and convention should not be considered synonymous with best practices.

CONCLUSION

CSIA-AA is a method for which it's easy to formulate (though far from easy to achieve) a "to-do" list that would incrementally improve accuracy, precision, and power: better inter-lab standardization; higher throughput; more frequent examination of analytically challenging AAs

like His; development of CSIA-AA for hydrogen and oxygen; position-specific analysis; and developing rigorous analytical methods robust against the common problems of sparse data split among many categories. Developing a central online repository for these data would also be prescient and entirely tractable given the "mere" thousands of samples that have been analyzed. Much as one can query a genetic sequence and immediately put it into a biological context, being able to query an isotopic fingerprint could be very informative. Data aggregated herein makes a start, but there is no substitute for a constantly-updated, easily accessible repository of CSIA-AA data. If values were presented with enough meta-data, untested hypotheses could be tentatively explored (e.g., are there isotopic signatures of endothermy?), gaps in our knowledge could be quickly identified (e.g., why so few samples from Archaea?), and reference datasets could be constructed (e.g., marine Phe isoscapes).

As all of these ends are pursued, consideration of biochemical-ecological mechanisms should be important and rewarding. Mechanisms turn isotopically-based predictive equations from useful black boxes to integrated frameworks in which exceptions and limitations may become far more obvious. AAs are not merely grist for analytical mills but rather the key links between biochemical signals and ecological meaning.

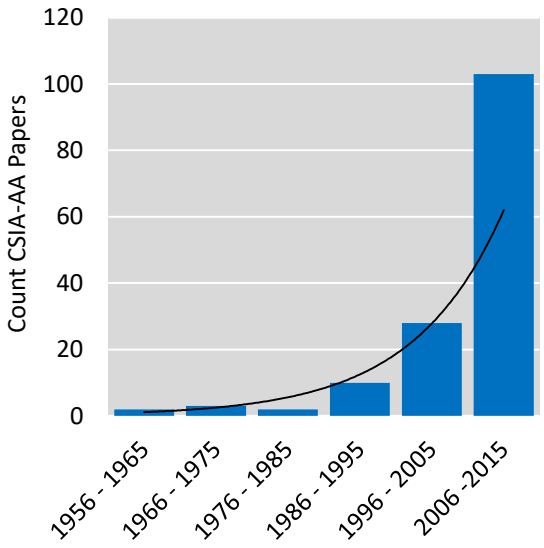

Figure 1-1. Publication dates of the resources aggregated for this study (median = 2012) fit with an exponential curve.

	Arg	Asp	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Tyr	Val
	0.82	0.85	0.83	0.69	0.69	0.79	0.76	0.71	0.81	0.71	0.85	0.67	0.66	0.58	0.82
Arg	*0.12*	0.84	0.79	0.61	0.60	0.50	0.51	0.78	0.56	0.68	0.87	0.59	0.54	0.53	0.77
Asp	0.89	0.31	0.87	0.71	0.74	0.79	0.81	0.74	0.79	0.79	0.90	0.69	0.72	0.54	0.85
Glu	0.92	0.38	0.94	0.69	0.79	0.80	0.78	0.72	0.75	0.77	0.86	0.65	0.73	0.50	0.83
Gly	0.40	0.61	0.30	0.47	0.76	0.62	0.64	0.54	0.39	0.55	0.67	0.78	0.68	0.46	0.68
His	0.38	*0.08*	0.27	0.35	0.40	0.73	0.75	0.79	0.59	0.63	0.87	0.76	0.73	0.59	0.84
Ile	0.92	0.27	0.92	0.90	0.48	0.43	0.87	0.81	0.84	0.82	0.81	0.67	0.73	0.79	0.84
Leu	0.95	0.48	0.89	0.91	0.43	0.37	0.95	0.80	0.67	0.86	0.83	0.58	0.71	0.72	0.88
Lys	0.47	0.67	0.62	0.60	0.59	0.50	0.42	0.51	0.78	0.77	0.70	0.72	0.78	0.77	0.79
Met	0.56	*-0.02*	0.32	0.35	*-0.04*	0.50	0.55	0.58	0.15	0.68	0.74	0.63	0.69	0.75	0.73
Phe	*0.04*	0.65	0.28	0.09	0.37	0.22	*-0.03*	0.09	0.70	-0.40	0.81	0.57	0.67	0.80	0.86
Pro	0.79	0.64	0.88	0.89	0.51	0.42	0.79	0.85	0.63	0.24	0.28	0.66	0.70	0.30	0.86
Ser	0.51	0.65	0.52	0.59	0.82	0.28	0.52	0.56	0.72	*0.05*	0.46	0.66	0.66	0.30	0.67
Thr	-0.63	*0.03*	-0.55	-0.63	0.22	-0.27	-0.75	-0.70	*0.00*	-0.24	0.16	-0.62	0.11	0.51	0.73
Tyr	0.57	0.62	0.70	0.72	0.34	0.38	0.62	0.70	0.72	*0.09*	0.53	0.75	0.52	-0.38	0.72
Val	0.91	0.40	0.85	0.92	0.52	0.44	0.91	0.92	0.44	0.43	0.10	0.84	0.55	-0.64	0.59
	Ala	Arg	Asp	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Tyr

$\delta^{13}C$ ↑

$\delta^{15}N$ ←

Figure 1-2. Spearman's rank correlation coefficients of $\delta^{13}C$ values (above diagonal) and $\delta^{15}N$ values for all pairs of AAs. Darker shading indicates stronger correlations. All but ten correlation coefficients (italicized) differ significantly from 0.

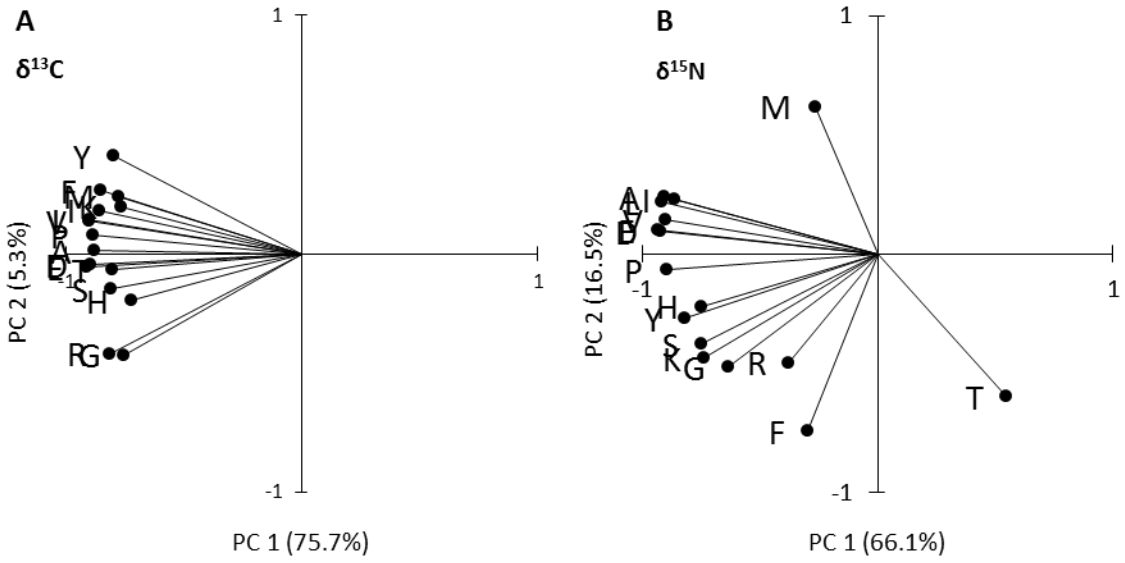

Figure 1-3. Factor loadings from VBPCA of datasets for **(A)** carbon, and **(B)** nitrogen. The conventional AA abbreviation letters are used: A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; P, proline; R, arginine; S, serine; T, threonine; V, valine; Y, tyrosine.

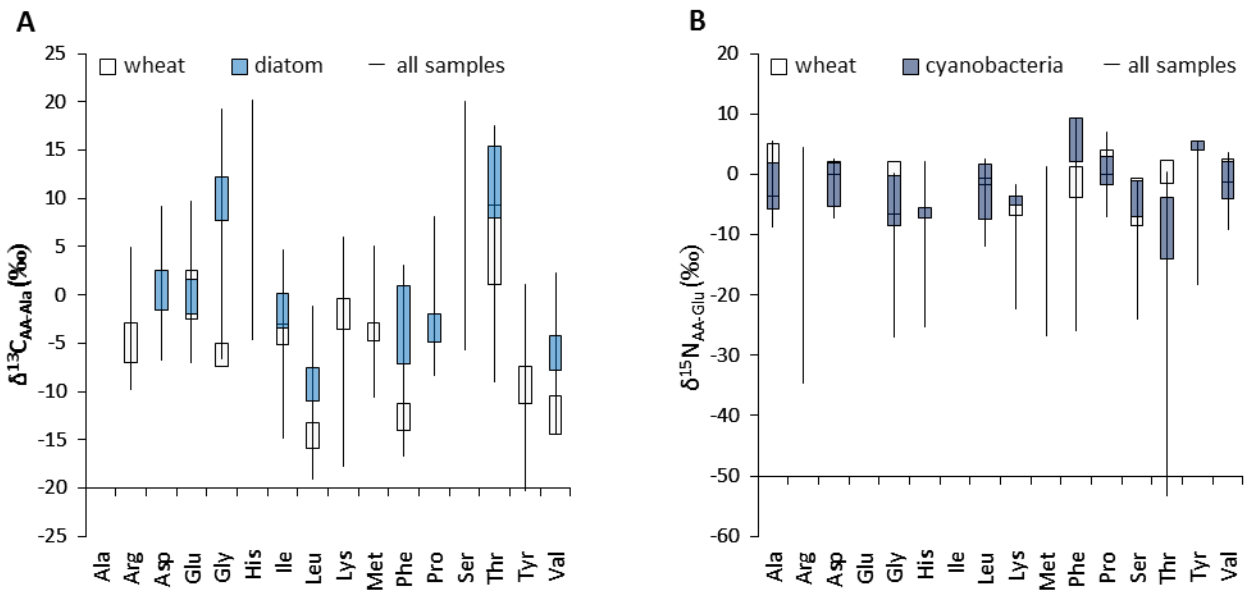

Figure 1-4. A graphical representation of the concept of "isotope fingerprints", which suggests that a taxon has a characteristic set of AA isotope ratios maintained across different environments. Broad bars span the full range of values observed for a single species across multiple studies and/or growing conditions. Lines span nearly the full range (2.5 – 97.5 percentiles) of values from the global dataset. Not all AAs were measured in all samples. **(A)** $\delta^{13}C$ values normalized to alanine. Data on wheat (*Triticum aestivum*) represent 18 different samples (Paolini et al. 2015), and data on diatoms (*Conticribra weissflogii*) represent 28 different samples (Larsen et al. 2015). **(B)** $\delta^{15}N$ values normalized to glutamic acid. Data on wheat represent 28 different samples (Bol et al. 2002, Styring et al. 2014, Paolini et al. 2015), and data on cyanobacteria (*Synechococcus* sp.) represent five different samples (Chikaraishi et al. 2009, McCarthy et al. 2013).

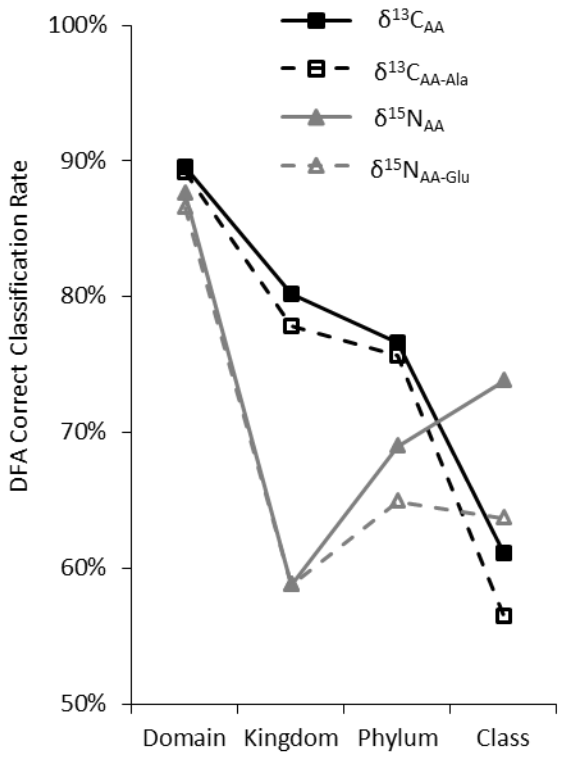

Figure 1-5. Rate of correct taxonomic classification of jackknifed samples with linear discriminant function analysis. All samples are from the taxonomic kingdoms Archaea, Bacteria, Chromista, Fungi, or Plantae—taxa capable of synthesizing all the proteinaceous AAs. $\delta^{13}C$ analysis was based on eight AAs or seven AAs normalized to alanine, and $\delta^{15}N$ analysis was based on seven AAs or six AAs normalized to glutamic acid. At a minimum, there were at least 160 samples with at least three samples per taxon for all DFA analyses.

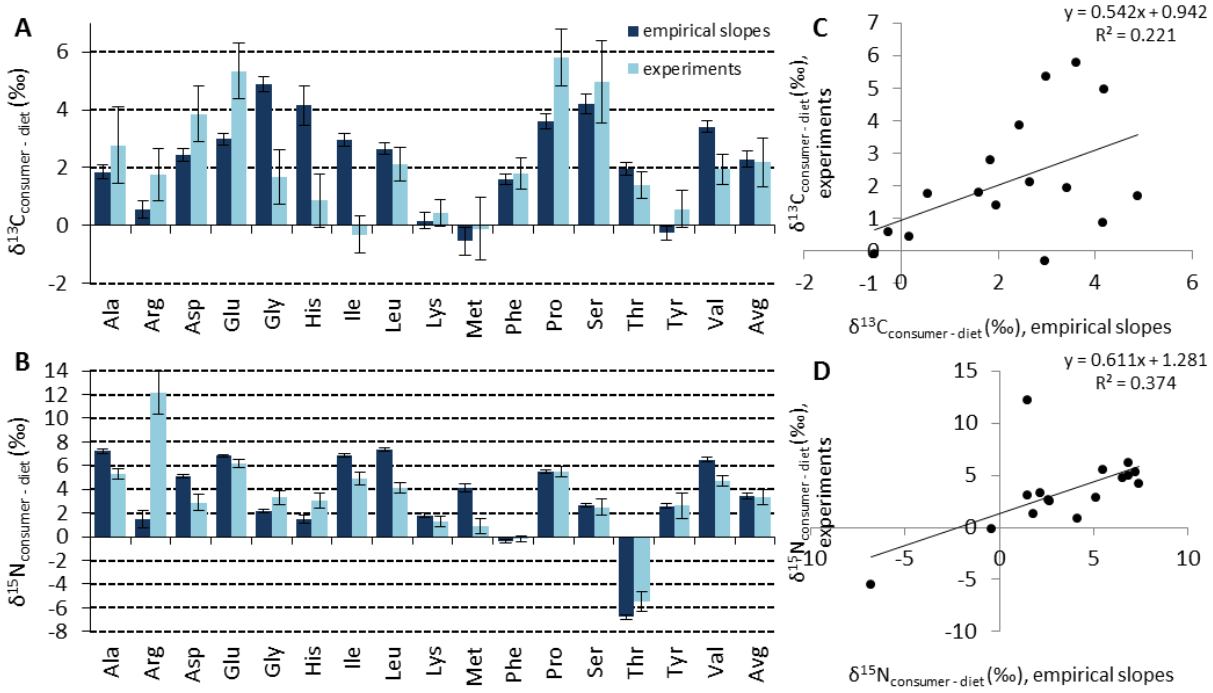

Figure 1-6. (A-B) Offsets (± 1 S.E.) between consumer biomass and consumer diet. Offsets based on the slope of the regression line of $\delta^{13}C$ or $\delta^{15}N$ values against empirical trophic position are labeled as "empirical slopes". Offsets determined by subtracting consumer $\delta^{13}C$ or $\delta^{15}N$ values from those of its diet are labelled as "experiments", though some data come from less-controlled field settings. **(C-D)** Agreement between offsets based on empirical slopes with offsets from experiments. Each point is an AA.

Table 1-1. *F*-values and significance of fixed effects (sample age, habitat, taxonomic class, trophic level) in models of AA $\delta^{13}C$ and $\delta^{15}N$ based on Type III ANOVA comparisons (* = p <.01, ** = p < .001, *** = p < .0001). The significance of analytical method, a random effect, is based on likelihood ratio tests. Conditional R^2 values for linear mixed models are based on the procedure described by Nakagawa and Schielzeth (2013).

	AA	Sample Age	Habitat	Taxonomic Class	Trophic Level	Method	R^2
$\delta^{13}C$	Ala	0.43	21.21***	9.42***	5.17*	***	0.488
	Arg	3.43	0.09	6.37***	3.51		0.264
	Asp	0.03	54.14***	11.43***	10.90***	***	0.562
	Glu	6.50*	42.58***	9.91***	11.85***	***	0.492
	Gly	0.00	82.21***	11.43***	2.63	***	0.578
	His	5.33*	76.45***	6.79***	0.08		0.687
	Ile	2.78	37.38***	9.16***	7.39**	***	0.562
	Leu	2.16	77.99***	9.20***	4.00*	***	0.535
	Lys	0.04	20.20***	5.68***	0.00	***	0.485
	Met	1.71	18.84***	5.38***	8.30**		0.613
	Phe	1.65	12.12***	11.90***	4.99*	***	0.451
	Pro	0.99	26.19***	13.12***	0.38	***	0.560
	Ser	7.92**	27.49***	9.67***	13.95***	***	0.570
	Thr	0.04	96.97***	12.77***	0.00	***	0.529
	Tyr	2.92	8.62**	6.39***	6.90**	***	0.520
	Val	8.26**	89.88***	13.01***	2.68	***	0.604
$\delta^{15}N$	Ala	4.52*	80.85***	17.99***	354.37***	***	0.835
	Arg	4.77*	9.16**	22.29***	16.92***		0.626
	Asp	0.25	13.31***	13.54***	213.72***	***	0.773
	Glu	3.76*	136.53***	14.13***	538.08***	***	0.780
	Gly	5.15**	86.67***	24.12***	2.31	***	0.549
	His	N/A	N/A	7.39***	0.03		0.393
	Ile	2.99**	15.71***	12.39***	271.93***	***	0.799
	Leu	4.49*	53.57***	19.19***	428.25***	***	0.846
	Lys	N/A	36.78***	23.80***	2.37	***	0.579
	Met	0.90	2.94	1.85*	8.21**	***	0.575
	Phe	1.98	15.41***	15.44***	0	***	0.758
	Pro	1.71	38.44***	16.30***	234.89***	***	0.410
	Ser	15.66***	32.32***	23.98***	4.67*	***	0.619
	Thr	0.18	16.11***	11.89***	573.90***	***	0.773
	Tyr	N/A	12.17***	10.76***	69.99***	**	0.537
	Val	2.01	60.39***	12.37***	229.58***	***	0.748

Table 1-2. Rankings of linear trophic position estimation models for all samples with $\delta^{15}N$ values for glutamic acid, phenylalanine, proline, and threonine (n = 606). These AAs were selected because all are commonly measured and have been explored for use in trophic position estimation (Chikaraishi et al. 2009, Bradley et al. 2014, McMahon et al. 2015, Nielsen et al. 2015).

Model parameters	R^2_{adj}	AICc	Delta AICc	AICc weight
$\delta^{15}N_{Glu\text{-}Phe}$, $\delta^{15}N_{Pro\text{-}Phe}$, $\delta^{15}N_{Thr\text{-}Phe}$	0.832	707.64	0.00	0.66
$\delta^{15}N_{Glu\text{-}Phe}$, $\delta^{15}N_{Thr\text{-}Phe}$	0.832	708.94	1.30	0.34
$\delta^{15}N_{mean[Glu\text{-}Phe,Pro\text{-}Phe,\text{-}(Thr\text{-}Phe)]}$	0.825	729.49	21.85	0.00
$\delta^{15}N_{Pro\text{-}Phe}$, $\delta^{15}N_{Thr\text{-}Phe}$	0.787	851.30	143.66	0.00
$\delta^{15}N_{Glu\text{-}Phe}$, $\delta^{15}N_{Pro\text{-}Phe}$	0.636	1179.92	472.28	0.00
$\delta^{15}N_{Pro\text{-}Phe}$	0.615	1212.85	505.21	0.00
$\delta^{15}N_{Glu\text{-}Phe}$	0.601	1234.88	527.24	0.00
$\delta^{15}N_{Thr\text{-}Phe}$	0.528	1337.63	629.99	0.00

Table 1-3. Summary of CSIA-AA applications with parameter estimations, potentially confounding effects, and a few suggested foundational and contemporary readings. Continues on next page.

Topic	Analytical Approach	Useful References, Notes
Trophic position	(1) Identify 1+ "source" AAs (*x*, with small change of Δ_x with trophic transfers, usually Phe) and 1+ "trophic" AAs (*y*, with large change of Δ_y with trophic transfers, usually Glu). (2) Determine average difference between source and trophic AAs for producers in food web of interest (β, conventionally 8.4 for C3 terrestrial, -0.4 for C4 terrestrial, and -3.4 for aquatic) (3) Interpret CSIA-AA data with equation: $TP = [(\delta^{15}N_x - \delta^{15}N_y - \beta) / (\Delta_x - \Delta_y)] + 1$	McClelland & Montoya (2002), McCarthy et al. (2007), *Chikaraishi et al. (2009), *Chikaraishi et al. (2011) Steffan et al. (2013), Germain et al. (2013), *Nielsen et al. (2015). Well-established method undergoing refinement. $\delta^{15}N$ the norm, but $\delta^{13}C$ may be useful.
Movements and migration	(1) Identify AAs that vary predictably among places of interest (e.g., different natal sites, latitudes, strata) and sample to characterize, usually using sessile and/or low trophic position organisms. For $\delta^{13}C$ and $\delta^{15}N$, organisms must interact (i.e., through material fluxes) to equilibrate with places. (2) Perform CSIA-AA on tissues fixed at certain time periods (e.g., scales, hair) or AAs with faster/slower turnover rates (*see* O'Brien et al. 2002, Downs et al. 2014, Bradley et al. 2014) to constrain inferential window. (3) Correct for consumer-diet offsets. Use mixing models, group assignment, or other analytical techniques depending on whether origin, duration in a place, or other spatial details are of interest.	Popp et al. 2007, McMahon et al. 2011, Choy et al. 2012,Vokhshoori & McCarthy 2014, Madigan et al. 2014, Lorrain et al. 2015. This general method is an application of the isoscape concept (*Hobson et al. 2010). Isoscapes best-characterized for Pacific Ocean. $\delta^{2}H$ and $\delta^{18}O$ values are among the most useful in bulk analysis but are only rarely studied using CSIA-AA.
Phylogenetic identity	(1) Identify relevant organisms in study system, analyzed with CSIA-AA under assumptions that more AAs are better, especially essential AAs, and that there is more existing data on $\delta^{13}C$ fingerprints. (2) Correct for consumer-diet offsets if necessary. Consider potential diagenetic effects if identifying source of environmental AAs (*see* Fogel & Tuross 1999, Keil & Fogel 2001, Larsen et al. 2015) (3) Use discriminant analysis, multinomial logistic regression, a mixing model such as Mix-SIAR, or other methods to determine phylogeny of organisms at base of food web.	Abraham & Hesse (2003), Scott et al. (2006), *Larsen et al. (2009), McCarthy et al. (2013), McMahon et al. (2015), Larsen et al. (2015), Paolini et al. (2015). $\delta^{13}C$ is heavily emphasized but $\delta^{15}N$ should be useful based on analysis in this study.
Habitat association	A. (1) If trophic position of organism is known, rearrange equation from topic "Trophic position" to solve for β, indicating aquatic or C3- or C4-terrestrial habitat association. A two end-member mixing model may be used to interpret intermediate values. B. (1) Characterize average aquatic and terrestrial fingerprints by analyzing representative, known-diet organisms; often relies on differences in Phe vs. Glu, Gly, Ser, or Val in tracheophytes vs. algae. (2) Use discriminant analysis, multinomial logistic regression, a mixing model such as Mix-SIAR, or other methods to assign organisms to habitats or partition habitat associations.	Keil & Fogel (2001), Corr et al. (2005), Honch et al. (2012), Larsen et al. (2012), *Ishikawa et al. (2014), *Naito et al. (2015), Webb et al. (2015) The literature is heavily focused on anthropological and archaeological data to resolve habitats on which ancient humans relied for food. Finer scale (C3/C4/arid/freshwater/marine) partitioning may be possible.

Table 1-3, cont.

