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The interplay between trophic ecology, environmental variability, and an endangered marine species

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Biology

by

Elizabeth Dorsey Hetherington

Committee in charge:

Professor Carolyn Kurle, Chair
Professor Lihini Aluwihare
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Professor Mark Ohman
Dr. Jeffrey Seminoff

2018

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University of California San Diego

2018

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ACKNOWLEDGEMENTS

Many people have contributed to my success over the last five years and none of this would be possible without the tremendous amounts of help I've had from people in my professional and personal life. First and foremost, I would like to thank my committee, and particularly Carolyn Kurle for welcoming me into her lab and providing me with a space to grow, develop, and pursue the research questions that interested me most, Jeff Seminoff for being a constant reminder that being a good scientist and a good person aren't mutually exclusive. Thank you for being kind, encouraging, and helpful, especially when I needed it most. I'd also like to thank Kelly Stewart for providing me with the amazing opportunity to work at SPNWR and for being an encouraging and fantastic female role model. Thank you to Peter Dutton, Joel Schumacher, everyone at U.S. Fish and Wildlife Service, and those involved with the Sandy Point leatherback project.

Some of the best and most satisfying moments of my tenure at UCSD involved collaborations and interactions with the people I've encountered along the way. Brian Popp and Jeff Drazen, for being great collaborators, taking me to sea, and making me feel at home when I was in Hawaii. Natalie Wallgrove and everyone in the Isotope Geochemistry Laboratory at the University of Hawaii: you are all GCIRMS wizards and I'm deeply thankful for your guidance and help in fixing my silly mistakes. Many thanks to the resources and personnel at Scripps Institution of Oceanography's Pelagic Invertebrate Collection, especially Linsey Sala, for teaching me some zooplankton identification and many hours spent listening to podcasts and talking about zooplankton while working under the microscope. Thank you to Andrew Johnson for the endless banter and laughs in an otherwise humorless environment. Thank you to Kelton McMahon for being a mentor and more importantly, a friend.

Thank you to the Kurle Lab members, UCSD faculty, graduate students, and postdocs who have been a great support network over the years. In particular, I'd like to thank Kyle Begovich for morning runs, Stephanie Nehasil for joining the Kurle lab and contributing so much to my last two years at UCSD, and of course, Marika Schulhof, my work wife and partner in Scandy love, battling trolls, coffee spills and 1990s nostalgia. We survived. We did it, friend.

Thank you to my master's advisor, Bob Olson, whose mentorship provided me with the skills and confidence that I needed to pursue a PhD and continue a career in science.

An especially **huge** thank you to my family and friends, the people who consistently helped in picking me up and dusting me off after every fall, setback, or disappointment: Mom, Dad, the unstoppable super powers of D3, the Lacy and LaTorre clans, especially Tish, the duo docs Fran & Jaime, Nadwa Ibrahim, Matthew Alvo, Greg Alvarez, my Mission Beach Crew, triathlon community, and all of the inspirational, nasty women in my life.

Saving the best for last: to my husband Dan Lacy, I would trade my life with you for nothing in the world. You have my word, in writing, I am done with school this time.

Funding was provided by the University of California San Diego, Division of Biological Sciences, University of California San Diego Frontiers of Innovation Scholars Program; University of California San Diego Cellular, Molecular, and Genetics Training Program; National Science Foundation Integrated Training for Continental Ecology Program; Goeddel Endowed Award; Boyd N. Lyon Memorial Scholarship, and the Jean Marie Messier Memorial Endowment.

Chapter 1, in full, has been submitted and accepted for publication of the material as it may appear in Oecologia, 2018, Hetherington, ED, Seminoff, JA, Robison, L, Dutton, P, Popp, BN, and Kurle, CM. The dissertation author is the primary investigator and author of this paper.

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Hetherington, ED, Ohman, MD, Popp, BN, Kurle, CM. Effects of formalin and ethanol preservation on amino acid nitrogen isotope values of consumer tissues. *In prep for Rapid Communications in Mass Spectrometry.*

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ABSTRACT OF THE DISSERTATION

The interplay between trophic ecology, environmental variability, and an endangered marine species

by

Elizabeth Dorsey Hetherington

Doctor of Philosophy in Biology

University of California San Diego, 2018

Professor Carolyn Kurle, Chair

A grand challenge of the 21st century is to understand the response of ecosystems and populations of species to environmental variability and intensifying climate change. My dissertation focuses on the potential for changing environmental conditions to influence marine food webs, foraging ecology, and ultimately population success of consumers. I combined biogeochemical tools (stable isotope analyses) of zooplankton and endangered leatherback turtles with measures of oceanography and environmental conditions to evaluate changes in foraging ecology and food web dynamics over time. My research specifically focuses on long-term trends in the foraging ecology and habitat use of Atlantic and Pacific leatherback turtles and how environmental variability in the Pacific may alter food web dynamics in a critical foraging area

for a declining leatherback population.

My first two chapters were focused on leatherback turtles, a cosmopolitan species with populations inhabiting tropical and temperate regions throughout the global ocean. In Chapter 1, I examined the trophic ecology of North Atlantic leatherbacks over an eighteen-year period to test the hypothesis that shifts in foraging ecology or environmental conditions in the North Atlantic have contributed to leatherback population recovery. In Chapter 2, I focused on a subgroup of the critically endangered Western Pacific leatherback population that forages in the California Current. Here, I addressed questions about their diet, habitat use, and the trophic structure of leatherback prey in the California Current Large Marine Ecosystem (CCLME). These two chapters allowed me to better understand whether the continuing decline of Pacific leatherbacks was related to dietary differences potentially driven by variability in environmental conditions between ocean basins as the North Atlantic population of turtles are steadily increasing. In Chapter 3, I investigated ecosystem responses to a multi-year, warm water anomaly (a marine heatwave and strong El Niño event) in the CCLME, which is a productive upwelling system that supports the biomass of many commercially and ecologically important species, including the leatherback population that Chapter 2 focused on. My findings illustrate mechanisms through which the amount of energy transferred to higher trophic level consumers is altered by environmental variability in the CCLME. In my first three chapters, I used stable isotope analyses, which can be a valuable tool for reconstructing patterns of trophic or foraging ecology over time. However, archived tissues that are used for analyses are often stored in chemical preservatives, which may affect their potential for use in isotope ecology. In Chapter 4, I conducted laboratory experiments to test the effects of common chemical preservatives on stable isotope values to better understand how we can best use preserved and archived tissues in

future studies.

My research provides insight into the trophic ecology and habitat use of an endangered marine consumer. Although I found no differences in trophic position between leatherback conspecifics, environmental conditions in the North Atlantic may have contributed to the recent increases in this population. My research elucidates the effects of a strong environmental perturbation on the California Current food web, which is a productive upwelling region used by many commercially important and protected species. This work provides trophic position estimates for two leatherback populations, several gelatinous zooplankton species, and calanoid copepods in the California Current, which can be incorporated into future ecosystem or habitat models and used for ecosystem-based management of marine resources. Furthermore, my results contribute to our understanding of temporal trends in foraging ecology and food web responses to environmental variability and anomalous warming, which is useful for predicting ecosystem responses to future climate change scenarios.

INTRODUCTION

Within ecosystems, food webs are used to represent dynamic collections of predator-prey interactions and provide a conceptual framework for understanding feeding relationships among species (Paine 1980, Polis & Strong 1996). Obtaining information about trophic, or predator-prey, interactions provides a better understanding of ecosystem structure and function, as it illustrates how energy flows through a system. In a food