Endosymbiont contributions to animals	A. (1) Perform CSIA-AA for $\delta^{13}C$ of essential AAs, which animals cannot synthesize. Bulk CSIA-AA should also be performed to determine the $\delta^{13}C$ of the total carbon pool (carbohydrates, lipids, AAs) (2) Compare consumer-diet offsets for essential AAs with expected values from experimental literature. When exceeded in the direction of bulk $\delta^{13}C$, endosymbiont contributions are suspected. Relative variation among AA $\delta^{13}C$ values makes overlap of all AAs with bulk $\delta^{13}C$ values unlikely. (3) Newsome et al. (2011) discuss modelling approaches to quantify contribution. B. (1) Characterize average dietary and endosymbiont fingerprints with representative samples. (2) Use discriminant analysis, multinomial logistic regression, a mixing model such as Mix-SIAR, or other methods to assign organisms to a food web or to partition reliance on dietary vs. microbial AAs.	Uhle et al. (1997), Christensen & Fogel (2011), *Newsome et al. (2011), Maeda et al. (2012), *Arthur et al. (2014), Ayayee et al. (2015a), Yamanaka et al. (2015) Contributions of microbes to plants less well studied, but see Yoneyama et al. (1998).
Autotroph growth conditions*	Identify environmental conditions of interest and compare organisms grown under range of conditions. The $\delta^{13}C$ and $\delta^{15}N$ values of Glu, Gly, Ile, Phe, Ser, and Thr may be particularly useful based on various studies listed under references. Given that AA biosynthesis is generally well-characterized, it may be possible to speculate on mechanisms behind differences (*see* Ohkouchi et al. 2015).	Nutrient limitation (Macko et al. 1987, Hofmann et al. 2003, McClelland et al. 2003, Smallwood et al. 2003). Environmental conditions (Larsen et al. 2015, Styring et al. 2014, Paolini et al. 2015). **More work needed.*
Diet quality*	Assume that dietary carbohydrates and lipids will have higher $\delta^{13}C$ values than AAs. Normalize non-essential AAs against 1+ essential AAs, and presume greater differences reflect greater reliance on carbohydrates and lipids. This approach only works in the simplest of systems because non-AA carbon is variable (e.g., C3- vs. C4-plants) and not necessarily correlated with protein source.	Hare et al. (1991), Howland et al. (2003), O'Brien et al. (2003), Jim et al. (2006), Newsome et al. (2014), Chi. **More work needed.*

Appendix 1-1. Definitions used for variables and classifications.

Age: The age of the specimen in terms of years between its death/collection and the date of isotopic analysis. If under 100 years, it is treated as "modern"; if older, "ancient". If a date span was provided rather than a specific date, the median date is used. All dates were rounded to the nearest 50 years.

Derivatization Method: Methodological grouping based on the derivatizing agent used to prepare AAs for isotopic analysis.

Habitat: The habitat associated with the specimen, either aquatic or terrestrial. This reflects where a species occurs most often (i.e., humans reliant on marine food webs would still be listed as terrestrial). Culture in a lab was also noted.

Literature Trophic Position: Trophic position rounded to the nearest half, with the convention of 1 for autotrophs, 2 for herbivores/detritivores, etc. If the author provided a trophic position, this value was used, so long as it did not come from an AA isotopic calculation but rather from empirical diet studies. If no trophic position was provided, general natural history references were consulted to generate a reasonable estimate. Two simplifying assumptions were made: (1)For humans, those with mainly terrestrial omnivorous diets are assigned a trophic position of 2.5, and those with marine omnivorous diets are assigned a trophic position of 3.5 to reflect the differences in aquatic vs. terrestrial food chain lengths, (2)All invertebrate filter-feeders and particulate-feeders (e.g., some bivalves, coral) were assigned a trophic position of 2 due to feeding largely on detritus or primary production.

Taxonomic Ranks: Domain as defined by Woese et al. (1990), with the ranks of kingdom, phlyum, class, order, family, genus, and species from Catalogue of Life (COL), or Integrated Taxonomic Information System (IT IS; USGS) if unavailable from COL. Algaebase (Guiry) was used for algae.

Appendix 1-2. List of the 144 publications with data utilized in our study.

Publications cited in main References section

Abelson & Hoering 1961; Abraham et al. 1998; Blair et al. 1985; Broek et al. 2013; Choy et al. 2010; Christensen & Fogel 2011; Corr et al. 2005; Décima et al. 2013; Dunn et al. 2011; Fogel & Tuross 1999; Gaebler et al. 1963; Gaebler et al. 1966; Gutierrez-Rodriguez et al. 2014; Hare et al. 1991; Hoen et al. 2014; Hofmann et al. 2003; Howland et al. 2003; Jim et al. 2006; Keil & Fogel 2001; Larsen et al. 2009; Larsen et al. 2012; Larsen et al. 2013; Larsen et al. 2015; Macko et al. 1987; Madigan et al. 2014; McCarthy et al. 2013; McClelland & Montoya 2002; McMahon et al. 2015; Newsome et al. 2014; O'Brien et al. 2003; Ohkouchi et al. 2015; Ostle et al. 1999; Paolini et al. 2015; Popp et al. 2007; Scott et al. 2006; Silfer et al. 1994; Steffan et al. 2013; Steffan et al. 2015; Styring et al. 2014; Vander Zanden et al. 2013; Vokhshoori & McCarthy 2014; Walsh et al. 2014

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Appendix 1-3. Precision of AA $\delta^{13}C$ and $\delta^{15}N$ values scaled to average of precision for all AAs in same sample. Values above/below 1 are less/more precise than average. This relative approach was used because of the wide variety of precision measurements used across studies.

	$\delta^{13}C$ precision/ average for sample	Rank, least to most precise	$\delta^{15}N$ precision/ average for sample	Rank, least to most precise
Ala	0.97	6	0.98	5
Arg	1.16	11	1.31	16
Asp	1.34	13	0.80	1
Glu	1.03	8	0.82	2
Gly	1.09	9	0.99	7
His	1.90	16	1.27	15
Ile	0.96	5	1.05	10
Leu	0.90	3	0.87	3
Lys	1.09	10	0.99	6
Met	1.38	14	1.17	11
Phe	0.87	2	1.05	9
Pro	0.91	4	0.98	4
Ser	1.19	12	1.04	8
Thr	1.52	15	1.17	12
Tyr	1.02	7	1.24	14
Val	0.86	1	1.21	13

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CHAPTER 2

Compound-specific $\delta^{13}C$ and $\delta^{15}N$ Analysis of Amino Acids: A Rapid, Chloroformate-based Method for Ecological Studies

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Abstract

Compound-specific stable isotope analysis of amino acids has proven informative to many ecological systems, but only a handful of analytical methods are routinely employed. We evaluated a simple, rapid procedure in which biological samples undergo short-duration acid hydrolysis and the resulting amino acids are derivatized with methyl chloroformate for gas chromatography/combustion/isotope-ratio mass spectrometry. Amino acid derivatives were separated on a polar gas chromatography column, combusted, and $\delta^{13}C$ and $\delta^{15}N$ values were measured. Tests of reproducibility and accuracy were conducted for amino acid reference mixtures and biological samples. A brief case study of turtles was used to assess whether isotopic data were consistent with *a priori* ecological expectations. The methyl chloroformate-based reaction successfully converted fifteen amino acids from acid hydrolysates of biological materials into separable derivatives. $\delta^{13}C$ and $\delta^{15}N$ values were had high average measurement precision ($\sigma < 1$ ‰). Reference materials were measured accurately, with good agreement between EA/IRMS and GC/C/IRMS determinations. Analysis of turtle blood samples yielded data consistent with their trophic ecology. This derivatization method is a rapid means of determining carbon and nitrogen isotopic ratios of amino acids present in the biological materials often sampled for ecological studies. While amino acids with charged or polar side-chains do not have uniformly high recoveries, the average precision of measurements is comparable to other, more established methods. Batches of samples may be prepared from many raw materials in less than a day, representing a significant reduction in preparation time over prevailing methods.
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INTRODUCTION

Measurement of carbon and nitrogen stable isotopes in biological materials is a powerful means of characterizing ecological relationships (Peterson & Fry 1987, Hobson & Wassenaar 1999, Martínez del Rio et al. 2009, Boecklen et al. 2011).While bulk $\delta^{13}C$ and $\delta^{15}N$ values for tissues and organisms are informative, compound-specific stable isotope analysis (CSIA) has the

potential to yield considerably more data from a given sample. CSIA of amino acids (AAs) is particularly promising because AAs constitute a significant portion of organismal biomass and exhibit a range of $\delta^{13}C$ and $\delta^{15}N$ values as a consequence of their various synthetic pathways and subsequent biochemical uses (Abelson & Hoering 1961, Gaebler et al. 1966, Macko et al. 1987). Much of this variation has proven ecologically meaningful, particularly in a food web context. Specific AA isotopic values reflect producers at the base of a food web (Larsen et al. 2009, Larsen et al. 2012) as well as a consumer's diet composition (Hare et al. 1991, Jim et al. 2006, McMahon et al. 2010), nutritional constraints (O'Brien et al. 2003, Newsome et al. 2011), and trophic level (McClelland & Montoya 2002, Chikaraishi et al. 2011). Measuring the suite of an organism's AA isotopic values, then, has the potential to reveal ecological information in a level of detail beyond what is attainable with isotopic measurements of carbon and nitrogen in bulk samples.

AAs are not amenable to separation via gas chromatography until their carboxyl, amino, and certain side-chain functional groups have been chemically modified, so derivatization procedures are required prior to analysis (Matthews & Hayes 1978). Two-stage procedures of esterification followed by acylation are routinely employed but quite involved (Silfer et al. 1991). The derivatization reactions and multiple purification steps of these procedures require hours for completion, and careful temperature control and anhydrous conditions are essential (Table 2-1; Silfer et al. 1991, Hofmann et al. 2003, Corr et al. 2007). Derivatization via silylation is a simple, single-step alternative, but silylated derivatives decrease oxidation reactor efficiency and may degrade capillary columns (MacKenzie et al. 1987, Molero et al. 2011). Furthermore, silylation adds a large amount of non-analyte carbon—at least six carbons per AA—which is expected to decrease the precision of $\delta^{13}C$ measurements (Docherty et al. 2001, Chikaraishi et al.

2010). With advantages and disadvantages inherent to all methods, it is of little surprise that many fields which utilize gas chromatography/combustion/isotope-ratio mass spectrometry (GC/C/IRMS) of AAs continue to debate the relative merits of different derivatization methods (Corr et al. 2007, Dunn et al. 2011, Villas-Bôas et al. 2011).

Procedures developed for gas chromatography/mass spectrometry (GC/MS) of AAs are a logical source of new protocols to investigate for GC/C/IRMS applications, and many of these protocols make use of chloroformates as derivatizing reagents (Alterman & Hunziker 2012). Alkyl chloroformates were first used for GC/MS when it was discovered that, in the presence of pyridine and a primary alcohol, chloroformates rapidly react with the heteroatom functional groups of AAs (Hušek 1991A, Hušek 1991B, Hušek 1998). Derivatization of AAs by alkyl chloroformates is extremely rapid, occurs at room temperature in an aqueous medium, and creates derivatives that are easily purified via liquid:liquid extraction into immiscible solvents. Indeed, the simplicity and efficiency of derivatization by alkyl chloroformates has made them the derivative of choice for commercially-available AA analysis kits for GC/MS (Badawy et al. 2008). The basic method is sufficiently versatile to have been adapted for microscale (Chen et al. 2010) and automated (Kaspar et al. 2008) analyses as well.

Several qualities of chloroformate-based derivatization are particularly well-suited to GC/C/IRMS of ecological samples. First, derivatization adds few non-analyte carbons to AAs when methyl or ethyl chloroformates are used, which is expected to improve the precision of measurements of $\delta^{13}C$ values relative to methods which add excessive non-analyte carbon (Docherty et al. 2001, Chikaraishi et al. 2010). For ecologists wishing to analyze both carbon and nitrogen isotopic values from the same sample, the minimal addition of non-analyte carbon will allow them to avoid preparing each sample by two separate methods, once for each element (e.g.,

Popp et al. 2007, Lorrain et al. 2009). The aqueous reaction medium in which derivatization proceeds allows aqueous solutions, such as the acid hydrolysates of tissues or biological liquids containing free AAs (e.g., blood, cellular lysates, urine), to be derivatized without prior drying (Hušek 1991B, Chen et al. 2010); other derivatization reactions must proceed under anhydrous conditions. Chloroformates do not lead to detectable racemization of AAs (Zampolli et al. 2007), which may be useful in studies of bacteria, extraterrestrial organic matter, or other systems in which preservation of the D-/L-configuration is desirable. Finally, the relative speed and simplicity of preparation (Table 2-1) is appealing.

Despite these potential advantages, the application of alkyl chloroformate-based derivatization for analysis of AA isotopic ratios has been scant (Montignon et al. 2001, Tea et al. 2007, Fromentin et al. 2012), and few studies have examined more than a few AAs or more than one element. From a pragmatic standpoint, the popularity of alkyl chloroformates for GC/MS studies and their potentially advantageous attributes for stable isotope analysis suggest that their utility for GC/C/IRMS warrants evaluation.

The central objective of this investigation was to determine the precision and accuracy associated with use of methyl chloroformate as a derivatizing reagent preceding GC/C/IRMS. Because ecologists are typically interested in amino acids from biological materials, we focused on the subset of AAs generated from acid-hydrolyzed protein samples. Importantly, we evaluated this method on a range of biological materials to demonstrate the precision of measurements and the scope of data that users may anticipate from future analyses. Finally, a brief case-study on freshwater turtles was undertaken to provide an evaluation of the method as applied to a real ecosystem with clear *a priori* expectations.

METHODS

Reagents and Reference Materials

L-amino acids at >98 % purity were purchased from Sigma Aldrich (St. Louis, MO, U.S.A.) and Alfa Aesar (Ward Hill, MA, U.S.A.). Two certified glutamic acid standards were obtained (USGS 40 & 41; USGS, Reston, VA, U.S.A.), and an artificially-enriched alanine standard (UCD-Ala-2) was prepared at the UC Davis Stable Isotope Facility.

Individual AAs were dissolved in 0.1 M hydrochloric acid to produce solutions for derivative identification. An approximately equimolar reference mixture of 15 AAs was also prepared, consisting of alanine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine at a concentration of 10 mM each in 0.4 M hydrochloric acid. These AAs were selected because they are not wholly degraded during the acid hydrolysis of proteins, the reason for which asparagine, cysteine, glutamine and tryptophan were excluded from the mixture. It should be noted, however, that these amino acids are capable of being derivatized by methyl chloroformate as free amino acids (Chen et al. 2010). Arginine was excluded because its guanidinium side-chain group is unreactive with alkyl chloroformates and its derivative does not readily elute from GC columns (Hušek 1991A). D-norleucine and/or 6-aminocaproic acid functioned as internal references to be added to samples and were prepared at a concentration of 25 mM in 0.4 M HCl. In all cases, solutions were stored in amber glassware at 4 °C with a nitrogen-purged headspace.

Solvents and reagents for acid hydrolysis and derivatization included ACS-grade 12.1 M hydrochloric acid, 50 % (w/w) sodium hydroxide, and HPLC-grade methanol purchased from Thermo Fisher Scientific (Waltham, MA, U.S.A.) as well as 99 % methyl chloroformate, HPLC-grade pyridine, and ACS-grade sodium sulfate purchased from Sigma Aldrich.

Acid Hydrolysis

Acid hydrolysis was used to release individual AAs from peptide and protein samples. 5-10 mg of ground, homogenized samples were placed in vials with 0.5 mL of 6 M hydrochloric acid. The vials were flushed with N_2, sealed with PTFE-tape and a heat-resistant cap, and placed in an oven at 150 $^{\circ}$C for 70 minutes. This short-duration, high-temperature hydrolysis minimizes the proportion of AAs predicted to undergo racemization relative to the more conventional hydrolysis method of heating a sample at 110°C for 24 hours (Csapó et al. 1997). After hydrolysis, samples were dried in a heating block at 60 $^{\circ}$C under a gentle stream of N_2, and the remaining solids were re-suspended in 200 µL 0.1 M hydrochloric acid and stored at -20 $^{\circ}$C.

Derivatization

The derivatization procedure using methyl chloroformate was modified from previously published procedures (Hušek 1991A, Hušek 1991B). Derivatization was conducted in a fume hood, and by working at the micro-scale, undue exposure to the various solvents and reagents was minimized. While all reagents should be used in strict adherence to exposure controls, methyl chloroformate, in particular, should be noted for its acute toxicity (GHS Category 1; Inhalation, Skin). 100 µL of analyte were placed in a GC vial with a 300 µL fixed insert. The remaining 100 µL of analyte were reserved in case re-analysis was required. 20 µL of the internal reference solution, 35 µL of methanol, and 30 µL of pyridine were added, and the reagents were briefly mixed. 15 µL of the derivatizing reagent, methyl chloroformate, were then added with mixing. After one minute, 100 µL of chloroform were added, and a vortex mixer was used until an emulsion formed. The solution was placed aside until the emulsion separated into aqueous and organic layers, usually in about 300 s. The organic layer was transferred into a new

vial with 300 µL fixed insert packed with ~0.1 mg sodium sulfate to absorb any residual water in the organic phase.

To test the efficiency of derivatization, samples of the derivatized and underivatized reference mixture were analyzed by the UC Davis Proteomics Center using an L8800 Hitachi AA Analyzer (Tokyo, Japan; Ozols, 1990). Briefly, the analyzer quantified AAs using ion-exchange chromatography followed by treatment with ninhydrin and colorimetric analysis of the ninhydrin-treated AAs. The limit of quantification for individual AAs by this method ranges from 10-50 pmol, well below the typical instrumental sensitivity for GC/C/IRMS (0.1-10 nmol; Sessions 2006). This procedure was used to compare the absolute concentration of unmodified AAs before and after addition of methyl chloroformate. To determine the relative recoveries of AAs during GC/C/IRMS, we compared peak areas from nitrogen analysis of each AA to the area of the largest AA peak, proline, accounting for differences in AA nitrogen content.

To confirm derivative structure, methoxycarbonyl (MOC) AA esters were analyzed by electron ionization using a Varian CP3800 gas chromatograph (Varian Analytical Instruments, Palo Alto, CA, U.S.A.) with a Saturn 2200 ion-trap mass spectrometer (Varian) under the same chromatographic conditions described below (see Methods, *Isotope-Ratio Mass Spectrometry*). Mass spectra of the MOC AA esters were collected under a scan range of *m/z* 50-350, scan time of 0.55 s, and emission current of 10 µA. Because mass spectra and fragmentation patterns for chloroformate-derivatized AAs have been well described by others (Huang et al. 1993, Zampolli et al. 2007, Chen et al. 2010) we simply compared the molecular ion values observed with published values (Chen et al. 2010) to determine if there were any discrepancies. Spectra acquired at this time were also used to search for any secondary products produced during

derivatization using published data and the NIST 08 Mass Spectral Library (National Institute of Standards and Technology, Gaithersburg MD, U.S.A.).

Isotope-Ratio Mass Spectrometry

CSIA of AAs was performed on a Thermo Trace gas chromatograph (Waltham, MS, U.S.A.) coupled to a Delta V Advantage isotope-ratio mass spectrometer via the GC Combustion Interface III (Thermo Electron, Bremen, Germany). Two capillary GC columns were tested: the relatively non-polar VF-5ms (5% phenyl-methyl stationary phase, 30 m x 0.25 µm i.d., 0.25 µm film thickness, Agilent Technologies, Santa Clara, CA, U.S.A.) and the high polarity VF-23ms (30 m x 0.25 µm i.d., 0.25 µm film thickness, cyanopropylphenylmethylpolysiloxane stationary phase, Agilent Technologies). Superior separation was achieved using the VF-23ms and this column was chosen for use with all subsequent MOC AA esters.

The injector temperature was set to 250°C, and the inlet liner was a deactivated Splitless FocusLiner (SGE Analytical Science, Melbourne, Australia). For $\delta^{13}C$ and $\delta^{15}N$ analysis analysis, 0.2-0.5 µL or 3-5 µL the sample were injected in splitless mode, respectively. Lower injector temperatures resulted in unacceptable losses of large MOC AA esters, especially those derived from lysine, histidine, and tyrosine. After testing constant flow rates from 1.5-3 mL/min for the carrier gas, helium, we initially found a constant flow of 2.8 mL/min provided superior separation. The column oven temperature was initially held at 80 °C for one minute, then increased to 250°C at a rate of 5.5 °C/min and held for another 5 min. Later, to improve ^{15}N sensitivity and the separation between phenylalanine and glutamic acid, the flow was reduced to 1.8 mL/min and the GC oven program modified to hold at 80 °C for one minute, increase to 180°C at a 4.0°C/min, and then to 250°C at a rate of 5.0 °C/min and a final hold of 5 min.

The combustion (3 NiO, 2 CuO, 1 PtO wires) and reduction (6 Cu wires) furnace temperatures were set to 950 °C and 650 °C, respectively. At these GC and combustion/reduction conditions, the presence of incomplete combustion—as would be indicated by strongly depleted measured (i.e., uncorrected) $\delta^{13}C$ values—was not apparent. Furthermore, differences in $\delta^{15}N$ and $\delta^{13}C$ values of MOC AA esters co-injected at 1.4 and 2.8 mL flow rates fell within analytical error, indicating no change in combustion efficiency with carrier gas flow rate. During $\delta^{15}N$ analysis, a liquid nitrogen trap was added after the reduction oven to remove CO_2 from the sample stream. Data were collected and analyzed with the ISODAT software package (Thermo Electron).

Data Analysis and Corrections

Elemental analysis/isotope-ratio mass spectrometry (EA/IRMS) was used to measure $\delta^{13}C$ and $\delta^{15}N$ values for comparison with GC/C/IRMS measurements. Multiple (n = 3) determinations for each AA were made with a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope-ratio mass spectrometer (Sercon Ltd., Crewe, U.K.). EA/IRMS results were evaluated using secondary reference materials calibrated against NIST Standard Reference Materials (IAEA-N1, IAEA-N2, IAEA-N3, USGS-40, and USGS-41). The long-term standard deviation for the instrument is 0.2‰ for $\delta^{13}C$ values and 0.3‰ for $\delta^{15}N$ values. Isotopic abundances are reported as δ values ("per mil", ‰) relative to the V-PDB scale (Vienna Pee Dee Belemnite) for $\delta^{13}C$ values, and atmospheric nitrogen for $\delta^{15}N$ values, as determined by the International Atomic Energy Agency (IAEA, Vienna, Austria).

Based on early indications of measurement precision, samples were measured as a single injection, not duplicate or triplicate injections as often reported in other analyses of biological materials (e.g., Corr et al. 2007, Popp et al. 2007, Larsen et al. 2009, Lorrain et al. 2009).

Replicates consisted of independent preparations, not merely resampling a derivatized sample. During measurement, provisional values were calculated by comparison to a pure reference gas (CO_2 or N_2). Final isotopic calibration of MOC AA esters was performed using one or both of the internal AA standards, norleucine and 6-aminocaproic acid.

The next step in determining isotopic values was correcting for the non-analyte carbon which was incorporated into AAs during derivatization. To account for this carbon and kinetic isotope effects, correction factors were calculated for each MOC AA ester after Docherty et al. (2001):

$$n_{cd}\delta^{13}C_{cd} = n_c\delta^{13}C_c + n_d\delta^{13}C_{dcorr} \text{ (Eqn 2-1)}$$

In this equation, n is the number of moles of carbon, C_c the compound of interest (AA), C_{cd} the derivatized compound (MOC AA ester), and C_{dcorr} the empirically determined correction factor. The correction factors were determined by measuring the $\delta^{13}C$ values of underivatized AA references with EA/IRMS and their derivatized forms. Because nitrogen is not present in the derivatization reagents, corrections for nitrogen addition were not required.

Prior to the analysis of biological samples, preparations of the reference mixture spanning a range of concentrations were measured. By measuring individual AAs across a range of concentrations, we were able to monitor and correct for instrument linearity (e.g., $\Delta\ \delta^{15}N$/peak area). Critically, a preparation at 10mM was measured after every five samples to allow for drift corrections on a compound-specific basis (Werner et al. 2001, Reinnicke et al. 2012). For $\delta^{13}C$ measurements, the linearity and drift-corrected isotopic values were averaged to generate the correction factors used in Eqn. 2-1 and applied to measured samples and quality

assurance/quality control standards. This overall strategy is prudent because of the variability of correction factors of individual AAs (Docherty et al. 2001) and because AAs differ in the presence of heteroatoms and functional groups, which may lead to different combustion efficiencies (Rennicke et al. 2012). A second mixture of pure AAs, concurrently prepared as MOC AA esters with each batch, was used to evaluate the final calibration of the individual amino acids.

In addition to measurement errors, we also calculated total analytical error for carbon, which arises from the propagation of error from measurements required to calculate empirical correction factors. From Docherty et al. (2001):

$$\sigma^2_c = \sigma^2_s (n_s/n_c)^2 + \sigma^2_{sd} ((n_s + n_d)/n_c)^2 + \sigma^2_{cd} ((n_c + n_d)/n_c)^2 \quad \text{(Eqn 2-2)}$$

In this equation, n is the number of moles of carbon, c is the underivatized compound, cd is the derivatized compound, d is the derivatizing reagent, s is the underivatized standard, sd is the derivatized standard.

Biological Materials

In addition to pure reference materials, biological materials were gathered from a variety of sources. We performed multiple preparations and analyses (n = 4) of North Atlantic right whale (*Eubalaena* glacialis) baleen, red algae (*Porphyra* sp. 'Nori') thallus, and chicken (*Gallus gallus*) egg to assess measurement precision on materials relatively high in protein, carbohydrates, and lipids, respectively. Additional biological materials, including white-tailed kite (*Elanus leucurus*), agricultural soils (Yolo series), and Humboldt squid beaks (*Dosidicus*

gigas), were obtained opportunistically to examine for co-elution, the prevalence of non-target peaks, and other chromatographic properties.

Finally, a brief ecological case study was undertaken using blood samples from freshwater turtles (*Trachemys scripta elegans* and *Emys marmorata*), a study system with clear, *a priori* expectations for trends in isotopic values (see Results & Discussion, *Case Study: Trophic Level and Diet Overlap*). This allowed us to assess whether isotopic data were congruent with expectations based on prior, empirically-based dietary studies, and provided an opportunity to gauge ecological realism of isotopic data generated from an exemplary natural system.

RESULTS & DISCUSSION

Chromatography and Derivative Structure and Stability

Derivative molecules were relatively small and polar, and their elution profile varied markedly with increasing polarities of GC columns. A polar VF-23 column provided the best chromatographic resolution. Peak tailing was minimized, and most fraction peaks showed baseline resolution (Fig. 2-1, Table 2-2). Partial overlap of isoleucine/leucine MOC AA esters was observed during nitrogen analysis, though only when injecting highly-concentrated samples. This corresponds with a tradeoff identified by Hušek (1991B): more polar GC columns improve separation of alanine and glycine while simultaneously diminishing separation of leucine and isoleucine. However, isoleucine/leucine are readily resolved by adjusting injection volumes, so the VF-23 column was still preferred over less polar columns in which alanine/glycine co-eluted completely, regardless of injection volume.

Mass-spectrometric analysis revealed strong agreement between structures generated under the present conditions and those reported by Chen et al. (2010). As expected, methyl

chloroformate resulted in the esterification of carboxyl groups and methyoxycarbonylation of amino groups of AAs. The phenol side-chain group of tyrosine also underwent methoxycarbonylation. The alcohol groups of serine and threonine react differently depending upon which alkyl chloroformate and primary alcohol are used for derivatization.[33,37,45] Under the present reaction conditions, the molecular ions for the MOC AA esters generated from serine and threonine were *m/z* 191 and *m/*z 205 , respectively, which is consistent with the methylation of their side-chain hydroxyl groups during derivatization (Chen et al. 2010).

The derivatization of glutamic acid resulted in two peaks because a portion of glutamic acid is cyclized into pyroglutamic acid during derivatization. The proportion of glutamic acid that is converted to pyroglutamic acid is dependent on reaction conditions, especially pH and temperature (Wilson & Cannan 1937, Shih 1985, Airaudo et al. 1987, Sacks & Brenna 2005). To avoid fractionation that might arise during conversions between forms, it would be ideal to convert all Glx to one form. Hušek (1991B) demonstrated that acidic reaction conditions are preferred in that most Glx takes the form of pyroglutamic acid. This form receives only one non-analyte carbon during derivatization (versus four for the native form), and the ester of pyroglutamic acid elutes in position with less potential for co-elution. Indeed, acidic derivatization conditions which promote the formation of pyroglutamic acid have been preferred in previous applications of alkyl chlorformates to isotope-ratio measurements of glutamic acid (Sacks & Brenna 2005).

We performed additional tests to insure that glutamic acid could be measured accurately because it is frequently used in ecological studies (see Results & Discussion, *Precision and Accuracy of $\delta^{13}C$ and $\delta^{15}N$ Analyses*). Assigning trophic position, for example, requires the comparison between trophic AAs, which become enriched in ^{15}N with trophic transfers, and

source AAs, which vary little following trophic transfers (McClelland & Montoya 2002, Popp et al. 2007). While a range of trophic AAs appear suitable for such calculations (Chikaraishi et al. 2009, Seminoff et al. 2012), initial applications of this technique have focused on comparisons between glutamic acid and phenylalanine (Chikaraishi et al. 2011, Popp et al. 2007, Lorrain et al. 2009). In order to accurately quantify the isotopic composition of glutamic acid by MCF derivatization, care should be taken to maintain consistent reaction conditions between samples.

Most peaks were distinct and readily identified, but baseline noise was observed in the region of the chromatogram preceding the lysine MOC ester (Fig. 2-1), especially with the high injection volumes required for $\delta^{15}N$ analysis. This noise was not observed to the same degree during $\delta^{13}C$ analysis. The largest peaks in this region were still quite small, generally <20 mV (GC/C/IRMS) or 40 ion counts (GC/MS) in peak intensity. Most could not be positively identified on the basis of their mass spectra (NIST spectral match probability <10%), but three ions were common to this region: *m/z* 94, *m/z* 79, and *m/z* 88. These ions correspond to the molecular ion of methyl chloroformate, pyridine, and the fragment representing the carboxy methyl ester residues of derivatized AAs, respectively (Chen et al. 2010). These peaks likely represent products of excess reagents and/or fragments from small carboxylic acids (Hušek 1998). Additionally, past research has shown that alkyl chloroformates generate a small amount of secondary derivatization products (Chen et al. 2010). It is possible that this background noise may contain these secondary products or other products formed during the large splitless injection; this may contribute to the overall analytical variation. Whatever the source of extraneous peaks, the accuracy and precision of $\delta^{13}C$ and $\delta^{15}N$ measurements of MOC AA esters were not unduly compromised by their presence (Table 2-2).

Because the time required for derivatization is quite short, samples were typically prepared immediately prior to analysis. If immediate analysis was not possible, samples were stored at -20 °C. The robust stability of ethoxycarbonyl (EOC) AA esters is well known, with stabilities reported from one week at room temperature up to several weeks at -20 °C (Hušek 1991B, Montigon et al. 2001), in marked contrast to the short-term stability of derivatives generated via *t*-butyldimethylsilylation (MacKenzie et al. 1987). In our experience, MOC AA esters from the reference mixture could be reliably stored for a week at -20 °C without significant differences in $\delta^{13}C$ and $\delta^{15}N$ values. Repeated measurements of $\delta^{13}C$ and $\delta^{15}N$ from the same preparations over two weeks generally fell within measurement error, but peak areas declined between initial preparation and seven days of storage, either due to analyte loss during storage or changes in combustion conditions (Fig. 2-2). We did not investigate the effects of longer-term storage because of the ease of preparing samples close to the time of analysis. This also eliminated any ambiguities over long-term storage effects in a relatively volatile solvent.

Amino Acid Recovery and Quantification

All AAs showed a high correlation between analyte concentration and peak area from GC/C/IRMS. A derivatized portion of the reference mixture was diluted to generate an eight-point calibration curve spanning an order of magnitude (2 – 20 mM concentrations of each AA). Coefficients of determination averaged 0.98 for carbon and 0.96 for nitrogen (Table 2-2).

All AA derivatization methods vary in their ability to generate recoverable derivatives for various AAs (Hofmann et al. 2003, Corr et al. 2007). Relative to the peak area for proline, the AA with the highest absolute recovery, AAs with non-polar side-chains averaged markedly higher recovery than AAs with polar or charged side-chains (Fig. 2-3). As a result, AAs with polar or charged side-chains generally had the highest limits of quantification. Histidine and

serine, especially, must be present at high concentrations in analyzed materials to generate MOC AA ester peaks of sufficient size for analysis (Table 2-2). The low recovery for histidine and serine is consistent with reports from alkyl chloroformates used elsewhere (Hušek 1991B, Chen et al. 2010), and a different derivatizing reagent should be used if measuring these AAs is critical to research objectives.

We were particularly interested in methodological refinements which might improve the recoveries of those AAs with polar or charged side-chains. While incomplete derivatization of some AAs was a possibility, ninhydrin-based quantification of AAs revealed that free (i.e., underivatized) amino groups fell below detectable limits after derivatization, suggesting complete or very nearly complete derivatization of amino groups. The incomplete methylation of side-chain hydroxyls of serine and threonine during derivatization remains a possibility.[37] Derivatives must be extracted from aqueous solution, and the efficiency of extraction may vary, contributing to differences in relative recoveries. While not precluding this possibility, previous evaluations of non-chloroform solvents have found that chloroform remains the most useful solvent for extracting a broad range of MOC AA esters (Hušek 2005).

Losses of polar and charged analytes may occur during GC/C/IRMS through adhesion to active sites within the GC, such as the inlet liner (Kaspar et al. 2008) or from incomplete combustion (Reinnicke et al. 2012). In both cases, the more active derivatives are predicted to be most affected. Measured peak intensity of MOC esters of AAs with polar or charged side-chains declined at injection temperatures below 250 °C, which is similar to results from previous studies of alkyl chloroformate AA derivatives (Hušek 1991A, Montigon et al. 2001), indicating that a reduction in residence time within the injector was critical. Maintenance of deactivation of inlet liners and the use of quartz wool was also found to positively influence the yield of polar and

charged AAs, as did the avoidance of metal unions within the GC (e.g. guard to analytical column; Fig. 2-4). Another potential sink for active analytes is the stainless-steel cross-piece (VICI, Houston, Texas, U.S.A.) within the Trace gas chromatograph and could not be ruled out, as suitable alternatives were not identified (Meier-Augenstein 2005). Incomplete combustion was ruled out as a source of low recovery, as determined by concomitant peak areas observed in co-collected FID data (Fig. 2-4).

Accuracy and Precision of $\delta^{13}C$ and $\delta^{15}N$ Analyses

Isotopic values of high-purity AA references were obtained with EA/IRMS. These values were compared with measurements of 10 replicate preparations of the reference mixture analyzed via GC/C/IRMS and calibrated only to the internal standard, norleucine (Table 2-2). Both $\delta^{13}C$ and $\delta^{15}N$ values obtained from EA/IRMS were linearly correlated with those from GC/C/IRMS (Fig. 2-5). An analysis restricted to AAs with aliphatic side-chains produced optimal results, generating a linear relationship with a slope not-significantly different from 1 for carbon ($p = 0.75$) and nitrogen ($p = 0.89$), with high coefficients of determination for both carbon ($R^2 = 0.997$, $p < 0.0001$) and nitrogen ($R^2 = 0.988$, $p < 0.0001$). The average absolute value of the offset between EA/IRMS and GC/C/IRMS was 2.22 ‰ for $\delta^{13}C$ and 1.42 ‰ for $\delta^{15}N$ values. AAs with charged or polar side-chains had the largest offsets; excluding just histidine and serine, for example, decreased the average offset between EA/IRMS and GC/C/IRMS $\delta^{13}C$ values from 2.22 ‰ to 1.17 ‰. Again, this underscores the importance of using compound-specific external reference materials to correct isotopic values.

To further test accuracy, we measured two certified glutamic acid reference materials (USGS-40 and -41) and two alanine working reference materials (UCD-ALA-1 and -2). Glutamic acid was tested because it has a charged side-chain and undergoes partial conversion to

pyroglutamic acid during derivatization, and we wished to test whether it could be measured over a range of $\delta^{13}C$ and $\delta^{15}N$ values. Alanine was selected as representative of AAs with non-polar side-chains. All measurements were accurate, falling well within the range of analytical error (Table 2-3).

We assessed the stability of $\delta^{13}C$ and $\delta^{15}N$ values from a trio of AAs with different side-chain functional groups (aspartic acid, leucine, lysine) over concentrations spanning one order of magnitude (0.2-20 mM). There was no directional change in $\delta^{13}C$ and $\delta^{15}N$ values over this span ($p>0.3$ for all MOC AA esters). For very small peaks (i.e., those generated by AA concentrations around or below 2.5 mM), linearity effects on $\delta^{15}N$ values were observed; the accuracy of $\delta^{15}N$ measurements below this concentration was dependent on the inclusion of linearity standards as previously described.

To evaluate precision, 10 replicate preparations of the reference mixture were analyzed. Standard deviations for measurements of both $\delta^{13}C$ and $\delta^{15}N$ averaged less than 1 ‰ (Table 2-2). Total analytical error for $\delta^{13}C$ values by the present method ranged from ±0.7 ‰ for phenylalanine to ±2.4 ‰ for glycine. These results fall into the same ranges reported by investigations of the most widely-used derivatization methods (Hofmann et al. 2003, Corr et al. 2007). It should be noted that precision may vary considerably between labs, even for the same derivatization method and similar GC/C/IRMS protocols. For example, Molero et al. (2011) obtained average $\delta^{15}N$ value precisions of 0.28 ‰ while Hofmann et al. (2003) obtained an average $\delta^{15}N$ value precision of 1 ‰ despite the fact that both used the same derivatization reagent. An evaluation of multiple methods of $\delta^{13}C$ analysis by Corr et al. (2007) reported total analytical error (Eqn. 2-2) that ranged from ±0.6 ‰ to ±2.3 ‰, depending on the method of analysis and AA derivative. Differences between analytical methods (e.g., single vs. duplicate or

triplicate measurements of each preparation), the type of error reported, and instrumentation used in other studies do not permit strict comparisons of accuracy and precision based purely on derivatization agent. A more systematic comparison of this and other derivatization methods is certainly warranted in the future.

Measurements of Biological Materials

The analysis of biological materials differed from analysis of reference mixtures in a few key aspects. Materials underwent acid hydrolysis and drying, non-AA compounds were present, and AAs were not present in equimolar concentrations. Despite these complicating factors, analyses of a variety of biological materials yielded 8-14 measurable AA peaks, including multiple essential/non-essential and trophic/source (McClelland & Montoya 2002, Popp et al. 2007) AAs (Fig. 2-6). The breadth of isotopic data generated has the potential to support a range of ecological investigations. Furthermore, samples were analyzed without prior knowledge of AA composition; by adjusting parameters (e.g., injection volume), we could often increase the number of resolved peaks in subsequent analyses.

While it is common to purify biological samples with strong-cation exchange chromatography prior to anhydrous derivatization procedures (e.g. esterification/acylation), minimal interference on the chromatograms of biological samples indicates that liquid:liquid extraction following derivatization can provide relatively pure organic fractions for analysis (Fig. 2-6). A strong-cation exchange purification step could certainly be integrated into the present method, but it does not seem to be an obligatory component of preparation for derivatization. A recent GC/MS study using ethyl chloroformate-based derivatization likewise demonstrated that AAs could be successfully derivatized and quantified in several biological materials despite the presence of other compounds (Mudiam et al. 2012). Particularly lipid-rich samples, though,

warrant more critical evaluation of the need for purification as chloroformates can derivatize a number of fatty acids (Castro et al. 1997,Goto et al. 2011).

Homogenized baleen, algae, and chicken egg samples were measured to assess precision achieved for biological materials. Four portions of each material were hydrolyzed, derivatized, and analyzed (Table 2-4). The analytical error associated with each AA was not uniform across the biological materials. This may arise from incomplete homogenization, different hydrolysis efficiencies among samples, the influence of the non-AA matrix on derivatization, or a number of other factors. Across AAs and materials, the average total analytical error was ±1.67 ‰ for $\delta^{13}C$ values and precision was ±0.88 ‰ for $\delta^{15}N$ values.

Case Study: Trophic Level and Diet Overlap

A case study on compound-specific nitrogen data was conducted to examine the utility of this method for analysis of diet and trophic level, increasingly popular applications. In California, the red-eared slider (*Trachemys scripta elegans*) is a non-native species listed on the IUCN list of "100 of the World's Worst Invasive Alien Species," (Lowe et al. 2000) and the western pond turtle (*Emys marmorata*) is a native species listed as an IUCN Red List vulnerable species (TFTSG 2012). There are concerns that invasive sliders are competing for resources with native pond turtles (Spinks et al. 2003, Thomson et al. 2007). Consequently, comparisons of diet are of considerable interest, and trophic positioning based on $\delta^{15}N$ of AAs may be a useful in helping to assess dietary overlap.

An exploratory analysis was conducted on turtles from the University of California, Davis Arboretum (Yolo County, California, U.S.A.), where the two species co-occur and are monitored as part of a long-term population study (Spinks et al. 2003). Differences in diet were predicted based on direct, stomach-content based studies from other regions which found that

adult pond turtles are largely carnivorous (Bury 1986, Ashton et al. 1997), and that sliders show a shift from carnivory as juveniles to greater herbivory as adults (Bouchard & Bjorndal 2006). Blood samples from turtles conformed with patterns seen in the literature. The higher the trophic level, the greater the value of $\delta^{15}N_{Glu\text{-}Phe}$ (Chikaraishi et al. 2009), and these values were indeed highest in pond turtles and juvenile sliders (Fig. 2-7). Likewise, all trophic AA $\delta^{15}N$ values were highest in pond turtles. Trophic AAs were more variable among juvenile red-eared sliders ($\delta^{15}N_{Glu}$ = ±2.24 ‰) than adult sliders ($\delta^{15}N_{Glu}$= ± 0.73 ‰) or pond turtles ($\delta^{15}N_{Glu}$= ±0.64 ‰), perhaps reflecting ontogenetic variation in feeding behaviors. Additional study is warranted before firm conclusions may be drawn, and alternative explanations for $\delta^{15}N$ differences cannot, as of yet, be ruled out. For instance, turtles may feed from different segments of the local food web (aquatic/terrestrial) and spatial variation in anthropogenic nitrogen (e.g. inorganic fertilizer, wastes) may obscure differences in $\delta^{15}N$ arising from diet (Chikaraishi et al. 2011). However, a difference in trophic level by age/species remains a parsimonious, empirically-grounded hypothesis for the patterns observed here and warrant further study.

CONCLUSION

The primary advantages of GC/C/IRMS using methyl chloroformate as a derivatizing reagent are: (1) simple, aqueous derivatization, (2) speed of preparation, especially when combined with rapid acid hydrolysis, (3) good chromatographic resolution of most of the proteinogenic AAs, and (4) apparent suitability for analysis of a range of biological samples. The accuracy and precision of carbon and nitrogen isotopic measurements are comparable to values reported in other studies. Importantly, this method provides reliable $\delta^{13}C$ and $\delta^{15}N$ measurements for multiple AAs from each of a range of ecologically-relevant categories, including essential,

non-essential, trophic, and source AAs. While all AA derivatization techniques have established advantages and disadvantages, CSIA of AAs using methyl chloroformate is a simple, robust analytical alternative for isotope ecologists and others to utilize on a diverse array of organisms and ecosystems.

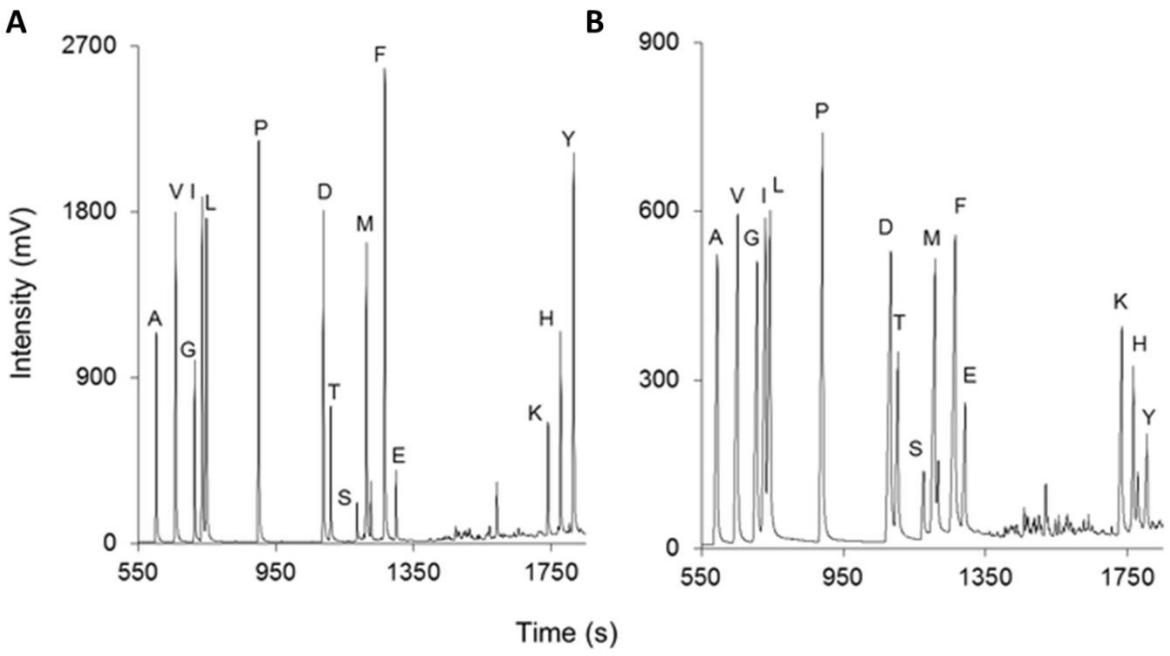

Figure 2-1. Typical chromatograms from GC/C/IRMS of an approximately equimolar AA standard solution for (A) carbon analysis and (B) nitrogen analysis. The conventional single-letter AA codes are used, A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; P, proline; S, serine; T, threonine; V, valine; Y, tyrosine.

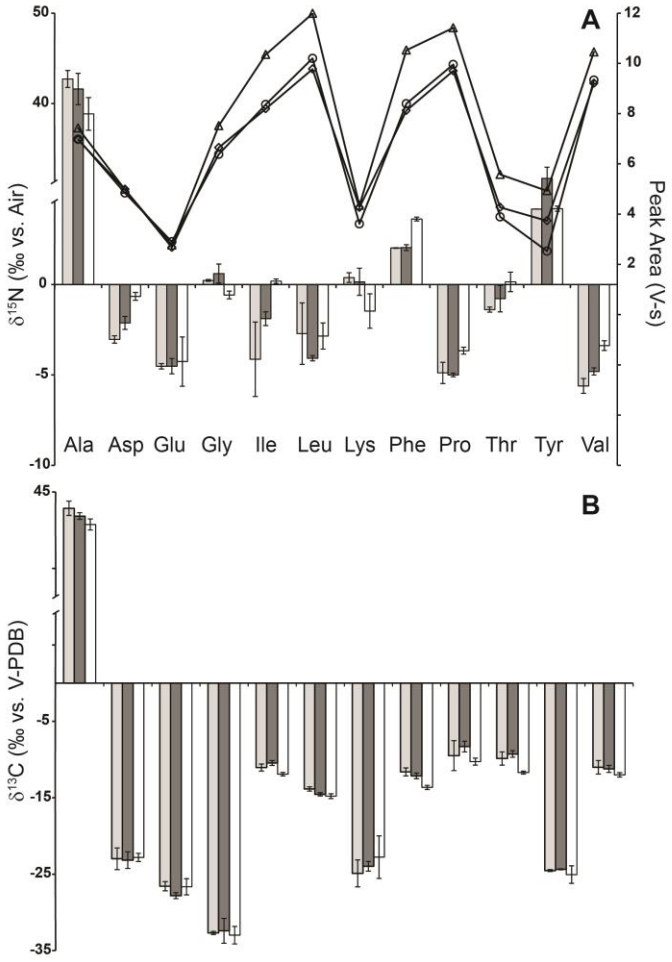

Figure 2-2. (A) $\delta^{15}N$ and (B) $\delta^{13}C$ measurements of AA MOC esters at 0 d (light gray bars), 7 d (dark gray bars), and 14 d (white bars) after preparation. Samples were stored at -20 °C. Points indicate total peak area at 0 d (triangles), 7 d (diamonds), and 14 d (circles). (n = 2).

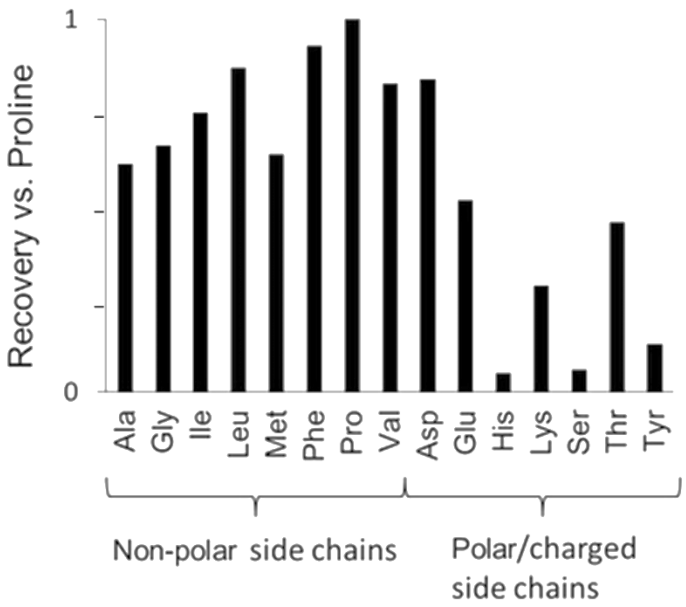

Figure 2-3. Average recoveries of AA MOC esters relative to proline during nitrogen analysis. (n = 5)

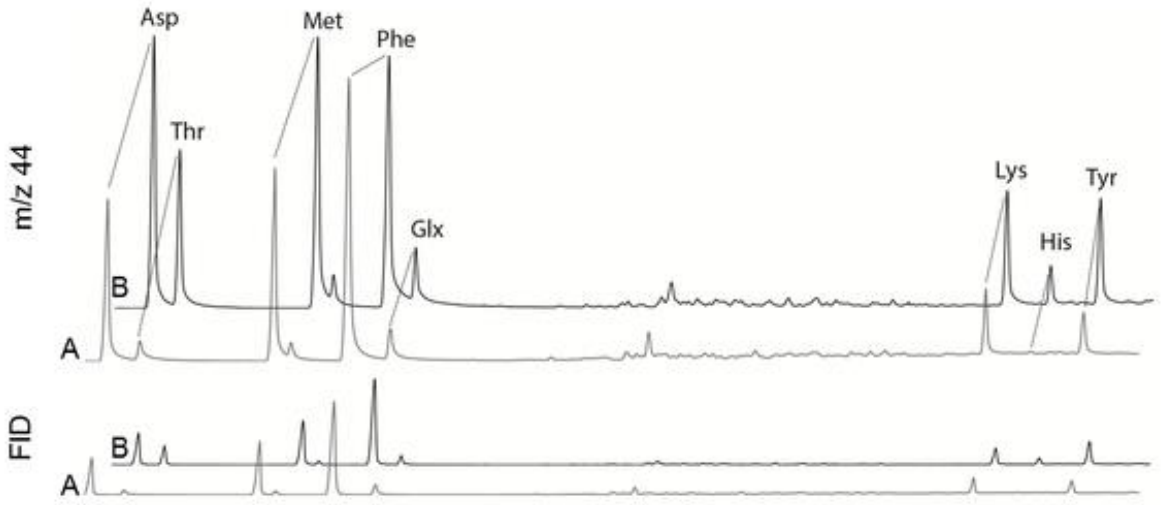

Figure 2-4. Increased signal intensity of AAs with charged or polar side-chains after replacement of (A) a low-mass metal union with (B) a deactivated pressfit union at the connection between the analytical column and retention gap. Chromatograms are from a 20 mM mixture of AAs analyzed for $\delta^{13}C$. Co-measured FID data are included to demonstrate differences in signal intensity were not due to changes in combustion. Phenylalanine remained essentially unchanged and may be viewed for comparison.

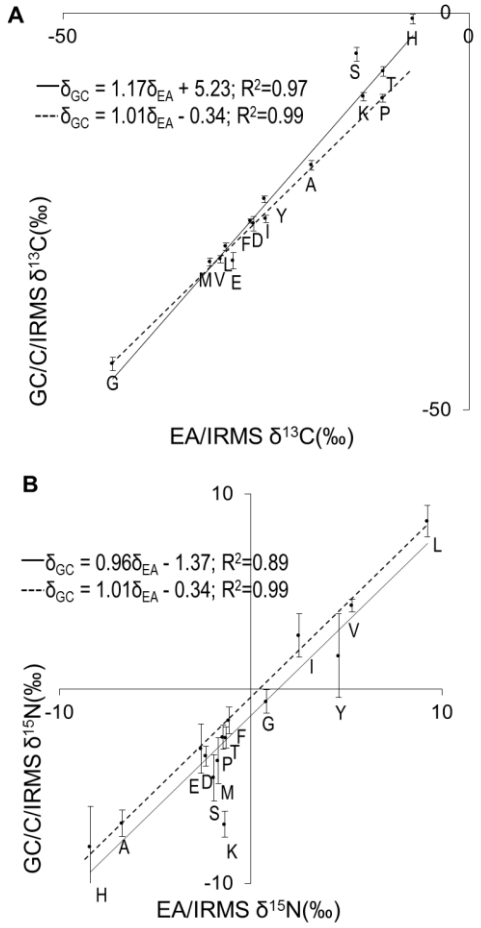

Figure 2-5. Isotopic measurements from GC/C/IRMS (n = 10) plotted against measurements from EA-IRMS (n = 3) for (A) carbon and (B) nitrogen. Solid lines are fitted to all AAs and broken lines are fitted to AAs with aliphatic side-chains only. Error bars are ± 1 SD.

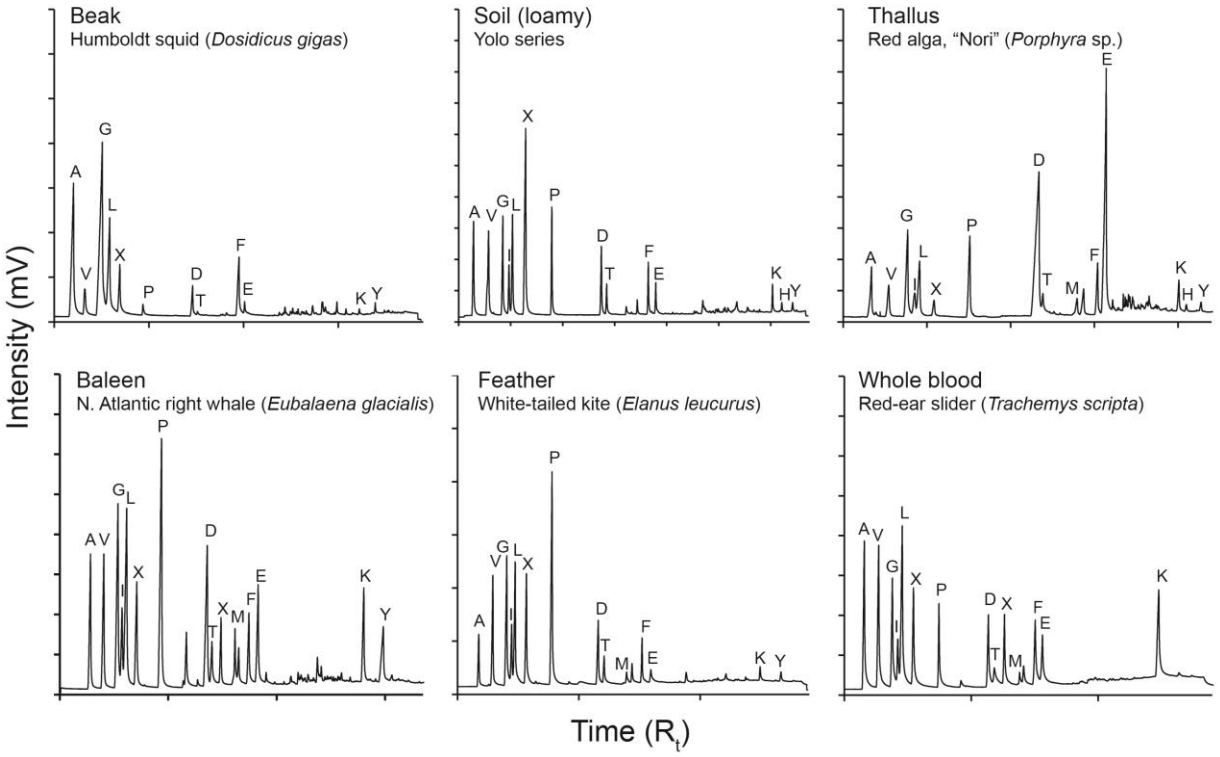

Figure 2-6. Chromatograms from $\delta^{15}N$ analysis of a range of biological materials. "X" denotes internal AA standards.

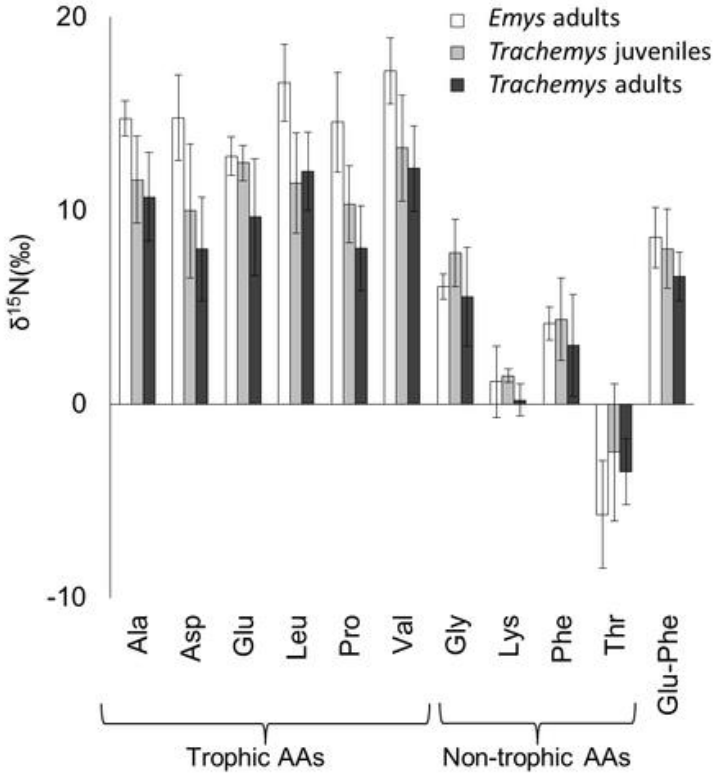

Figure 2-7. Average $\delta^{15}N$ values of blood AAs from co-occurring species/ages of turtles with standard deviation (n = 4 per group). Trophic AAs are those that tend to increase with trophic level, non-trophic AAs exhibit different patterns with trophic level. Glu-Phe indicates trophic level with greater differences corresponding to higher trophic postion.

Table 2-1. Comparison of the reaction times and steps required for widely-used AA derivatization methods (1-4) with this study's method(5). While samples for ecological studies often require hydrolysis, lipid removal, and other preparatory steps prior to derivatization, these steps are excluded from the comparison as they will vary by study system. (A) Methods come from comparative papers by Hofmann et al.[20] and Corr et al.[21] In cases where there were multiple evaluations of the same basic method (1-3), the faster approach is shown. (B) Derivatization reaction times omit active preparation time (e.g., mixing), which was rarely quantified; thus, these are underestimates of total preparation time. (C-D) Total volume of solvents that must be evaporated over the course of derivatization and liquid-liquid extraction steps are reported as proxies for additional preparation time. (E) In cases where authors evaluated multiple different GC protocols, the time for one complete cycle of the protocol yielding the greatest number of resolved AAs is shown.

A. AA derivatization method	B. Derivatization time, no. stages	C. Solvent to dry, no. drying steps	D. Liquid:liquid extraction steps	E. GC time
1) Esterification, acetylation	70 m, 2 stages	2.6 mL, 4 steps	0	62 m
2) Esterification, trifluoroacetylation	70 m, 2 stages	2.5 mL, 4 steps	0	53 m
3) Esterification, pivaloylation	90 m, 2 stages	3.7 mL, 4 steps	1	62 m
4) *t*-Butyldimethylsilylation	30 m, 1 stage	0 steps	0	88 m
5) Esterification, methoxycarbonylation	1 m, 1 stage	0 steps	1	45 m

Table 2-2. Characteristics of MOC AA ester measurements. (A) Empirically-determined limit of quantitation (S/N = 10/1). (B) R^2 for derivative concentration versus peak area using an eight-point calibration curve of the standard solution spanning 0.2 - 20 mM. (C) Predicted values are based on the mass-balance adjusted average EA/IRMS delta values for carbon and average EA/IRMS delta values for nitrogen (n = 3). (D) Average observed delta value (n = 10) calibrated against norleucine; compound-specific corrections were not applied in this analysis to illustrate raw offsets. (E) Measurement error. (F) Total propagated analytical error for carbon (Eqn. 2-2) with norleucine used as the derivatized standard (i.e., not compound-specific corrections).

AA	Side-chain Character	Elut. Time (m)	Carbon							Nitrogen					
			LOQ[A] (mM)	R^2,[B] (conc. v. area)	$\delta^{13}C$ Pred.[C]	$\delta^{13}C$ Obs.[D]	Δ	σ^E	σ^F	LOQ[A] (mM)	R^2,[B] (conc. v. area)	$\delta^{15}N$ Pred.[C]	$\delta^{15}N$ Obs.[D]	Δ	σ^E
Ala	Non-polar	9.8	0.25	0.99	-19.40	-19.22	0.19	0.61	1.52	0.25	0.99	-6.72	-6.91	-0.19	0.68
Val	Non-polar	10.9	0.25	0.99	-30.59	-31.01	-0.42	0.44	1.01	0.25	0.99	5.30	4.27	-1.03	0.32
Gly	Non-polar	11.8	0.25	0.99	-43.88	-44.22	-0.34	0.85	2.41	0.25	0.99	0.82	-0.65	-1.47	0.60
Ile	Non-polar	12.2	0.25	0.98	-29.93	-29.43	0.49	0.45	0.96	0.25	0.97	2.53	2.73	0.19	1.11
Leu	Non-polar	12.5	0.25	0.99	-25.00	-25.92	-0.92	0.43	0.94	0.25	0.96	9.24	8.60	-0.64	0.81
Pro	Non-polar	15.0	0.25	0.99	-10.61	-10.76	-0.15	0.51	1.09	0.25	0.98	-1.44	-2.49	-1.04	0.61
Asp	Charged	18.2	0.25	0.99	-22.67	-25.44	-2.77	0.75	2.00	0.25	0.98	-2.34	-3.45	-1.11	0.51
Thr	Polar	18.5	0.75	0.98	-10.56	-7.39	3.17	0.63	1.56	1.50	0.95	-1.30	-2.51	-1.21	0.53
Ser	Polar	19.8	2.00	0.97	-13.82	-5.19	8.63	0.87	2.29	4.00	0.93	-1.92	-4.57	-2.65	1.19
Met	Non-polar	20.3	0.25	0.99	-31.90	-31.37	0.52	0.47	1.05	0.25	0.97	-1.69	-3.69	-1.99	1.16
Phe	Non-polar	21.2	0.25	0.98	-26.88	-26.23	0.65	0.26	0.70	0.25	0.99	-1.14	-1.62	-0.49	0.68
Glu	Charged	21.6	1.50	0.93	-29.01	-27.54	1.47	1.57	1.98	2.50	0.88	-2.60	-3.07	-0.47	1.28
Lys	Charged	29.2	0.50	0.98	-13.00	-10.56	2.44	0.53	1.28	0.50	0.98	-1.36	-6.97	-5.61	0.67
His	Charged	29.8	0.75	0.97	-10.17	-0.82	9.35	0.61	1.40	3.00	0.93	-8.39	-8.12	0.27	2.46
Tyr	Polar	30.5	0.25	0.99	-25.19	-23.46	1.72	0.43	0.97	0.50	0.93	4.59	1.70	-2.90	2.15
Average, non-polar			0.31	0.99			-0.09	0.58	1.44	0.41	0.98			-0.81	0.65
Average, charged/polar			0.79	0.97			3.54	0.68	1.38	1.57	0.94			-1.98	1.37
Overall Average			**0.53**	**0.98**			**1.60**	**0.63**	**1.41**	**0.95**	**0.96**			**-1.35**	**0.98**

Table 2-3.

Measurements of glutamic acid USGS reference materials as pyroglutamic acid and of alanine working reference materials. All were prepared as 10 mM solutions with (n = 10) replicate preparations. (A) Known values are those reported with the reference materials for Glu and based on EA/IRMS measurements for Ala (n = 5). (B) Total propagated analytical error for carbon (Eqn. 2-2) and the standard deviation for nitrogen are reported.

	Material	Known[A]	GC/C/IRMS	Δ	σ[B]
$\delta^{13}C$	USGS-40	-26.39	-26.23	0.16	0.99
	USGS-41	37.63	37.71	0.08	1.14
	UCD-Ala-1	-19.18	-19.44	-0.26	0.92
	UCD-Ala-2	41.40	41.46	0.06	1.01
$\delta^{15}N$	USGS-40	-4.52	-4.24	0.28	0.43
	USGS-41	47.57	47.86	0.29	1.24
	UCD-Ala-1	-3.61	-2.98	0.63	0.55
	UCD-Ala-2	43.73	43.84	0.11	1.06

Table 2-4. $\delta^{13}C$ and $\delta^{15}N$ values of AAs from North Atlantic right whale (*Eubalaena glacialis*) baleen, red algae (*Porphyra* sp. 'Nori') thallus, and domestic chicken (*Gallus gallus*) whole egg. (n = 4; n.d. = "not determined" due to low abundance in sample). Total propagated analytical error is reported for carbon, and the standard deviation is reported for nitrogen.

Material		Ala	Asp	Glu	Gly	Ile	Leu	Lys	Phe	Pro	Thr	Tyr	Val
E. glacialis baleen	$\delta^{13}C$	-20.2±1.87	-16.30±1.80	-8.40±1.62	-9.98±2.75	-20.02±1.58	-28.92±0.69	-18.18±0.82	-28.99±1.87	-19.42±1.27	-9.80±2.24	-31.41±1.47	-24.12±1.06
	$\delta^{15}N$	13.48±0.98	11.05±0.90	15.45±1.80	1.32±0.76	16.34±0.64	14.86±0.81	0.23±0.73	7.06±0.70	14.59±0.71	-24.88±1.87	6.78±0.21	18.43±0.45
Porphyra sp. thallus	$\delta^{13}C$	-11.46±1.49	-2.62±2.78	-6.31±1.23	-8.40±4.63	-18.60±1.03	-23.94±0.77	-13.63±1.13	-18.50±1.31	-9.63±1.41	-5.23±2.02	-25.61±0.74	-19.75±1.18
	$\delta^{15}N$	6.38±0.89	10.09±0.93	8.92±0.42	-3.1±2.27	5.75±1.80	4.24±0.98	0.17±0.93	7.67±0.79	7.25±0.95	7.91±0.74	n.d.	8.22±1.19
G. gallus egg	$\delta^{13}C$	-6.82 ±1.71	-8.75±2.12	-14.76±2.19	-8.79±4.13	-16.37±1.45	-23.92±0.90	-15.59±2.22	-21.90±1.40	-11.44±0.95	-9.12±1.89	-19.07±1.05	-21.28±1.35
	$\delta^{15}N$	4.71±0.24	4.45±0.69	7.50±1.72	4.58±0.45	6.11±0.56	3.57±0.16	-3.66±0.51	3.31±0.55	5.55±1.29	-1.23±0.52	3.61±1.38	5.62±0.43

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CHAPTER 3

A Mobile Songbird Greatly Extends a Stream's Aquatic Signature at Little Evident Cost

Robert Walsh, John Eadie

Abstract

Cross-habitat subsidies are an important feature of food webs, but the spatial extent of their influence is generally limited due to marked declines in availability with distance from the donor habitat. The behavior and mobility of subsidy-exploiting consumers is often overlooked as a means of extending the bounds of subsidy influence. For five years, we studied the tree swallow (*Tachycineta bicolor*), a generalist aerial insectivore, and how its use of emergent aquatic insect subsidies varied by distance from a perennial creek. The species' reliance on nestboxes allowed for use of aquatic prey to be studied over the breeding season using an array of boxes placed near and distant from water. Given the limited productivity of seasonally arid uplands, we hypothesized that swallows nesting closer to water would have greater access to subsidies and experience higher reproductive success. Instead, use of aquatic prey exceeded proportional availability. Emergent aquatic insects from a stream covering <5% of swallow foraging areas constituted, depending on the type of analysis, 66.1% – 74.6% of nestling diet. Neither of the estimates of aquatic prey use had a statistically significant decline relating to the distance of tree swallow nestboxes from water, which ranged from 25 to 425 meters. Various measures of reproductive success were also relatively uniform over a range of distances from water. Models of reproductive success gave greater importance to effects of site as well as to year and clutch initiation date than to distance from water. Aquatic prey is a critical subsidy to tree swallows, and it appears to be utilized based on regional versus fine-scale availability.

INTRODUCTION

Apart from some indirect effects, food web interactions require spatial overlap; an organism cannot eat what is not there. Yet the intervals between trophic interactions afford time for resources and organisms to move, sometimes broadly, creating the potential for spatial discrepancies between production and consumption. Influential works grounded in empirical food webs (Polis & Strong 1996, Polis et al. 1997) inspired a surge of research on the widespread occurrence food web subsidies: energy, matter, or organisms that move to an ecosystem different

from the one where they were produced. Subsidies impact the dynamics, composition, and stability of communities in recipient habitats (Huxel & McCann 1998, Holt 2002, Leroux & Loreau 2008, Yang et al. 2008, Harvey et al. 2017). After years of substantial progress, recent reviews have identified a need for better understanding dynamics, including the transmission of subsidies into recipient habitats (Schindler & Smits 2017) and consumer responses to gradients of subsidy availability (Richardson & Sato 2015). Integrating animal movements may be particularly important for progress (Earl & Zollner 2017). Herein, we examine spatial alignment between resources, diet, and reproduction in a riparian songbird to better understand how the mobility of resources and their consumers impacts the landscape extent of food web subsidies.

One of the most prolific areas of food web subsidy research has been exchanges between aquatic and terrestrial ecosystems in riparian zones (Baxter et al. 2005, Marczak et al. 2007B, Richardson & Sato 2015). Surface waters accumulate detritus, sediment, and other terrestrial materials from their broader watersheds because of the directional pull of gravity to their generally low-lying, "concave" landscape position (Lindeman 1942, Leroux & Loreau 2008). Upstream stretches subsidize downstream ones (Vannote et al. 1980, Harvey et al. 2017). While floods may export resources and organisms into uplands, aquatic-to-terrestrial subsidy movement often relies on organisms such as amphibians and emergent aquatic insects (Paetzold et al. 2008, Schindler & Smits 2017). The ability of organisms to move against prevailing physical forces may be substantial in terms of both biomass and spatial extent (Doughty et al. 2016). A coarse generalization about riparian subsidies borne out by meta-analyses (Macarelli et al. 2011, Bartels et al. 2012) is that aquatic systems receive relatively larger amounts of lower-quality (detrital), passively-transported terrestrial subsidies, whereas terrestrial systems receive relatively smaller amounts of higher-quality (organismal), actively-transported aquatic subsidies.

Mobile consumers may markedly extend the reach of aquatic subsidies into terrestrial landscapes. This can take the form of carrying resources (e.g., bears dragging salmon carcasses upland, Gende et al. 2004), or of assimilating resources, such that the aquatic subsidy is effectively integrated into the terrestrial consumer's biomass or waste (e.g., fish-fed flies dispersing into forests, Francis et al. 2006). Whatever the form, coupling the movements of both subsidies and subsidized consumers allows for a potentially greater extent of aquatic resources in uplands. Indeed, theory predicts that consumer movements mediate dynamics of subsidized food webs in part by spreading impacts across a broader landscape (Callaway & Hastings 2002, McCann et al. 2005, Earl & Zollner 2014). Subsidies may also affect upland dynamics via trophic cascades and other complex or indirect effects on community interactions (Murakami & Nakano 2002, Knight et al. 2005, Yang et al. 2008), but our focus is on the spread of the "aquatic signature" (Muehlbauer et al. 2014) itself as a unique resource in recipient terrestrial ecosystems.

Despite the considerable potential, the actual importance of terrestrial consumers in transmitting aquatic subsidies to uplands remains an open question (Schindler & Smits 2017). An important problem is the cost of movement. Many organisms simply inhabit zones rich in aquatic subsidies, avoiding major movement costs (Polis et al. 1996, Murakami & Nakano 2002). Aquatic-terrestrial boundaries are exploited by species (Marczak et al. 2007A) and individuals (Quevedo et al. 2009) that specialize on subsidies. These patterns would limit the extent of subsidies to the shoreline region. However, even heavily subsidized riparian consumers commonly use or avoid areas for reasons other than simple subsidy availability (e.g., thermal regulation, Daniels 1987; territorial exclusion, Uesugi & Murakami 2007; obligate roosting, Power et al. 2004). Variable consumer life history makes organism-mediated movements of

aquatic resources a viable possibility (Power & Rainey 2000, Kautza et al. 2016). It may be that the non-resource-seeking behaviors of organisms most significantly alter the subsidy landscape.

In the present study, emergent insects are the aquatic subsidy of interest and tree swallows (*Tachycineta bicolor*) are the focal terrestrial consumer. Emergent aquatic insects make aquatic resources available to a host of terrestrial consumers when they leave their natal waters as flighted adults to disperse and mate (Nakano & Murakami 2001, Baxter et al. 2005, Wesner 2010). Some insects move well into uplands (Jackson et al. 1989, Popova et al. 2017), although syntheses spanning multiple habitats have determined that the vast majority stay over water or within a few meters of the shoreline (Gratton et al. 2009, Muehlbauer et al. 2014). Thus, movements of terrestrial insectivores might be required to transfer aquatic subsidies farther upland. Tree swallows are an ideal candidate for directing these transfers because they are central place foragers that must return to their nest to feed young. Their acceptance of artificial nestboxes means that their landscape position, and therefore their proximity to water and aquatic subsidies, can be manipulated. This was especially relevant at our California study sites. The dry Mediterranean climate often means that terrestrial insect productivity is moisture-limited (Bolger et al. 2005, McCluney & Sabo 2009) while aquatic insects can continue to emerge during dry summers so long as streams flow (Power et al. 2004, Rundio & Lindley 2012). We established arrays of nestboxes set out at increments up to hundreds of meters from a perennial stream to systematically probe tree swallow use of subsidies of aquatic insects. Apart from allowing important control over spatial variables, nestboxes also afforded the opportunity for close monitoring of nesting swallows and their young to assess diet and fitness impacts of disparate access to aquatic subsidies.

Given the probable importance of aquatic insect subsidies to tree swallows, we predicted that (1)Tree swallows would preferentially settle more densely near water, (2)Swallows would feed on more aquatic prey when nesting near water, and (3)Swallows would have higher reproductive success when nesting near water. Evidence in support of these predictions would suggest limited transmission of aquatic subsidies upland due to maximizing a consumer's spatial overlap with aquatic insects in line with prevailing, bottom-up views of subsidy responses. Deviations from these predictions would suggest a number of other possibilities, including that aquatic insects subsidies were unimportant for tree swallows, that subsidy availability was not the primary determinant of the extent and nature of subsidy use on the landscape, or that individuals settled in areas with the resources they preferred or specialized on.

METHODS

All research was conducted under UC Davis IACUC protocol 15331, California Department of Fish and Wildlife scientific collecting permit, and USGS Bird Banding Laboratory banding permit 23383. R Version 3.4.3 (R Core Team 2018) was used for statistics.

Study Sites

Research was conducted along Putah Creek, Yolo and Solano Counties, California, U.S.A, from 2010 to 2014. Over these years, the perennial creek had a mean discharge of 14.55 m^3/s based on daily flow data from April through July (USGS 2016), the tree swallow breeding season. Total precipitation during the same four-month period averaged just 2.68 cm as temperatures climbed rapidly to a mean daily high of >35 °C by July (NOAA 2016). The warm, dry breeding season is typical for the Putah Creek watershed region and its Mediterranean climate.

We established three grids of nestboxes as the primary focus of the study. All nestboxes were built from untreated lumber to dimensions suitable for tree swallows (12.7 cm x 14 cm floor, 25.4 cm height, 3.8 cm entrance hole). Nestboxes were mounted on 1.5 m poles fitted with an overturned waste-bin to deter nest-raiding rodents and snakes. To create the nestbox grids, lines of 11 nestboxes each were placed in rows parallel to the creek with the first row 25 m from the creek and each subsequent row 100 m farther upland up to 425 m from the creek (Fig. 3-1A). The grid sites, called Bobcat Ranch (BR), Stevenson Bridge (SB), and South Fork (SF), were well separated from one another (>14 km from next nearest grid) , but each was built within 1 km of smaller, established nestbox trails to facilitate rapid colonization by tree swallows (Fig. 3-1B). These smaller nestbox trails were established in 2000 and were monitored throughout the study period for complementary data.

Land cover at all of the nestbox grid sites comprised the creek, a narrow belt of riparian deciduous forest, and uplands dominated by grassland; there was considerable agricultural activity in the vicinity of two sites (Table 3-1). Land management practices varied at each site at the discretion of landowners. Over the five-year study period, all sites had one or two years of sheep or cattle grazing that overlapped at least partially with the tree swallow breeding season. Summer mowing of dried grasses took place annually at the Stevenson Bridge and South Fork grids. The character of Putah Creek also varied by site. Bobcat Ranch was farthest upstream with relatively cold, fast water from releases from Monticello Dam. The other two sites followed another, much shallower dam and had relatively warmer waters.

Insect Availability

Diurnal flying insects constitute the vast majority of the nestling tree swallow diet (reviewed by Winkler et al. 2011), and the goal was to assess the composition and abundance of

this prey pool. Several types of sticky traps were used as the initial sampling method, but unsatisfactory biases emerged. Some taxa commonly observed and known to be eaten by swallows never appeared on traps (e.g., Odonates); low capture rates required deployments exceeding a day and we could not discern the proportion of nocturnal captures (i.e., prey unavailable to swallows); and predators pulled at least some insects off the traps. Thus, from 2013 to 2014, sweep nets were used to monitor the composition of the flying insect prey pool at various distances from water. Within one to six hours of sunrise, a very fine-mesh insect net was swept briskly side-to-side through the air 125 times along the shore of Putah Creek and then again along each line of nestboxes composing the nestbox grids. Care was taken to avoid sweeping vegetation so that only insects in flight were captured. Specimens were stored in 70% ethanol for identification and further analysis. A total of three rounds of insect sampling occurred each year to track insects during peaks in egg-laying (late April), hatching (mid-May), and fledging (June). This method allowed for fine spatial resolution of insect occurrence, only sampled day-flying insects, and succeeded in capturing even strong-flying species that had been absent from sticky traps. While the sampling window was short and insects flying at heights > 2 m were out of sampling range, the method gave the potential to characterize prey availability in at least a general sense.

Insects were identified to a taxonomic level that allowed for assignment to aquatic or terrestrial habitat, with Arnett et al. (2000) and Merritt et al. (2008) used as authorities. Taxa for which an aquatic environment was indispensable for at least one life stage were considered aquatic (Appendix 3-1). Midges (Chironomidae) were a particularly important consideration because of their abundance in insect surveys and in tree swallow diets. Even within a single genus, the habitats used by various species may run the gamut from marine to freshwater to

plant-associated to truly terrestrial (e.g., *Metriocnemus* s, Pinder 1995). Because this challenging family is aquatic overall, all chironomids were classified as aquatic. Such a pragmatic simplification had the potential to make aquatic resources appear more available than they actually were (i.e., even a terrestrial moist-soil midge would be counted as aquatic biomass), but it is a bias already known and likely evident in the aquatic insect subsidy literature (Muehlbauer et al. 2014). The Bohart Museum of Entomology (University of California, Davis) also provided guidance in taxonomically finer identifications of some insects, including all those used for stable isotope analysis. Once a habitat for a specimen was identified, its dry weight was determined by measuring length to the nearest 0.1 mm and using the appropriate aquatic or terrestrial insect length-mass equations of Sabo et al. (2002).

Though justified by our study aims, the switch from sticky traps to sweep netting invalidated comparisons of insect abundance between early and later years. Thus, all sweep netting years and sites were pooled to characterize the overall insect abundance patterns along Putah Creek. Using the 'nlme' package (Pinheiro et al. 2016), we fitted linear, exponential, and power models to describe diurnal emergent aquatic insect biomass as a function of distance from water. AIC values and adjusted R^2/pseudo-R^2 values were used to compare fits, and the best-performing model was selected to characterize the general pattern of aquatic insect availability.

Settlement and Foraging Behavior

Each nestbox was monitored at least twice a week beginning in March and ending in July or August, depending on activity. Nestboxes were considered settled when a pair of tree swallows built a nest and laid at least one egg. If a clutch was depredated, abandoned, or failed, and the same or a different pair subsequently laid at least one egg, that was considered a second settlement event. Nestboxes were counted as potentially contested if the presence of a

heterospecific competitor at the nestbox was noted on three or more occasions within a given nesting season. We compared the total number of nesting attempts at a given box as dependent on distance to water, with site-year as a random effect with package 'lme4' (Bates et al. 2014). We also compared the proportion of years a nestbox was used when not contested using binomial error models to clarify the potential importance of heterospecifics to limiting settlement.

The potential time and energetic costs associated with various settlement locations were determined by consulting the literature on metabolism and flight behavior in Hirundinidae. Costs were calculated as a function of the number of trips that a pair of tree swallows might make to Putah Creek, the most prey-rich area at all sites. Birds were not spatially tracked, precluding the calculation of true costs, but the potential-cost approach served as a heuristic device for considering how demands on pairs varied across the landscape.

While full foraging trips could not be tracked, it was possible to observe the initial departures of tree swallows actively provisioning nestlings. From 2011-2013, we randomly selected focal nests with nestlings >8 days old for 15 minutes of observation during which we noted the direction of departure for every foraging trip made by the parent swallows. On each trip, the swallow was observed for at least 3 – 6 seconds before assigning a foraging departure direction. Flights to switch perches, interact with other swallows, etc. were excluded from analysis. Flight direction was assigned on a compass scale in which 0° indicated movement directly to the creek, 90°/270° indicated movement parallel to the creek either upstream or downstream, and 180° indicated movement directly away from the creek. Foraging departure directions were analyzed with the 'circular' package (Agostinelli & Lund 2013), which accounted for the circular and directional nature of observations. Rao's spacing tests were used

to determine if there was significant directionality in the departure directions of swallows with a null assumption that directions of foraging trips were uniformly spread in all directions.

Diet

During routine visits to monitor tree swallow nests, insect prey were opportunistically collected from any adult captured inside its nestbox before it could provision nestlings. This usually involved Prey is carried as a bolus comprising up to dozens of small insects (Quinney & Ankney 1985), and both full and partial boluses were retrieved. The samples were frozen or preserved in ethanol for subsequent identification, counting, and weighing of the insects in a manner similar to that done for sweep net survey insects. Adults transfer prey to young rapidly, so boluses were difficult to obtain in quantity, with $n = 28$ boluses comprising $n = 465$ insects collected. Given the limited scope of this sampling, Mann-Whitney U-tests were used to assess differences in the amount of aquatic prey biomass in boluses collected at pooled water-proximate nests (0 – 224 m from Putah Creek) and pooled water-distant nests (225 – 425 , from Putah Creek).

Overall diet characterization of nestling tree swallows was based on compound-specific stable isotope analysis of amino acids (CSIA-AA) of carbon in nestling feather. This approach breaks down protein samples into constituent AAs, each of which is measured isotopically to produce a suite of isotopic values for ecological inference rather than a single bulk value. A small, distal piece of tail feather was collected from each nestling at an age of approximately 12 – 14 days. These samples reflected diet or use of body reserves at an age of approximately 9 – 11 days, when the portion collected grew. Feathers from individual nestlings were homogenized to create one sample per nest. Next, samples were randomly selected in a stratified manner such that one to two nests were picked to represent each distance from water for every site-year of the

project, if available. Only nests with two or more nestlings reaching 12 days old had adequate feathers for analysis. Thus, samples should be viewed as random samples of diet in partially to fully successful nests.

To facilitate interpretation of isotopic data from nestling feathers, additional sampling occurred to develop a reference database of known aquatic and terrestrial resources and organisms. This included 30 reference samples of basal resources (e.g., oak leaves, diatoms, particulate organic matter), 22 reference species of aquatic and terrestrial insects, and 12 reference species of aquatic and terrestrial birds. All basal resources sampled represented collections from $n = 9$ individuals or patches from each site where present; insects samples consisted of at least three individuals per species collected at study sites in May; and bird samples were feathers of contemporary (1980 or more recent) California specimens housed at the Museum of Wildlife and Fish Biology (UC Davis). In all cases, all the components of each sample were dried and homogenized in an electric coffee mill or with a mortar and pestle. The intent was to encompass natural variation within a sample (i.e., material came from multiple individuals and areas) rather than to run multiple exemplars of the same species. This was done under the assumption that interspecific variation would exceed intraspecific variation, such that references based on multiple species would more fully reflect the scope of variation among resources, insects, and birds. Experiments have shown that primary producer species maintain a consistent isotopic "fingerprint" even when sampled across very different environments (Paolini et al. 2013, Larsen et al. 2015), further justifying a sampling approach that maximized taxonomic breadth over individual depth to characterize the riparian food web.

All feather and reference samples were analyzed as discussed in Walsh et al. (2014). Briefly, samples were hydrolyzed, the resulting amino acids were derivatized into volatile forms,

and the derivatives were analyzed by gas-chromatogrpahy/combustion/isotope ratio mass spectrometry. This resulted in carbon isotope ratios for twelve or more amino acids per sample. We focused on isoleucine (Ile), leucine (Leu), Phenylalanine (Phe), and Valine (Val), as these all had good measurement precision and are essential amino acids which cannot be synthesized by animals, simplifying their interpretation as largely reflective of food source (Howland et al. 2003, Jim et al. 2006). Each was normalized by subtracting the overall average from the $\delta^{13}C$ value to generate $\delta^{13}C_{norm}$ values. This is done to enhance pattern detection over variable background variation in environmental $\delta^{13}C$ value.

Mixing models were required to convert isotope ratios of amino acids to estimates of aquatic prey in diet. These models make assumptions about end members (i.e., isotope values characteristic of aquatic versus terrestrial food webs), and discrimination factors (i.e., how the characteristic isotope values change as an organism assimilates its diet as biomass). To minimize the number of assumptions made, we used the average isotope values from strictly aquatic and strictly terrestrial reference birds samples as aquatic and terrestrial end members, and no directional discrimination factors were included (e.g., McMahon et al. 2015), as the reference birds presumably converted amino acids from diet to biomass using similar physiological mechanisms (i.e., discrimination factors were already accounted for in the end members). This approach did rely on assumptions about the diets of birds, so we only used species for which various *Birds of North America* (Cornell, various years) accounts specified diet as being > 90% aquatic. We used the 'MixSIAR' package (Stock & Semmens 2013), which takes an entirely Bayesian approach to mixing model analysis. This was advantageous over determinant linear mixing models in that analytical imprecision was easily incorporated and an informative prior for diet based on bolus sampling could be used to improve the models. Models were run with at least

100,000 iterations (burn-in = 50,000, thin = 50). Convergence was assessed with Gelman-Rubin tests on three chains and Geweke tests. Inferences about diet used the package's own Bayesian mixed effects models.

Reproductive Success and Survival

Every 2-3 days, any birds present at nestboxes were noted, eggs and/or nestlings were counted, and adults more than half-way through incubation and/or unbanded nestlings >11 days old were measured and banded. If a nest had been depredated or disturbed, efforts were made to determine the agent. Visits to each nest were generally very brief to minimize interference, though up to two hours were allotted per nesting attempt to capture adults.

GLMMs were used to assess reproductive success with site-year as a random effect in package 'mle4'. We chose this over site and year as separate random effects because each had very few levels and many events that could potentially alter swallow reproductive success were particular to one site in one year. In GLMMS, we used normal errors for models of nestling condition (wing-controlled body weight residuals), Poisson errors for models relating to clutch size and number of nestlings fledged, and binomial errors were used for models of proportion of nestlings. We considered the use of structural equation modeling to integrate multiple explanatory and response variables reflective of key latent variables (e.g., site quality, reproductive success), but coercing clearly categorical variables, such as site, into continuous or ordinal ones proved problematic from the perspective of interpreting results.

To examine whether adults differed in apparent survival based on distance from water, we built Cormack-Jolly-Seber mark-recapture models with program MARK (White & Burnham 1999). A 5-year encounter history was made for all breeding adults captured at least once (n = 239). Capture methods and effort were relatively uniform throughout the study period, so the

probability of recapture was specified to remain constant. We examined models that included distance from water, site, and distance*site, estimating capture and survival rates with maximum likelihood analysis. AIC_c values were used to compare support for the different models.

RESULTS

Insect Availability

The abundance of emergent aquatic insects declined in a rapid, non-linear fashion with distance from water. Of the diurnal flying insects captured and identified in net sweep surveys (*n* = 812), 47.4% of individuals were emergent aquatic taxa. A negative power model best described how their biomass varied along a transect perpendicular to Putah Creek (Fig. 3-2A). Based on this power model, 50% of the total emergent aquatic insect biomass within a perpendicular transect 0.5 – 425 m from water occurred in the first 146 m. A comparison of AIC scores suggested that an exponential model was nearly as successful in describing the spatial pattern of emergent aquatic insect abundance. Based on the exponential model, 50% of the total emergent aquatic insect biomass within a perpendicular transect 0.5 – 425 m from the water occurred in the first 15 m. The majority of aquatic biomass consisted of dragonflies and damselflies (Odonata; 46.6%) and true flies (Diptera, primarily Nematocera; 41.7%). Emergent aquatic insect taxa present at lower abundances were caddisflies (Trichoptera; 5.9%), mayflies (Ephemeroptera; 4.2%), stoneflies (Plecoptera; 1.6%), and beetles (Coleoptera; < 1%).

Mean terrestrial insect abundance at the various sampling intervals fell within a more restricted range (8.29 – 22.45 mg dry mass per 125 net sweeps) than was observed for emergent aquatic insects (1.25 – 31.22 mg dry mass per 125 net sweeps). A negative power function best described the abundance of flying terrestrial insects with distance from water. However, this was

largely driven by sampling along the insect-rich shoreline; if samples 0.5 m from water were excluded, a linear model was adequate and the amount of terrestrial insect biomass did not vary significantly with distance from water (slope = -0.007, $F = 0.319$, $p = 0.574$; Fig. 3-2B). The majority of terrestrial insects captured were true flies (primarily Cyclorrhapha; 38.7%), wasps and ants (Hymenoptera; 26.4%), and true bugs (Hemiptera; 25.6%). Present at lower abundances were beetles (Coleoptera, 3.5%), lacewings (Neuroptera, 3.4%), and butterflies and moths (Lepidoptera, 2.4%).

Settlement and Foraging Behavior

Tree swallows found the study sites rapidly, with nearly one nest attempt per nestbox per season by the second year of the study (Table 3-2). Birds were present in at least small numbers year-round, but most activity was observed from March to August (Appendix 3-2). The cumulative number of nesting attempts at a given nestbox was primarily explained by study site ($F = 23.45$, $p < 0.001$), and some additional variation was explained by distance from water ($F = 5.44$, $p = 0.02$). However, its impact was not in the predicted direction as nestboxes farther from water were more heavily settled than nestboxes adjacent to water (Fig. 3-3). We noted that the presence of competitor species including western bluebirds (*Sialia mexicana*), ash-throated flycatchers (*Myiarchus cinerescens*), and house wrens (*Troglodytes aedon*) was highest closest to water. Thus, data were re-examined as cumulative number of nesting attempts per nestbox per year of availability, with any years during which a competitor was present at a given nestbox treated as if it were unavailable. With this approach, distance to water no longer played a significant role in explaining settlement choices.

Foraging trips to Putah Creek had the potential to impart substantial time and energetic costs on adult tree swallows nesting in upland nestboxes. Tree swallow pairs studied using

electronic loggers at another California breeding site provisioned nestlings an average of 312 times per day (Rose 2009). With a flight speed of 6.6 m/s for adult tree swallows in the chick rearing stage (Blake & Chan 2006), this would lead to hours of commuting time if even a small fraction of upland bird foraging trips went to the prey-rich creek (Fig. 3-4A). In flight, Hirundines expend an average of 3.89 times as much energy as they do when roosting (average estimate based on Hails (1979) and Table 1 therein), with roosting in Tree Swallows estimated at 1.49 kJ/h (Williams et al. 1988). Compared to birds nesting at 25 meters from Putah Creek, birds at distances of 125, 225, 325, and 425 meters from the creek would have to expend, respectively, 5, 9, 13, and 17 times as much energy to make an equal number of trips to the creek (Fig. 3-4B).

When provisioning nestlings, swallow departure directions were non-random, with an average orientation towards Putah Creek (Fig. 3-5). Of 219 directed foraging trips undertaken by 67 individuals, the average departure direction was 18.62° (95% C.I. 5.73° - 32.09°), or generally towards Putah Creek (set to 0°). Departures from most distances showed a similar orientation. The most notable exception was birds departing from nestboxes 425m from water at Stevenson Bridge. We had limited sampling at this distance, and departures averaged 273.48°, which is approximately parallel to creek (95% C.I. 205.30 – 347.39). Rao's spacing tests confirmed significant deviations from the null expectation of a random distribution of foraging departure orientations ($p < 0.01$ for all distances). For individuals observed making more than one foraging trip within a given observation period ($n = 54$), the probability of a departure direction mirroring the prior departure within ±15° (roughly the precision of observation) was 0.44; the probability expected if all directions were equally likely would be 0.08.

Diet

Emergent aquatic insects were the most important prey item in tree swallow boluses by both count and biomass (Fig. 3-6). Eight different orders of arthropods were documented, but true flies, mayflies, and flying ants (Formicidae) were the most frequent prey. The average bolus collected contained 16.6 insects with a dried weight of 24.32 mg. Of multi-item boluses, the majority (63.6%) were monotypic in the sense that all insects belonged to a single family, though there may have been multiple different genera/species present. There were no significant differences in the overall composition of boluses collected from tree swallows at boxes adjacent to and distant from Putah Creek (Table 3-3). Birds near water ate more mayflies than did upland birds, but the difference was suggestive, not significant (Mann-Whitney, $U = 71.0$, $p = 0.167$).

Aquatic and terrestrial basal resources, insects, and birds differed in their patterns of essential amino acid $\delta^{13}C_{norm}$ values, which served as the basis for diet inferences (Appendix 3-3). Samples that had been *a priori* classified as aquatic had relatively high $\delta^{13}C_{norm}$ values for Leu and Val and low $\delta^{13}C_{norm}$ values for Ile and Phe relative to terrestrial samples. In PCAs for each type of reference material (Fig. 3-7), PC1 largely separated aquatic from terrestrial samples and explained most of the variance (50.5 – 84.1%). A few exceptions to reliable sorting included wild oat (*Avena fatua*) and English walnut (*Juglans regia*), which fell on the more aquatic side of PC1, though not clustered among the aquatic samples. Other grasses and trees fell on the terrestrial side. Submerged, conditioned tree leaves occupied an intermediate position. In general, plant samples were more challenging for CSIA-AA than other samples because their low protein content meant some AA measurement peaks were very small. Among insects, predatory species such as Odonates, assassin bugs (*Zelus* sp.), and lacewings (Chrysopidae) occupied an intermediate position. Because they were wild-collected, whether they had fed on

aquatic or terrestrial prey was unknown. PCA including tree swallows showed considerable variation among samples nests, but most fell on the more aquatic side of the PC1 axis.

The amount of aquatic prey in tree swallow diets across all sites, years, and distances was estimated at 66.1% (95% B.C.I. 54.2 – 78.1%; Fig. 3-8) based on CSIA-AA. The aquatic signature in tree swallow biomass declined at a rate of 0.02% per meter from water in diet models with distance from water as a continuous fixed effect and site-year as a random effect. Estimates of diet overlapped broadly at all distances from water, and site-year contributed substantially more to variation in diet than did distance from water ($\sigma_{site\text{-}year}$ = 1.11, 95% B.C.I 0.53 – 2.25; $\sigma_{distance}$ = 0.27, 95% B.C.I 0.01 – 1.10). Further examination of site and year revealed that Bobcat Ranch had the lowest rate of aquatic prey use, and prey use differed markedly by year. In particular, 2011 had very low aquatic prey use and 2013 had very high aquatic prey use; there was no overlap in the medians or 95% B.C.I. of these years (Fig. 3-8).

Reproductive Success and Survival

Controlling for variation by site-year, none of the indicators of reproductive success varied significantly with distance from water, except when depredated nests were included. That is, the only importance of distance from water as a variable arose from there being higher depredation in upland nestboxes, which resulted in slightly lower numbers of nestlings fledged. The primary predators were a group of raccoons (*Procyon lotor*) that were able to clear the predator baffles and raid the majority of upland nests at Stevenson Bridge in 2013; they did not locate the nests nearest water. Otherwise, clutch sizes, nestling body condition, and number of nestlings fledged was relatively invariant with distance from water, though there were marked differences among sites (Fig. 3-9). Site and clutch initiation day accounted for much of the variation in numbers of fledglings and fledgling body condition (Table 3-4). While isotopic

estimates of nestling diet from individual nests were only broadly constrained, we plotted nestling body condition against these values to look for a direct relationship between diet and reproductive success, but no relationship was apparent (Fig. 3-10).

The survival of adult tree swallows was very similar at all distances from water. Overall apparent survival was estimated at 0.43 (95% CI: 0.29 - 0.58) and recapture probability at 0.44 (95% CI: 0.26 – 0.64). Estimates by distance from water were widely overlapping. Survival varied more by site, with South Fork adults having the greatest survival rate (0.53, 95% CI: 0.36 - 0.70) and Bobcat Ranch adults having the poorest survival (0.20, 95% CI: 0.08 – 0.42). Stevenson Bridge adult survival estimates were intermediate between the other sites (0.34, 95% CI: 0.19 – 0.54). Model comparison suggested that a simple model containing only site had much better support than models including distance from water and models without any environmental variables (Table 3-5).

DISCUSSION

Mobile consumers are an important focus in food webs because their omnivory and mobility allow them to link dynamics across a broad landscape (Polis et al. 1997, McCann et al. 2005, Sitters et al. 2015). Aquatic subsidies can be a valuable resource, inciting aggregations of terrestrial consumers (Murakami & Nakano 2002, Marczak et al. 2007B, Spiller et al. 2010), but our findings highlight how consumers can dramatically translocate subsidies through movements to meet non-trophic life history requirements. Tree swallows flew toward Putah Creek to forage on abundant aquatic insects but returned upland to feed young, creating hotspots of aquatic energy and nutrients that were coincident with appropriate nesting structures, not reflective of local resource availability. Scales adequate to track a decline in aquatic insects were inadequate

to detect a response by tree swallows, the diet and reproductive success of which were better explained at a coarse level than a fine one (site vs. distance from water). Birds hundreds of meters from water were still very much part of an aquatic food web. Overall, (1) Swallow foraging trips were more important than aquatic insect motility at increasing the terrestrial extent of aquatic subsidies, (2) There was an apparent lack of time and energetic constraints on commuting swallows, which may have reflected low costs of movement or reproductive elasticity, and (3) Reliance on aquatic insects was high even with non-trivial abundances of terrestrial insects, which has implications for the conservation of swallows and potentially other declining aerial insectivores.

The Landscape Extent of Aquatic Subsidies

The primary basis for our study design was the assumption that aquatic insect abundance declined sharply with distance from water (Gratton et al. 2009, Muehlbauer et al. 2014), and this was indeed the case. Like the negative power model we used to describe insect abundance along Putah Creek, other models and empirical descriptions of aquatic insect abundance have relatively "long tails" extending into uplands (Jackson et al. 1989, Petersen et al. 2004, Wesner et al. 2010). These tails usually reflect a trace presence rather than important penetration of upland habitats, with some exceptions (Popova et al. 2017). Along Putah Creek, about an order of magnitude more aquatic prey occurred at the shoreline than in uplands, and terrestrial insect abundance was relatively uniform at all distances apart from the riparian zone, where insect diversity and abundance are often distinct (Ramey & Richardson 2017). The placement of nestboxes therefore seemed adequate to allow for settlement along a strong subsidy availability gradient.

Even with a steep decline in aquatic insect availability with distance from water, tree swallows settled uniformly at all distances. Most swallows arrived in February or March, when grasslands were still usually green and productive from recent rain. This could have led to a preference for uplands, which would not only have insect prey but also more sunlight than the tree-shaded creek, facilitating warming and incubation. Tree swallows are known to be sensitive to nestbox microclimate during nestbox selection (Ardia et al. 2006), so this possibility cannot be excluded. The only strong signal was lower overall settlement at Bobcat Ranch, one of many indications that site mattered more than position within site.

The incongruity between local insect abundance and tree swallow diet suggested directed foraging on swarming aquatic insects. Boluses obtained from adults were usually monotypic, a pattern that was not observed in our indiscriminate sweep net sampling. For example, four of the 14 boluses obtained at nestboxes far from Putah Creek (>224 m) were composed entirely of mayflies, yet in thousands of net sweeps at the same distance from water, no mayflies were captured. Creek-directed foraging movements confirmed selection of feeding grounds closer to water. A tendency to mirror prior directions of departure was consistent with the notion that birds located swarming insects and made repeated visits to feed on them. We had predicted that aquatic prey use would be high given the moisture-limited uplands of our California study sites, yet rates of aquatic insect consumption have been generally high in other, far more mesic tree swallow nesting sites (Blancher & McNicol 1991, Mengelkoch et al. 2004, Kautza et l. 2016), especially during emergence events (St. Louis et al. 1990) and inclement weather (McCarty et al. 1997). Whether this indicates that aquatic prey are preferred or merely more clumped so as to make for more efficient foraging would require additional research.

Given the surprising mismatch between landscape location, prey availability, and prey use, it is important to critically consider the CSIA-AA that served as the basis for most dietary inferences. For all types of reference samples, the major axis in PCA separated species based on their membership in primarily aquatic or terrestrial food webs. Similar factor loadings likely reflected some fundamental biochemical differences between unicellular aquatic and tracheophytic terrestrial producers, telltale "isotopic fingerprints" that transmit through food webs (Larsen et al. 2013). The method is still growing in use compared to bulk stable isotope analysis, so it was encouraging that specific aspects behind loadings (e.g., Leu ≈ Phe in aquatic food webs, Leu < Phe in terrestrial food webs) echo patterns in analogous producers in a different riparian food web studied with the same CSIA-AA method (Thorp & Bowes 2017). Even a different CSIA-AA method applied to a different system found that Phe and Val successfully separated aquatic from terrestrial consumers, much like they did in our system (Webb et al. 2015).

Because we used known aquatic/terrestrial birds as the end members of our mixing model, the most straightforward way to interpret "aquatic" in this study was having biochemical characteristics consistent with secondary/tertiary consumers known to feed on aquatic animals. We did not discern particular aquatic producers, but the isotopic signature was similar to certain green algae (Larsen et al. 2013, Thorp & Bowes 2017). The overall isotopic estimate of 66.1% aquatic diet was slightly lower than the 74.6% aquatic diet estimate from boluses. This may reflect imprecision due to relatively few bolus samples or suggest that our classification scheme tended to over-classify organisms as aquatic (e.g., all midges). It is also worth considering that the isotopic analysis detected the important subsidy "boomerang" (Scharnweber et al. 2014) of terrestrial leaves to aquatic insects which then emerge back to terrestrial environments. These

insects would be classified as aquatic despite a significant terrestrial diet component. Riparian food webs are inherently complex and challenging to study with chemical tracers (Jardine et al. 2015), but there is clear potential for CSIA-AA to help clarify some linkages. In particular, the clear differences among sites and years in terms of aquatic insect consumption warrant further investigation to determine the cause of the shifts.

The role of tree swallows in translocating an aquatic signature to uplands may seem like the singular result of some idiosyncratic life history traits, but this case study bears broader relevance. Other organisms have directly analogous behaviors, such as insectivorous bats roosting in upland trees or caves (Power & Rainey 2004). More generally, consumers often disperse subsidies in a uniquely concentrated, directional manner that can create hotspots on the landscape not predicted by resource use matching local availability (Bauer & Hoye 2014, Sitters et al. 2015). A great deal of food web subsidy theory is sensitive to consumer behaviors, such as dietary preferences, movement, and response times (Huxel & McCann 1998, McCann et al. 2005, Earl & Zollner 2014). Tree swallows provide empirical support for the view that consumers do move broadly and feed omnivorously in the larger landscape, which is often required for stabilizing food web effects to emerge (McCann et al. 2005, Rooney et al. 2008).

Aquatic Subsidies and Reproduction

Tree swallows had similar reliance on aquatic prey regardless of distance from water, but clear foraging costs were not evident. Barring a failure to detect massive influxes of aquatic insects into uplands, birds had to exert time and energy to obtain these insects at or close to Putah Creek. Due to our sweep netting at low altitude, this is a possibility, though others have found little difference between aquatic insect abundance near the ground and many meters above ground (Brown & Brown 1996). Predatory terrestrial insects could have fed on aquatic prey and

moved upland, but most terrestrial prey in boluses occupied low trophic positions. How, then, could swallows pass on no costs of resource acquisition to nestlings, which were uniform in number and size across distances to water? Interestingly, we are not the first to predict that costs would be imposed but find little evidence of them. Murphy et al. (2000) asked the provocative question, "Is reproduction by tree swallows cost free?" in light of multiple experiments that have increased the size of tree swallow broods to no consistent deleterious effects on adults or young. The authors speculated that swallows simply have a capacity to raise more young than they typically do, which may serve as a buffer against taxing instances of bad weather or mate loss, or may indicate that swallows are limited by egg-laying capacity, not chick-rearing. In short, swallows often have a capacity for greater time and energetic expenditures than they typically exert (Winkler et al. 1996). With flight energetic costs less than half of those of other birds (Hails 1979), the commuting penalties imposed by settlement location may not have fully tested their energetic capacity.

While distance from water did not seem to impact reproductive success, productivity was not uniform along Putah Creek. Site was the most important explanatory variable in models of reproductive success. Bobcat Ranch had fewer nesting attempts, lower average clutch sizes, poorer body condition of nestlings, and lower apparent adult survival than the other two sites. Conversely, South Fork had the most nesting attempts, highest average clutch sizes, best body condition of nestlings, and highest apparent adult survival. Stevenson Bridge was intermediate. The correlations between metrics of reproductive success suggest overall high-quality and low-quality sites rather than pronounced reproductive trade-offs (e.g., fewer chicks of larger size or vice-versa). Subsidies may have played a role in these patterns. As Putah Creek flows from upstream dams to the Central Valley floor, it slows, warms, and gathers runoff from a landscape

dense in farmland. Aquatic insect emergence is tied strongly to temperature (Merritt et al. 2008) and a combination of warming and eutrophication has been shown to enhance export of aquatic insect subsidies to terrestrial systems (Greig et al. 2012). Indeed, diet of swallows had the highest aquatic signatures at Stevenson Bridge and South Fork, which were in the downstream reaches, and they ate the fewest aquatic insects from the cooler, swifter stretch at Bobcat Ranch.

One unexpected implication of our finding that swallows diets were roughly similar was that birds farther from the subsidy would be predicted to be more reliant upon it. That is, in order to make up for the additional energetic costs of flying to/from Putah Creek, birds far from the creek would need to eat more insects, which could mean that they would need to consume more aquatic prey overall than birds next to water. This is speculative and would hinge on whether birds near water rested instead of commuting; if all birds flew just as much, needs would be identical. Flight costs did not appear to markedly affect settlement behavior, diet, or reproductive success at the scale we studied, which could suggest that there is more of a threshold effect than a continuous decline in aquatic prey use.

Aquatic Insect Subsidies as a Focus for Conservation

Several studies have documented a decline in the reproductive success for birds faced with shortfalls in emergent aquatic insects due to human activities. House martins (*Delichon urbica*) fledged one-third fewer young when living at wetlands sprayed with *Bti* to control mosquitoes (Poulin et al. 2007); young pied flycatchers (*Ficedula hypoleuca*) had 10% lower survival along regulated rivers compared to undammed ones (Strasevicius et al. 2013); and tree swallows suffered a variety of ill effects when nesting next to experimentally acidified lakes that altered insect populations (St. Louis & Barlow 1993). Other human activities, such as introducing fish which feed on emerging insects, may also play a role (Epanchin et al. 2010). In

all of these studies, birds have been generalist insectivores, yet terrestrial insects did not compensate for shortfalls in aquatic prey.

Potentially complementary characteristics may explain the value of aquatic insects as prey. Emerging swarms of many taxa are highly concentrated near water (Muehlbauer et al. 2014), which may make for profitably concentrated foraging. Quality can also matter a great deal (Marcarelli et al. 2011). Aquatic insects are rich in polyunsaturated fatty acids, which benefit the growth or fitness of a variety of terrestrial species (Gill & Valivety 1997, Martin-Creuzberg et al. 2017). Carefully-controlled lab studies have demonstrated that consumption of these fatty acids significantly enhanced the growth of young tree swallows (Twining et al. 2016). Field studies to show the same effect are generally lacking. Dodson et al. (2016) found that prothonotary warbler (*Protonotaria citrea*) nestlings fed on aquatic mayflies grew more quickly than those fed on caterpillars, but there were potential confounds. In our study, individual-nest estimates of aquatic prey consumption did not vary meaningfully with metrics of reproductive success. However, swallows ate so much aquatic prey overall that they may have exceeded any threshold levels of polyunsaturated fatty acids required to avoid negative effects.

Emergent aquatic insects are unique in that they may impose costs on consumers as they also subsidize them. Insects are potential vectors of mercury and other aquatic contaminants to their watersheds (Cristol et al. 2008, Walters et al. 2008), but evidence of ill effects on survival and fitness is often limited. For example, the survival of swallows was estimated to be only 1% lower along heavily mercury-contaminated waters than along relatively clean waters (Hallinger et al. 2011), and researchers could only detect a slight negative effect of mercury on the production of fledglings by young, inexperienced female swallows despite exposure to very high levels of the metal (Brasso & Cristol 2008). Some aquatic insects can also be problematic

because they feed on blood and/or spread parasites, such as mosquitoes (Culicidae) and blackflies (Simuliidae). Tomás et al. (2008) found no significant effects of blackflies on blue tits (*Cyanistes caeruleus*) or their nestlings, but very high infestations could feasibly be problematic. Blackflies and similar insects potentially have a dual role as food and parasite (Malmqvist et al. 2004). On balance, however, it seems that tree swallows' reliance on aquatic insects can be so overwhelming that aquatic insects are a net positive in spite of potential, but often localized and particular, associated risks.

In the past decade, marked declines in the number of aerial insectivorous birds have been noted, with differing explanations (Nebel et al. 2010, Hallmann et al. 2014). Tree swallows are among this group (Shutler et al. 2012). Dramatic declines in flying insects have also been noted across a range of locations (Hallmann et al. 2017), and the two phenomena may be related. The movement of insects and birds are both important to determining the spatial extent of aquatic subsidies, and declines in either group would be predicted to lessen the organism-mediated relay of aquatic subsidies upland. In particular, the presence of surface water was crucial to supporting upland consumers, so these species should be considered when creating water use and conservation plans.

CONCLUSION

Even when food web subsidies are a consumer's primary diet, consumer responses are more complex than simply tracking maximum resource availability. The movements of emergent aquatic insects make aquatic resources available to consumers, and movements of species like tree swallows distributes these resources patchily across the terrestrial landscape. This study demonstrates a marked incongruity between swallow diet and the aerial insect pool around their

nests. Overall, birds at all distances from water averaged similarly heavy use of aquatic prey and similar reproductive success. The dense concentration of insects near water and/or their high quality apparently made them a profitable foraging target at all scales in this study. It is often difficult to detect subsidy use when alternative prey, such as terrestrial insects, are also available, but two forms of dietary analysis confirmed the importance of aquatic subsidies. Nestling swallows hundreds of meters upland were strongly linked to algae-based food webs.

Future work to better understand individual variation in use of aquatic resources would be useful, as would work to more fully integrate timing into the study of aquatic prey use. The aquatic insect prey pool represented multiple different species pulses, and finer-scale monitoring could uncover whether any of these were of particular importance in consideration of the timing of settlement and chick-rearing. Overall, the study of subsidized food webs must embrace these complexities, and new tools such as CSIA-AA are poised to help on the empirical side of research.

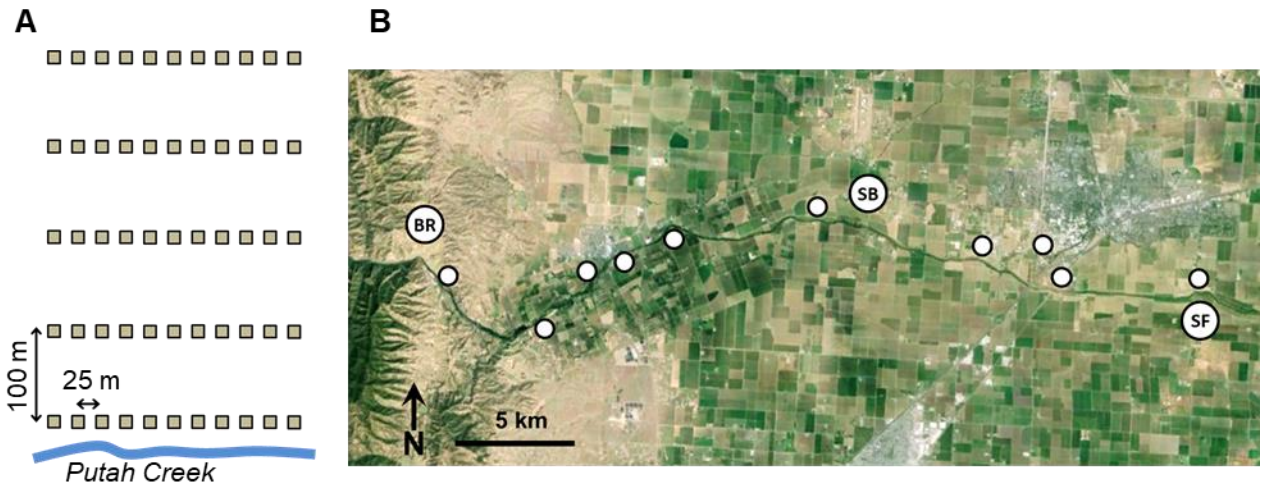


Figure 3-1. A. Layout of a nestbox study grid site. The farthest upland row of boxes was only present at Stevenson Bridge. **B.** Aerial photo of the study area with large, labeled circles for nestbox study grids (BR, Bobcat Ranch; SB, Stevenson Bridge; SF, South Fork) and small, unlabeled circles for smaller, pre-established nestbox trails.

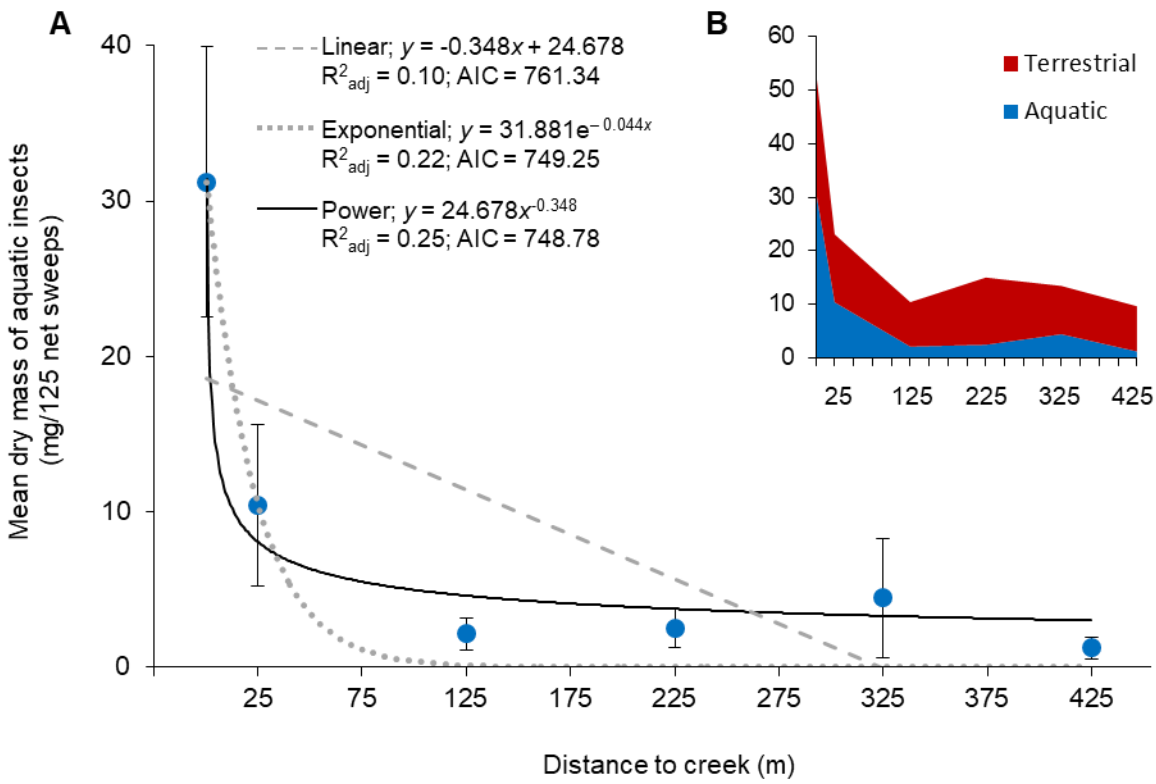


Figure 3-2. A. Abundance of emergent aquatic insects as a function of distance from Putah Creek. For clarity, only the mean dry mass (± s.e.) of emergent aquatic insects captured is shown for each sampling distance, not all data points used to fit curves. **B.** Figure 2A with mean dry mass of terrestrial insects stacked above mass of aquatic insects.

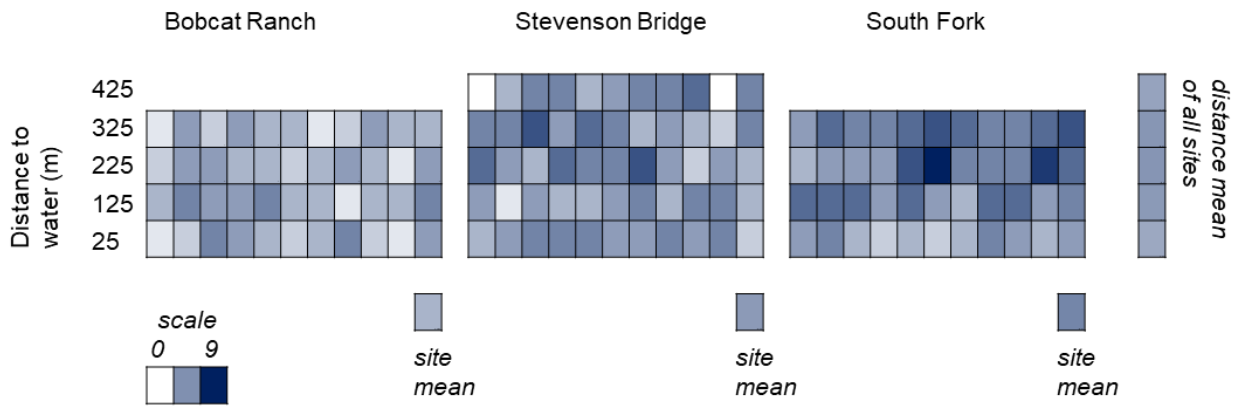


Figure 3-3. Count of cumulative nesting attempts by tree swallows, 2010 – 2014. Each rectangle corresponds to a nestbox in the nestbox grid with the space between removed.

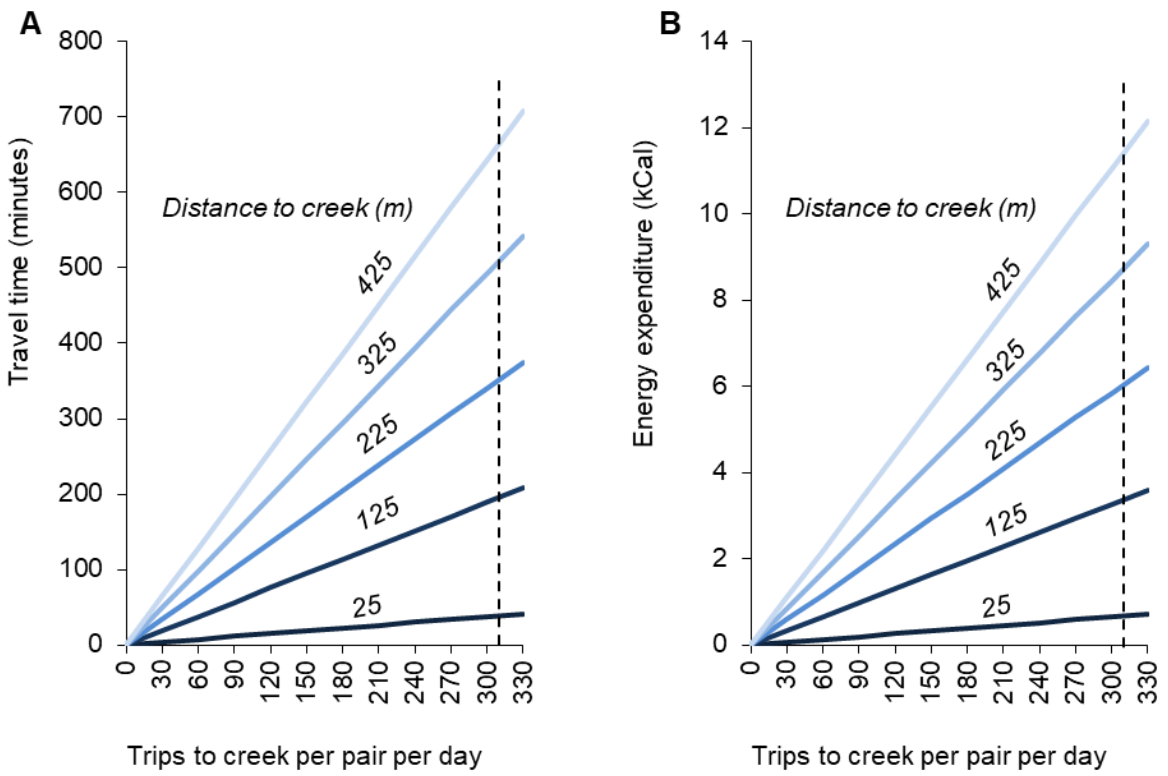


Figure 3-4. Predicted costs incurred in terms of (A) commute time and (B) energy to reach prey-rich Putah Creek by tree swallow pairs at varying distances from water. Costs are to reach the nearest point of the shoreline of Putah Creek by the shortest possible path. The dashed lines represent n = 312 combined foraging trips per day by a pair, the average in another California population observed by Rose (2009).

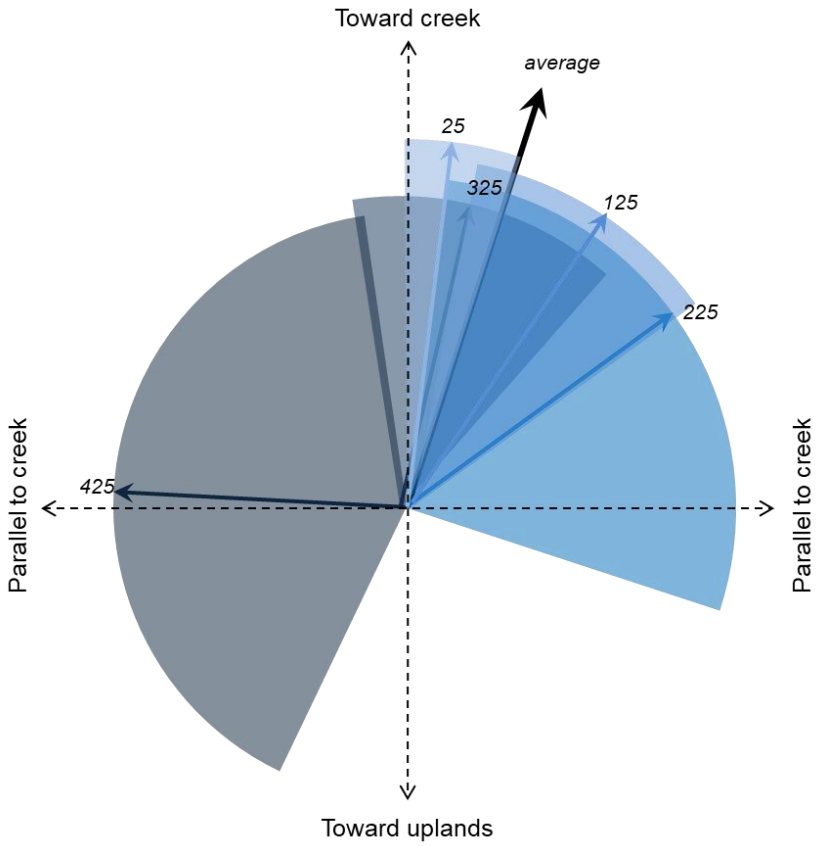


Figure 3-5. Typical foraging departure directions of tree swallows provisioning nestlings. Arrows indicate mean foraging departure directions of birds at varying distances from water (italicized numbers), with shaded regions for the 95% C.I. Only the direction of departure is meaningful; arrows are of different lengths solely to improve visual clarity.

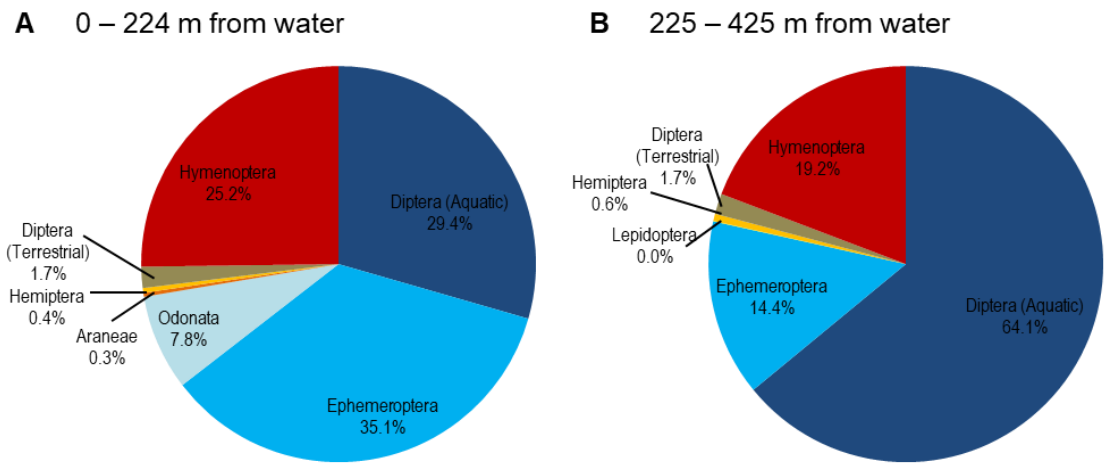


Figure 3-6. Average composition of tree swallow boluses (A) near and (B) distant from water. Composition is based on dry biomass.

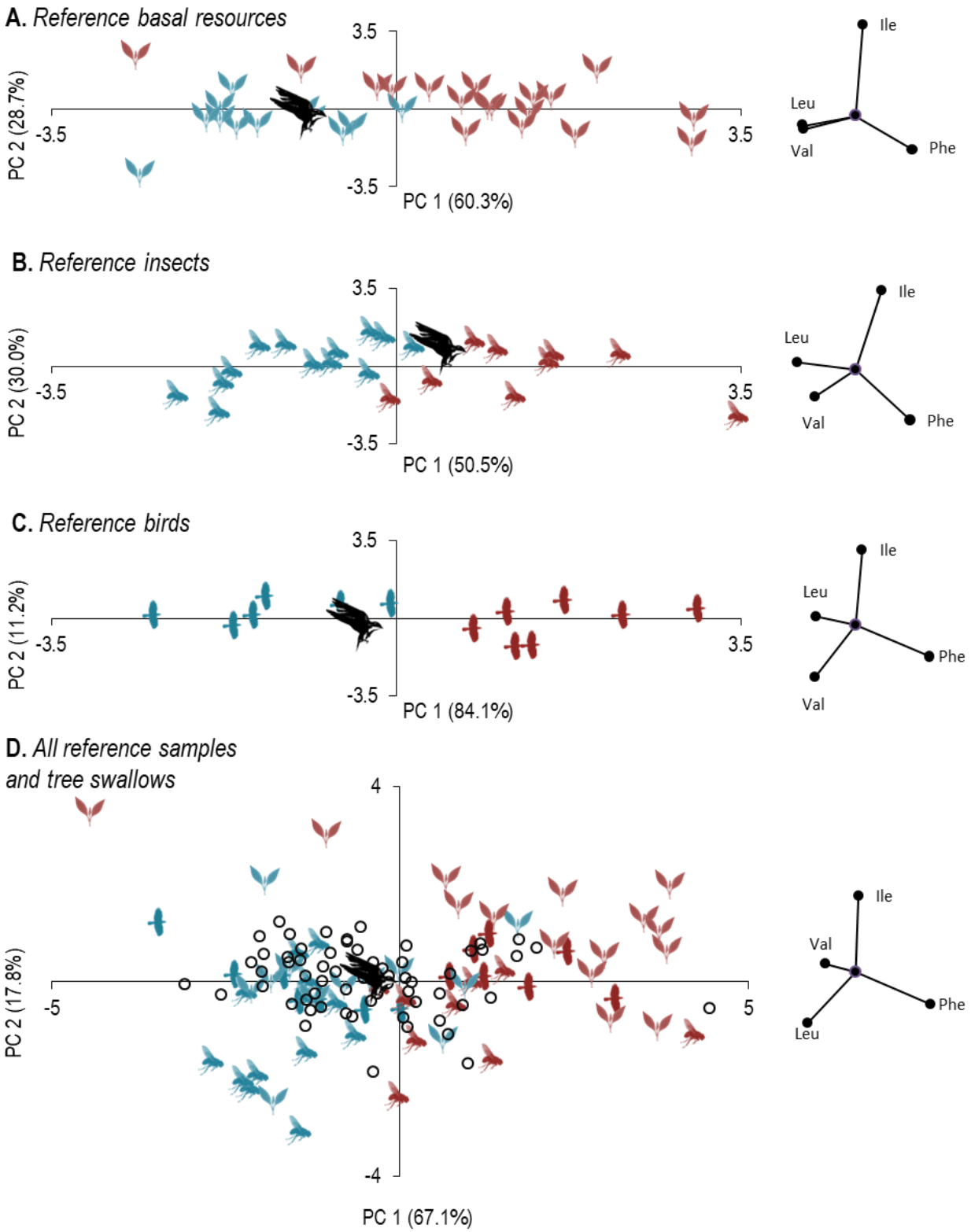


Figure 3-7. PCAs for reference basal resources, insects, and birds (A-C), with loadings to the right. Samples which were classified *a priori* as aquatic appear in blue, terrestrial in red. The tree swallow figure indicates where the mean of all swallows fell, included as a single sample in each analysis. Open circles in the final plot (D) are for all tree swallow samples.

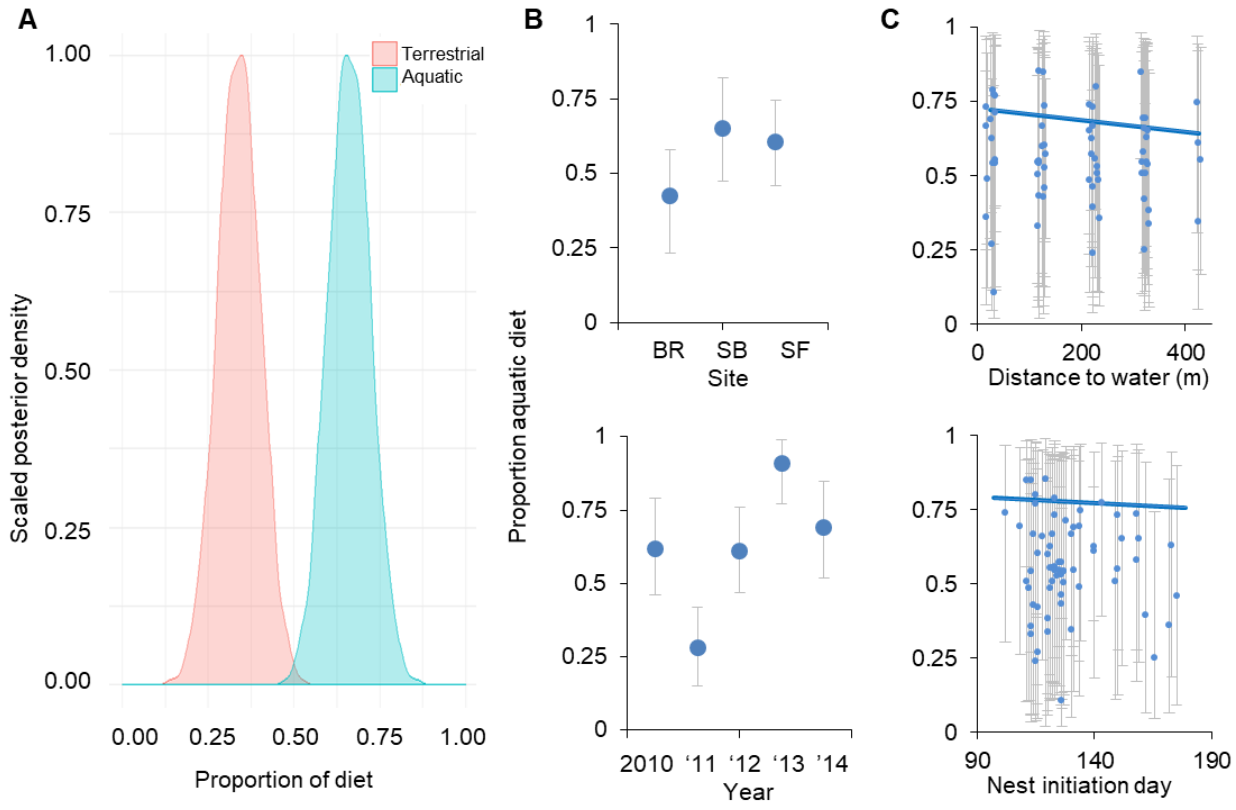


Figure 3-8. (A)Overall estimate of tree swallow diet across all sites and years. (B)Diet estimates with 95% B.C.I. for nestbox sites and study years. (C)Diet estimate curves with individual point estimates and 95% B.C.I. at increasing distances from water and as the nest season progressed.

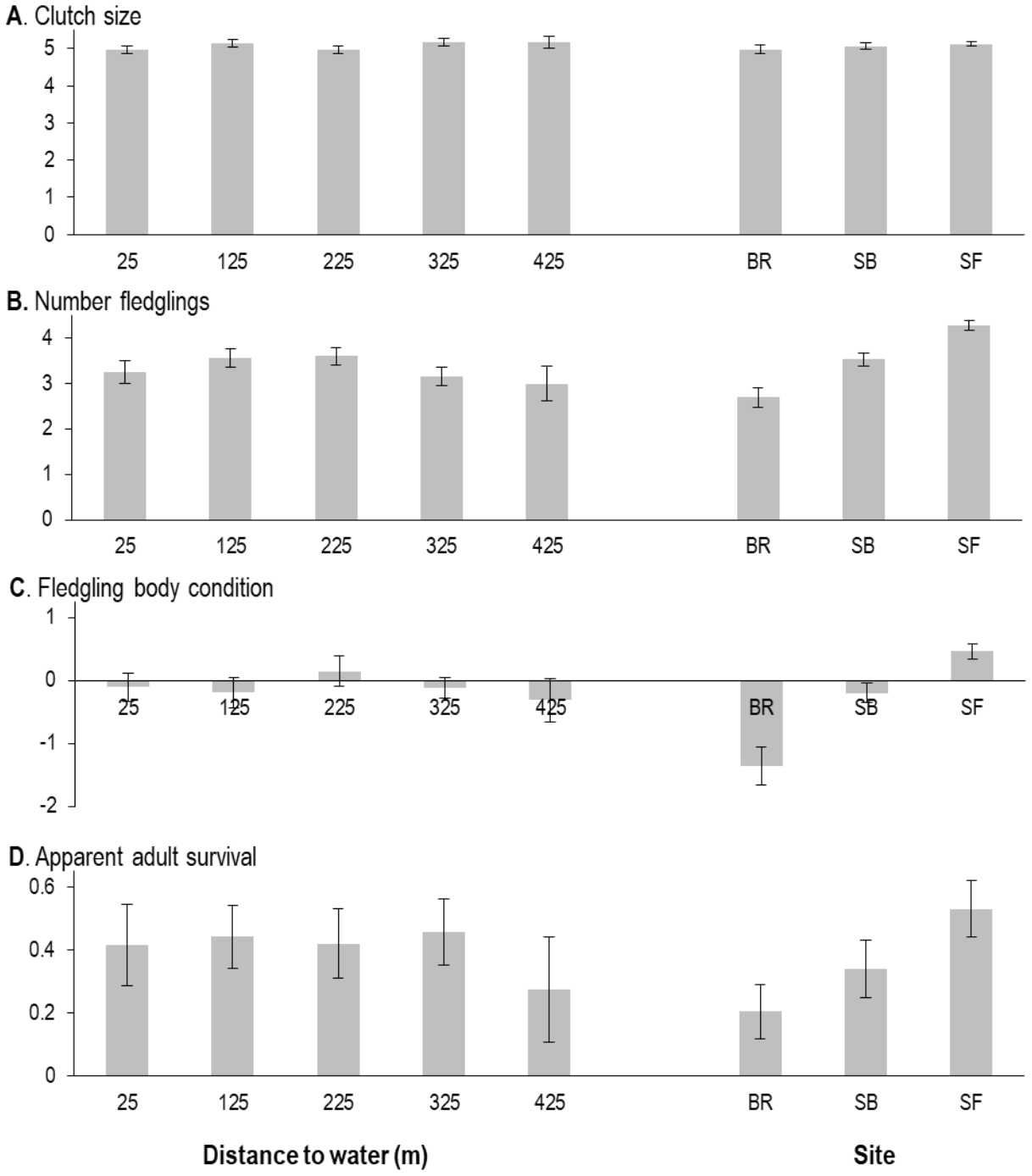


Figure 3-9. Summary statistics (mean ± 1 SE) related to reproductive success as parsed by distance to water or by site. Note that for the distance of 425 m from water, data were collected at only one site, SB, which had the highest rate of depredation.

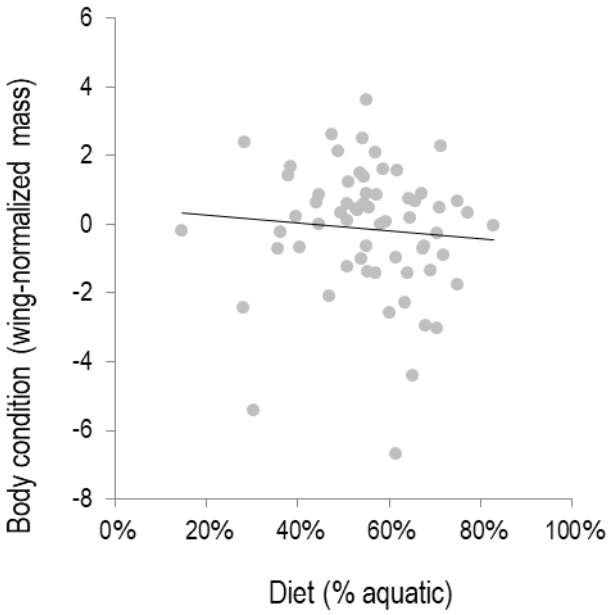


Figure 3-10. Body condition of fledglings as a function of CSIA-AA estimate of diet. The slope of the best-fit line was not significantly different than 0.

Table 3-1. Characteristics of nestbox study grid sites. Land cover is described for a 500 m radius circle drawn from the center of each site, the approximate scale most relevant to characterizing tree swallow diet (Mengelkoch et al. 2004, Kautza & Sullivan 2016). Cover types were outlined in aerial photos by hand and ground-truthed.

Site (abbrev.), land owner	**Latitude, longitude, elevation**	**Description of nesting fields**	**Regional land cover**				
			Putah Creek	*Riparian Forest*	*Grass-land*	*Agri-culture*	*Roads & other*
Bobcat Ranch (BR), Audubon California	38.518°N 122.056°W 67 m	Hilly. Annual grasses and forbs, very few scattered blue oaks (*Quercus douglasii*).	2.6%	12.2%	74.7%	0%	10.6%
Stevenson Bridge (SB), UC Davis	38.539°N 121.850°W 32 m	Flat. Perennial blue wildrye (*Leymus triticoides*), annual grasses and forbs.	1.5%	12.8%	32.0%	39.1%	14.6%
South Fork (SF), City of Davis	38.517°N 121.688°W 10 m	Flat. Mostly annual grasses and forbs, many scattered young valley oaks (*Quercus lobata*).	3.4%	7.2%	49.4%	34.1%	5.9%

Table 3-2. Mean values (+/- s.d.) of various metrics from tree swallow breeding attempts. We did not test for significant differences between means for this table, but for each metric, the highest-ranked site, year, and distance from water are highlighted in gray. The distance of 425 m reflects data from only the Stevenson Bridge study site.

		Settlement & Community			Phenology		Breeding Adults		Reproduction					
Factor	Group	Mean nest attempts per nestbox per year	Prop. nestboxes with heterospecific competitor presence	Prop. nestboxes depredated or damaged	Mean clutch initiation date	Prop. nestboxes with possible double broods (initiated post June 1)	Prop after-second-year females	Prop. adults captured that are re-captured in subsequent years	Mean clutch size	Mean brood size	Fledglings per nest attempt	Nestling wing-controlled mass residual (g)	Nestling fat stores (scale: 0 min - 7 max)	Prop. nestlings that are re-captured in subsequent years
Overall	N/A	**0.67 (0.66)**	**0.19 (0.01)**	**0.09 (0.01)**	**130 (18)**	**0.11 (0.03)**	**0.57 (0.04)**	**0.21 (0.03)**	**5.08 (1.03)**	**4.67 (1.33)**	**3.70 (1.96)**	**-0.08 (1.80)**	**2.92 (0.93)**	**0.02 (0.00)**
Site	Bobcat Ranch	0.41 (0.55)	0.24 (0.03)	0.03 (0.01)	130 (14)	0.03 (0.01)	0.41 (0.09)	0.11 (0.05)	4.98 (1.12)	4.36 (1.67)	2.70 (2.08)	-1.36 (2.16)	2.71 (1.19)	0.02 (0.01)
	Stevenson Bridge	0.69 (0.67)	0.20 (0.02)	0.17 (0.02)	131 (20)	0.13 (0.03)	0.47 (.07)	0.12 (0.03)	5.07 (1.05)	4.75 (1.25)	3.53 (2.04)	-0.20 (1.70)	2.79 (0.93)	0.02 (0.01)
	South Fork	0.89 (0.65)	0.12 (0.02)	0.05 (0.01)	130 (18)	0.15 (0.03)	0.71 (0.05)	0.42 (0.06)	5.13 (0.98)	4.74 (1.20)	4.31 (1.57)	0.46 (1.49)	3.10 (0.78)	0.01 (0.01)
Year	2010	0.27 (0.45)	0.09 (0.02)	0.01 (0.01)	130 (13)	0.02 (0.01)	0.50 (0.25)	0.25 (0.22)	5.15 (0.84)	4.67 (1.58)	4.33 (1.78)	0.72 (1.25)	3.12 (.57)	0.02 (0.01)
	2011	0.91 (0.75)	0.20 (0.03)	0.10 (0.03)	133 (22)	0.25 (0.04)	0.66 (0.08)	0.22 (0.05)	5.14 (1.00)	4.45 (1.60)	3.70 (1.87)	-0.19 (1.79)	3.17 (0.79)	0.05 (0.01)
	2012	0.78 (0.63)	0.20 (0.03)	0.02 (0.01)	131 (16)	0.11 (0.03)	0.48 (0.07)	0.29 (0.05)	5.04 (1.04)	4.76 (1.10)	3.99 (1.77)	-0.28 (1.95)	3.04 (1.05)	0.01 (0.01)
	2013	0.80 (0.64)	0.23 (0.04)	0.28 (0.04)	126 (17)	0.08 (0.02)	0.67 (0.09)	0.14 (0.04)	5.04 (1.16)	4.90 (1.15)	2.87 (2.23)	-0.15 (1.81)	2.34 (0.92)	0.00 (0.00)
	2014	0.56 (0.59)	0.22 (0.03)	0.01 (0.01)	131 (16)	0.07 (0.02)	0.50 (0.08)	N/A	5.03 (0.98)	4.60 (1.16)	3.97 (1.78)	0.10 (1.73)	2.96 (0.81)	N/A
Distance to water (m)	25	0.46 (0.59)	0.27 (0.03)	0.02 (0.01)	131 (16)	0.05 (0.02)	0.44 (0.09)	0.20 (0.07)	4.96 (0.84)	4.41 (1.47)	3.63 (1.98)	-0.11 (1.60)	3.19 (0.87)	0.01 (0.01)
	125	0.65 (0.60)	0.21 (0.03)	0.07 (0.02)	127 (15)	0.05 (0.02)	0.65 (0.07)	0.22 (0.05)	5.14 (1.05)	4.87 (0.95)	4.11 (1.70)	-0.19 (2.01)	2.83 (0.97)	0.02 (0.01)
	225	0.76 (0.69)	0.14 (0.03)	0.12 (0.03)	130 (18)	0.13 (0.03)	0.54 (0.07)	0.21 (0.05)	4.96 (1.12)	4.60 (1.41)	3.54 (1.96)	0.15 (2.11)	2.80 (1.00)	0.02 (0.01)
	325	0.76 (0.70)	0.15 (0.03)	0.08 (0.02)	132 (19)	0.16 (0.03)	0.63 (0.08)	0.22 (0.05)	5.18 (1.03)	4.71 (1.30)	3.57 (2.03)	-0.12 (1.47)	2.94 (0.89)	0.02 (0.01)
	425	0.76 (0.67)	0.13 (0.05)	0.20 (0.05)	132 (20)	0.18 (0.03)	0.47 (0.13)	0.15 (0.10)	5.17 (0.84)	4.71 (1.67)	3.60 (2.28)	-0.31 (1.57)	2.95 (0.74)	0.02 (0.02)

Table 3-3. Characteristics of boluses intercepted from adult tree swallows provisioning young. Apparently partial bolus recoveries included.

	All nests	**Nests 0 – 224 m from creek**	**Nests 225 – 425 m from creek**	**Comparison, 0 – 224 m vs. 225 – 425 m**
Total boluses, prey items analyzed	28 boluses, 465 prey items	14 boluses, 172 prey items	14 boluses, 293 prey items	N/A
Mean number of prey items (S.D.)	16.60 (21.33)	12.29 (11.72)	20.93 (27.70)	Mann-Whitney *U* test U = 100.5, p = 0.93
Mean dry mass, mg (S.D)	24.32 (24.49)	30.58 (28.42)	18.06 (18.81)	Mann-Whitney *U* test U = 69.0, p = 0.190
Mean aquatic biomass, mg (S.D.)	18.15 (24.00)	22.13 (28.62)	14.17 (18.52)	Mann-Whitney *U* test U = 82.5, p = 0.49
Monotypic boluses (single-item boluses excluded)	14/22	6/12	8/10	Fisher's exact test p = 0.12

Table 3-4. Effects on (A)Number of fledglings produced and (B)Fledgling body condition. Site and clutch initiation date were the only significant predictors.

(A) ***Number of fledglings***

	Df	Sum Sq	Mean Sq	F-value, p
Year	4	28.91	7.23	0.074
Site***	2	130.27	65.14	<0.001
Distance to water	1	0.24	0.24	0.790
Clutch initiation day***	1	43.76	43.76	<0.001
Parent size	1	2.43	2.43	0.395
Residuals	217	724.70	3.340	

(B) ***Fledgling body condition***

	Df	Sum Sq	Mean Sq	F-value, p
Year	4	19.20	4.80	0.124
Site***	2	63.82	31.91	<0.001
Distance to water	1	0.50	0.50	0.664
Clutch initiation day***	1	29.01	29.01	<0.001
Parent size	1	1.65	1.65	0.428
Residuals	172	449.00	2.61	

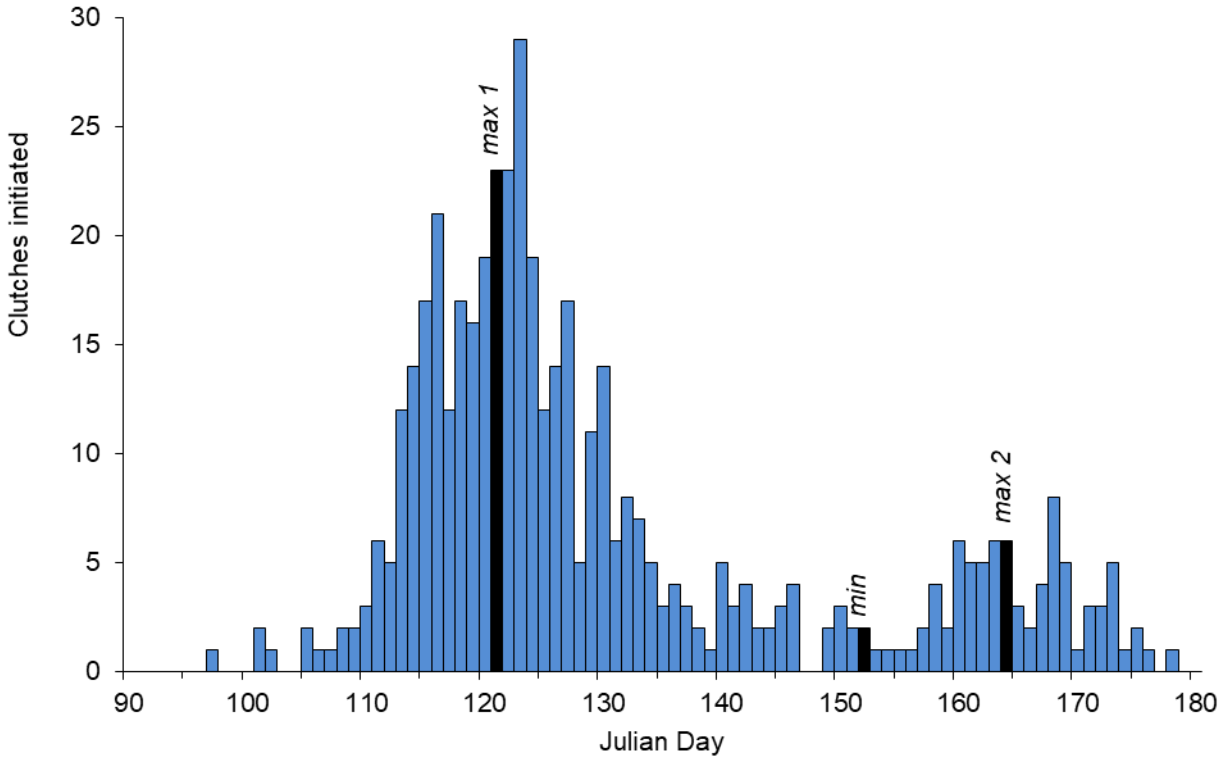
Table 3-5. Comparison of mark-recapture models. ϕ is survival probability, p is recapture probability (constrained to be constant), and subscripts represent the variables over which survival was allowed to vary, including by nestbox site and distance to water.

Model	Parameters	AIC_c	$dAIC_c$
$\phi_{site}\ p$	4	281.587	0.000
$\phi\ p$	2	287.184	5.597
$\phi_{distance*site}\ p$	14	292.954	11.367
$\phi_{distance}\ p$	6	294.572	12.985

Appendix 3-1. Habitat classification scheme for insects by order and some sub-ordinal groupings. In many cases, habitat assigned is a gross simplification (e.g., some parasitic wasps parasitize aquatic insect larvae, but all Hymenoptera are labeled as terrestrial). If any taxa of ambiguous aquatic/terrestrial origin were especially common in samples (e.g., large craneflies (Tipulidae) in spring sampling) efforts were made to distinguish them as truly aquatic or terrestrial. Taxa likely to be encountered in California were included in the list, but not all were encountered.

1. Odonata (Aquatic)
2. Ephemeroptera (Aquatic)
3. Plecoptera (Aquatic)
4. Orthoptera (Terrestrial)
5. Thysanoptera (Terrestrial)
Hemiptera
    6. Terrestrial Hemiptera (all but taxa below)
    7. Aquatic Hemiptera (Gerromorpha; Nepomorpha)
8. Hymenoptera (Terrestrial)
9. Raphidioptera (Terrestrial)
10. Megaloptera (Aquatic)
11. Neuroptera (Terrestrial)
Coleoptera
    12. Terrestrial Coleoptera (all except aquatic taxa, below)
    13. Aquatic Coleoptera (Microsporidae; Adephaga section Hydradephaga; Hydrophilidae; Elmidae; Dryopidae; Psephenidae)
14. Trichoptera (Aquatic)
Lepidoptera
    15. Terrestrial Lepidoptera (all except aquatic taxa, below)
    16. Aquatic Lepidoptera (Cambridae/Pyrallidae)
Diptera
    17. Aquatic lower Diptera/"Nematocera" (all except terrestrial taxa, below)
    18. Terrestrial lower Diptera/"Nematocera" (Bibionomorpha; Psychodomorpha)
    19. Terrestrial lower Brachycera (all except aquatic taxa, below)
    20. Aquatic lower Brachycera (Tabanidae, Athericidae, Empididae, Dolichopodidae)
    21. Terrestrial Cyclorrhapha (all except aquatic taxa, below)
    22. Aquatic Cyclorrhapha (Sciomyzidae, Ephydridae)

Appendix 3-2. Summary histogram of tree swallow nesting at all sites, 2010 – 2014. The minimum dividing the two peaks was determined as the midpoint of the sliding 7-day period with the lowest sum-total nest initiations (day 152; June 1 in non-leap years). The maxima were determined as the midpoints of the sliding 7-day periods with the highest sum-total nest initiations. The first maximum mainly reflects first/only clutches (day 121; May 1 in non-leap years), and the second maximum is a combination of second clutches, replacement clutches, and late clutches (day 164; June 13 in non-leap years).



Appendix 3-3A. Isotope values of basal resources.

Code	Sample	Type	Habitat	Notes	$\delta^{13}C$ (‰)								
					Ala	Asp	Glu	Gly	Ile	Leu	Phe	Pro	Val
VEG1	Benthic cyanobacteria (Cyanophyceae)	Basal resource	Aquatic	Putah creek	-6.8	-2.5	-18.5	-6.6	-19.9	-22.9	-21.9	-16.1	-18.2
VEG2	Phytoplankton 60-242 µm	Basal resource	Aquatic	Putah creek	-24.3	-36.3	0.0	-17.3	-29.0	-35.5	-33.6	-27.1	-31.5
VEG3	Periphyton, rocky substrate	Basal resource	Aquatic	Putah creek	-19.2	-11.8	0.0	-14.6	-26.6	-32.5	-31.4	-29.2	-29.2
VEG4	Periphyton, silty substrate	Basal resource	Aquatic	Putah creek	-22.7	-26.9	-24.7	-18.6	-28.7	-36.0	-33.1	-28.5	-32.0
VEG5	Filamentous green algae (*Cladophora* sp.)	Basal resource	Aquatic	Putah creek	-20.8	-15.2	-20.6	-16.2	-28.6	-34.1	-35.2	-23.2	-33.8
VEG6	Orange epiphytic diatoms (Bacillariophyceae)	Basal resource	Aquatic	Putah creek	-15.2	-11.7	-13.9	-14.5	-24.2	-32.8	-32.2	-16.1	-28.3
VEG7	Chartreuse epiphytic diatoms (Bacillariophyceae)	Basal resource	Aquatic	Putah creek	-12.0	-7.9	-20.7	-8.3	-20.8	-27.3	-26.3	-20.9	-23.1
VEG8	Beige epiphytic diatoms (Bacillariophyceae)	Basal resource	Aquatic	Putah creek	-16.5	-7.8	-27.4	-14.2	-22.1	-28.2	-27.8	-29.5	-24.8
VEG9	Water milfoil (*Myriophyllum* sp.)	Basal resource	Aquatic	Putah creek	-15.3	-9.5	-19.8	-8.9	-20.0	-25.7	-22.9	-15.1	-23.9
VEG10	Waterweed (*Elodea* sp.)	Basal resource	Aquatic	Putah creek	-23.0	-17.2	-29.6	-15.2	-27.6	-34.3	-32.8	-23.0	-32.4
VEG12	Aquatic moss (Bryophyta)	Basal resource	Aquatic	Putah creek	-22.8	0.5	-24.9	-17.5	-27.4	-34.8	-30.6	-31.7	-30.4
VEG13	Submerged, decaying leaves (Salicaceae, most)	Basal resource	Aquatic	Putah creek	-20.3	-33.5	-22.3	-19.3	-25.8	-35.4	-29.5	-18.4	-29.1
VEG11	Floating water-primrose (*Ludwigia peploides*)	Basal resource	Terrestrial	Putah creek	-24.5	-16.5	-27.0	-19.1	-29.4	-38.2	-28.9	-26.7	-33.8
VEG14	Valley oak (*Quercus lobata*)	Basal resource	Terrestrial	Putah creek	-22.0	-32.9	-23.6	-13.1	-26.9	-36.8	-28.8	-25.3	-31.8
VEG15	English walnut (*Juglans regia*)	Basal resource	Terrestrial	Putah creek	-19.4	-13.0	-31.6	-11.4	-25.1	-35.4	-33.7	-18.8	-30.6
VEG16	Northern California walnut (*Juglans hindsii*)	Basal resource	Terrestrial	Putah creek	-24.0	-15.1	-31.4	-16.8	-27.6	-37.0	-32.9	-27.6	-34.1
VEG17	Olive (*Olea euorpaea*)	Basal resource	Terrestrial	Putah creek	-17.0	-7.7	-21.2	-8.0	-22.9	-31.5	-25.9	-19.5	-29.2
VEG18	Fremont's cottonwood (*Populus fremontii*)	Basal resource	Terrestrial	Putah creek	-26.9	-16.2	-36.1	-13.9	-27.8	-39.3	-32.5	-25.7	-37.4
VEG19	Red willow (*Salix laevigata*)	Basal resource	Terrestrial	Putah creek	-25.2	-17.8	-35.7	-14.4	-27.4	-37.7	-30.3	-24.8	-34.0
VEG20	Himalayan blackberry (*Rubus armeniacus*)	Basal resource	Terrestrial	Putah creek	-22.0	-13.4	-25.0	-10.1	-29.3	-36.9	-30.4	-25.7	-33.3
VEG21	Sunflower (*Helianthus annuus*)	Basal resource	Terrestrial	Putah creek	-23.2	-17.7	-24.2	-18.7	-30.1	-37.7	-31.0	-24.2	-35.7
VEG22	Milk thistle (*Silybum marianum*)	Basal resource	Terrestrial	Putah creek	-29.5	-12.7	-26.1	-12.6	-32.0	-42.2	-38.1	-25.7	-37.1
VEG23	Tomato (*Solanum lycopersicum*)	Basal resource	Terrestrial	Putah creek	-18.7	-9.8	-23.6	-5.0	-25.7	-34.8	-29.5	-19.8	-31.6
VEG24	Alfalfa (*Medicago sativa*)	Basal resource	Terrestrial	Putah creek	-21.1	-14.0	-16.4	-9.5	-25.5	-34.0	-22.9	-19.8	-31.4
VEG25	Hairy vetch (*Vicia villosa*)	Basal resource	Terrestrial	Putah creek	-25.6	-14.7	-28.6	-10.2	-29.6	-40.0	-29.0	-31.1	-36.3
VEG26	Torrent sedge (*Carex nudata*)	Basal resource	Terrestrial	Putah creek	-28.9	-19.9	-3.3	-17.8	-28.7	-39.3	-33.5	-29.6	-34.9
VEG27	Purple needlegrass (*Stipa pulchra*)	Basal resource	Terrestrial	Putah creek	-25.2	-33.5	-11.3	-11.4	-26.6	-37.1	-29.8	-24.1	-32.6
VEG28	Ripgut brome (*Bromus diandrus*)	Basal resource	Terrestrial	Putah creek	-24.6	-12.5	-23.5	-15.7	-27.4	-36.8	-33.0	-21.5	-33.0
VEG29	Wild oat (*Avena fatua*)	Basal resource	Terrestrial	Putah creek	-25.1	-13.0	-29.5	-12.0	-28.7	-37.7	-40.6	-26.6	-34.1
VEG30	Blue wildrye (*Elymus glaucus*)	Basal resource	Terrestrial	Putah creek	-23.7	-28.1	-19.0	-9.2	-26.1	-35.3	-29.0	-22.3	-31.7

Appendix 3-3B. Isotope values of insects.

Code	Sample	Type	Habitat	Notes	$\delta^{13}C$ (‰)								
					Ala	Asp	Glu	Gly	Ile	Leu	Phe	Pro	Val
INS1	Burrower mayfly (*Hexagenia* sp.)	Insect	Aquatic	Putah creek	-20.7	-18.6	-21.3	-14.0	-28.6	-30.8	-30.7	-22.0	-29.5
INS2	Squaregill mayfly (Caenidae)	Insect	Aquatic	Putah creek	-21.0	-18.7	-21.0	-14.0	-26.9	-29.9	-30.5	-21.5	-28.2
INS3	Familiar bluet (*Enallagma civile*)	Insect	Aquatic	Putah creek	-23.0	-20.7	-21.6	-18.9	-26.8	-31.9	-30.5	-21.3	-29.8
INS4	American rubyspot (*Hetaerina americana*)	Insect	Aquatic	Putah creek	-27.6	-24.8	-25.9	-20.3	-27.9	-33.7	-32.9	-24.1	-30.8
INS5	Striped meadowhawk (*Sympetrum pallipes*)	Insect	Aquatic	Putah creek	-27.3	-24.6	-26.1	-17.3	-30.2	-33.6	-34.2	-24.7	-32.3
INS10	Water boatman (Corixidae)	Insect	Aquatic	Putah creek	-23.7	-19.4	-24.2	-20.4	-26.2	-33.5	-32.5	-22.6	-30.5
INS12	Aquatic cranefly (Tipulidae)	Insect	Aquatic	Putah creek	-24.7	-21.7	-23.8	-22.5	-26.8	-33.7	-33.2	-22.3	-31.3
INS13	Midges, small (Chironomidae)	Insect	Aquatic	Putah creek	-23.8	-22.5	-22.7	-21.1	-28.3	-34.1	-33.3	-24.3	-32.7
INS14	Small Shorefly (Ephydridae)	Insect	Aquatic	Putah creek	-21.7	-19.7	-20.5	-13.4	-26.2	-30.5	-30.5	-20.0	-29.6
INS16	Elegant spotted marshfly (*Poecilographa decora*)	Insect	Aquatic	Putah creek	-28.2	-25.7	-25.5	-17.5	-32.1	-35.1	-36.3	-24.2	-34.7
INS18	Caddisfly (Trichoptera)	Insect	Aquatic	Putah creek	-25.8	-20.3	-22.1	-19.8	-24.3	-27.5	-29.7	-18.6	-28.2
INS21	Midges, large (Chironomidae)	Insect	Aquatic	Putah creek	-25.1	-23.1	-26.8	-21.2	-27.6	-33.8	-33.1	-24.6	-31.1
INS22	Speckled mayfly (*Callibaetis* sp.)	Insect	Aquatic	Putah creek	-29.8	-27.3	-31.2	-22.4	-33.0	-38.4	-39.2	-27.8	-36.7
INS6	Grasshopper (Acrididae)	Insect	Terrestrial	Putah creek	-22.6	-21.2	-22.7	-16.7	-29.5	-35.1	-31.1	-21.3	-32.5
INS7	Treehopper (Membracidae)	Insect	Terrestrial	Putah creek	-21.6	-18.8	-20.0	-18.0	-26.5	-34.4	-26.0	-19.5	-29.6
INS8	Western elderberry bug (*Boisea rubrolineata*)	Insect	Terrestrial	Putah creek	-23.6	-21.4	-25.3	-20.7	-26.4	-34.4	-30.1	-21.6	-31.3
INS9	Green assassin bug (*Zelus* sp.)	Insect	Terrestrial	Putah creek	-22.6	-20.7	-23.0	-13.9	-26.8	-32.4	-29.3	-20.5	-28.8
INS11	Green lacewing (Chrysopidae)	Insect	Terrestrial	Putah creek	-29.6	-24.2	-25.5	-20.8	-26.9	-34.4	-30.5	-24.9	-31.2
INS15	Dung-associated fly (Ephydridae)	Insect	Terrestrial	Putah creek	-25.1	-23.0	-24.6	-15.2	-28.6	-32.3	-30.8	-22.3	-32.0
INS17	Cabbage white butterfly (*Pieris rapae*)	Insect	Terrestrial	Putah creek	-29.0	-26.4	-26.4	-24.5	-30.6	-37.3	-34.3	-24.2	-35.4
INS19	European honeybee (*Apis mellifera*)	Insect	Terrestrial	Putah creek	-18.7	-20.0	-22.0	-15.1	-25.9	-32.4	-30.9	-15.6	-30.8
INS20	Velvet tree ant (*Liometopum occidentale*)	Insect	Terrestrial	Putah creek	-23.2	-12.7	-14.9	-18.1	-24.3	-30.7	-28.7	-18.5	-29.0

Appendix 3-3C. Isotope values of birds.

Code	Sample	Type	Habitat	Notes	$\delta^{13}C$ (‰)								
					Ala	Asp	Glu	Gly	Ile	Leu	Phe	Pro	Val
AMDI	American dipper (*Cinclus mexicanus*)	Bird	Aquatic	Museum specimen	-18.3	-12.0	-14.9	-11.1	-18.9	-24.8	-25.1	-13.0	-21.6
BEKI	Belted kingfisher (*Ceryle alcyon*)	Bird	Aquatic	Museum specimen	-18.1	-14.1	-18.3	-13.0	-18.3	-25.0	-26.6	-14.9	-21.2
EAGR	Eared grebe (*Podiceps nigricollis*)	Bird	Aquatic	Museum specimen	-16.6	-14.2	-19.0	-13.2	-19.7	-25.4	-24.9	-15.8	-23.0
BUFF	Bufflehead (*Bucephala albeola*)	Bird	Aquatic	Museum specimen	-27.6	-23.4	-20.3	-23.3	-27.8	-34.0	-31.5	-20.9	-31.1
LBDO	Long-billed dowitcher (*Limnodromus scolopaceus*)	Bird	Aquatic	Museum specimen	-26.1	-21.9	-20.8	-25.0	-25.6	-31.5	-29.7	-19.7	-28.7
NSHO	Northern shoveler (*Anas clypeata*)	Bird	Aquatic	Museum specimen	-19.7	-16.7	-14.9	-17.0	-19.4	-25.3	-23.9	-13.7	-21.5
OSPR	Osprey (*Pandion haliaetus*)	Bird	Aquatic	Museum specimen	-22.4	-17.8	-16.5	-9.5	-20.7	-26.9	-26.7	-15.4	-24.5
LOSH	Loggerhead shrike (*Lanius ludovicianus*)	Bird	Terrestrial	Museum specimen	-22.9	-16.4	-18.9	-15.3	-23.5	-30.9	-27.9	-18.0	-28.8
BUSH	Bushtit (*Psaltriparus minimus*)	Bird	Terrestrial	Museum specimen	-25.1	-22.1	-20.0	-17.8	-23.3	-31.6	-28.3	-20.7	-29.7
NUWO	Nuttall's woodpecker (*Picoides nuttallii*)	Bird	Terrestrial	Museum specimen	-19.3	-15.8	-17.2	-12.3	-18.6	-27.5	-23.7	-17.1	-25.0
BUOR	Bullock's oriole (*Icterus bullockii*)	Bird	Terrestrial	Museum specimen	-23.0	-22.1	-24.0	-16.6	-25.8	-33.5	-29.4	-23.5	-30.1
BUOW	Burrowing owl (*Athene cunicularia*)	Bird	Terrestrial	Museum specimen	-28.4	-23.9	-21.1	-23.0	-25.5	-34.5	-28.7	-20.8	-30.5
GRRO	Greater roadrunner (*Geococcyx californianus*)	Bird	Terrestrial	Museum specimen	-20.8	-16.6	-15.3	-15.7	-20.2	-28.3	-22.0	-13.9	-25.3
COPO	Common poorwill (*Phalaenoptilus nuttallii*)	Bird	Terrestrial	Museum specimen	-24.5	-17.5	-19.3	-24.5	-23.7	-31.6	-26.4	-14.4	-27.6

Appendix 3-3D. Isotope values of tree swallows nestlings.

					$\delta^{13}C$ (‰)								
Code	**Sample**	**Type**	**Habitat**	**Notes**	**Ala**	**Asp**	**Glu**	**Gly**	**Ile**	**Leu**	**Phe**	**Pro**	**Val**
Bo131	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	BR101 2013	-25.8	-21.2	-22.3	-18.6	-27.5	-34.2	-33.7	-20.3	-30.1
Bo132	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	BR204 2013	-25.0	-21.0	-20.6	-20.7	-26.8	-33.4	-32.7	-20.1	-31.9
Bo133	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	BR304 2013	-25.5	-20.7	-21.9	-20.6	-26.2	-33.1	-32.0	-19.2	-31.9
Bo134	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	BR404 2013	-25.3	-22.0	-22.1	-19.3	-27.0	-34.4	-33.9	-20.8	-31.3
BR102	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	BR201 2010	-27.4	-23.2	-22.3	-20.2	-25.9	-34.0	-32.4	-21.6	-31.5
BR111	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	BR102 2011	-27.1	-24.0	-21.6	-22.1	-26.1	-34.6	-30.4	-22.6	-32.4
BR112	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	BR206 2011	-26.7	-23.2	-21.9	-20.3	-26.0	-34.4	-30.5	-22.4	-31.4
BR113	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	BR304 2011	-27.0	-23.3	-20.2	-20.2	-25.5	-34.4	-29.5	-22.0	-31.3
BR114	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	BR402 2011	-27.7	-24.3	-23.8	-20.7	-25.9	-34.9	-30.1	-22.2	-31.3
BR122	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	BR204 2012	-26.5	-22.1	-19.9	-19.4	-25.1	-32.8	-30.9	-20.2	-28.9
BR123	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	BR305 2012	-25.3	-20.8	-19.9	-20.2	-25.2	-32.4	-31.1	-20.5	-29.2
BR124	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	BR407 2012	-24.4	-21.8	-19.7	-18.7	-24.9	-32.2	-30.1	-20.1	-28.9
BR141	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	BR103 2014	-26.5	-23.7	-20.7	-22.2	-25.8	-34.4	-26.4	-21.9	-31.3
BR141	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	BR104 2014	-24.8	-19.3	-26.2	-17.2	-26.6	-32.8	-32.5	-20.9	-29.2
BR142	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	BR107 2014	-23.8	-19.7	-24.3	-17.3	-24.9	-32.9	-30.1	-19.8	-29.4
BR143	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	BR310 2014	-26.5	-21.9	-21.2	-23.5	-26.95	-33.3	-32.6	-21.8	-30.6
BR144	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	BR404 2014	-27.3	-22.2	-26.1	-24.7	-25.9	-34.5	-30.3	-21.1	-30.8
BR144	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	BR408 2014	-26.7	-20.1	-21.9	-20.9	-26.6	-33.4	-32.1	-21.7	-30.0
SB101	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SB107 2010	-26.2	-25.8	-22.6	-18.0	-26.7	-33.5	-32.5	-21.6	-29.8
SB102	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SB209 2010	-27.2	-23.6	-21.7	-19.5	-27.3	-34.1	-32.5	-22.3	-31.7
SB103	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SB305 2010	-24.7	-24.3	-22.3	-17.4	-27.4	-33.1	-31.4	-21.2	-30.0
SB111	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SB107 2011	-28.1	-24.8	-23.8	-22.3	-26.6	-34.9	-31.3	-23.0	-32.2
SB112	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SB206 2011	-27.3	-23.8	-21.2	-22.8	-26.7	-32.9	-30.1	-22.7	-31.0
SB113	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SB303 2011	-27.8	-24.8	-24.0	-17.1	-27.9	-34.6	-32.2	-23.5	-32.2
SB114	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SB407 2011	-27.5	-24.7	-21.2	-23.0	-27.2	-34.1	-31.1	-22.8	-31.6
SB115	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SB501 2011	-28.4	-24.7	-21.8	-24.6	-27.8	-33.0	-30.1	-23.3	-32.1
SB121	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SB109 2012	-24.7	-23.3	-20.6	-18.4	-26.0	-32.0	-31.1	-21.1	-29.2
SB122	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SB206 2012	-23.5	-22.0	-20.3	-17.7	-25.7	-32.5	-30.3	-19.8	-29.3
SB123	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SB306 2012	-25.3	-21.9	-21.1	-16.4	-27.1	-34.3	-30.0	-21.7	-30.8
SB124	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SB400 2012	-23.1	-19.8	-19.1	-16.9	-25.1	-30.7	-29.3	-19.8	-28.2
SB125	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SB504 2012	-25.2	-20.4	-22.8	-17.5	-27.9	-33.0	-32.0	-19.8	-29.7
SB141	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SB109 2014	-24.1	-19.35	-20.9	-15.6	-24.85	-31.9	-29.45	-19.45	-28.6
SB142	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SB205 2014	-27.5	-23.7	-20.2	-22.1	-25.5	-32.9	-30.6	-21.1	-30.5
SB142	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SB209 2014	-24.8	-20.1	-21.0	-24.9	-26.2	-30.2	-30.8	-20.6	-34.2
SB143	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SB305 2014	-25.4	-21.2	-21.5	-18.9	-24.8	-31.8	-29.7	-20.0	-29.9
SB143	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SB308 2014	-26.8	-20.1	-22.3	-20.2	-27.1	-33.4	-32.3	-21.9	-30.4

SB144	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SB403 2014	-25.9	-22.7	-22.6	-19.6	-25.8	-33.3	-30.1	-20.3	-30.7
SB144	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SB405 2014	-24.5	-20.4	-20.6	-17.4	-25.3	-32.1	-30.0	-20.9	-29.0
SB145	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SB504 2014	-23.3	-20.4	-22.1	-16.7	-23.5	-31.1	-30.0	-18.3	-28.2
SB145	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SB508 2014	-25.2	-20.2	-20.9	-17.8	-25.9	-32.6	-30.3	-21.4	-29.4
SF102	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SF201 2010	-26.0	-22.4	-20.9	-19.7	-26.2	-33.1	-30.8	-20.9	-30.0
SF103	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SF304 2010	-25.9	-22.2	-20.9	-18.1	-26.5	-33.1	-30.2	-20.4	-29.8
SF104	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SF410 2010	-22.7	-21.6	-18.6	-18.1	-23.6	-31.0	-29.7	-19.3	-29.1
SF111	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SF101 2011	-28.8	-25.0	-21.6	-22.7	-28.6	-34.2	-33.5	-24.4	-32.4
SF112	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SF200 2011	-27.9	-24.9	-22.3	-17.9	-28.3	-34.1	-31.2	-23.9	-32.2
SF113	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SF303 2011	-28.8	-25.8	-23.1	-19.2	-28.8	-35.6	-32.4	-24.4	-33.7
SF114	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SF405 2011	-28.8	-25.3	-21.3	-22.7	-28.8	-34.5	-32.3	-24.5	-32.7
SF121	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SF101 2012	-26.1	-20.5	-20.8	-21.2	-26.4	-33.1	-31.6	-20.4	-31.5
SF122	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SF206 2012	-26.4	-20.7	-22.7	-19.6	-26.8	-33.1	-31.7	-19.8	-30.4
SF123	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SF300 2012	-25.0	-19.7	-20.6	-17.3	-26.5	-32.7	-31.0	-19.5	-30.4
SF124	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SF401 2012	-25.1	-19.3	-20.9	-17.4	-26.5	-32.5	-30.2	-19.8	-30.4
SF141	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SF107 2014	-25.4	-17.9	-21.1	-19.2	-26.6	-32.0	-32.3	-21.1	-31.4
SF141	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SF110 2014	-24.1	-19.5	-21.0	-16.6	-26.4	-32.8	-30.7	-21.1	-29.9
SF142	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SF201 2014	-26.5	-23.3	-22.0	-22.2	-27.0	-32.6	-32.6	-21.5	-31.7
SF142	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SF207 2014	-25.3	-20.3	-20.2	-19.2	-26.4	-33.7	-31.3	-22.0	-30.2
SF143	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SF301 2014	-28.8	-26.0	-24.1	-25.4	-28.2	-35.2	-35.6	-23.7	-33.2
SF143	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SF309 2014	-23.6	-19.0	-20.4	-15.5	-26.3	-33.3	-30.5	-21.5	-30.0
SF144	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SF404 2014	-27.5	-24.4	-22.4	-23.9	-27.3	-33.9	-34.0	-22.8	-32.5
SF144	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SF405 2014	-25.3	-21.0	-21.1	-18.7	-26.2	-33.8	-32.2	-22.6	-30.5
SF131	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SF105 2013	-25.1	-22.3	-24.2	-23.7	-25.7	-33.1	-33.9	-20.5	-32.9
SF132	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SF204 2013	-26.4	-22.6	-24.4	-19.6	-27.3	-32.9	-33.5	-20.3	-31.5
SF133	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SF304 2013	-23.8	-19.7	-18.7	-15.3	-25.9	-32.6	-31.7	-21.1	-28.5
SF134	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SF402 2013	-24.5	-20.9	-22.7	-18.9	-26.7	-33.7	-33.2	-20.2	-31.2
SB131	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SB109 2013	-26.5	-22.2	-24.1	-16.5	-29.2	-35.5	-35.7	-23.0	-32.5
SB132	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SB209 2013	-24.8	-20.8	-21.2	-23.3	-26.3	-31.5	-32.8	-19.2	-34.1
SB133	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SB310 2013	-25.0	-21.5	-22.1	-16.8	-26.8	-31.9	-33.4	-19.9	-31.0
SB134	Tree swallow (*Tachycineta bicolor*)	Nestbox grids	Unclassified	SB407 2013	-25.5	-21.9	-20.4	-16.8	-26.4	-31.5	-33.5	-20.3	-30.2
	Tree swallow, average				***-25.9***	***-22.0***	***-21.7***	***-19.7***	***-26.4***	***-33.2***	***-31.5***	***-21.2***	***-30.8***

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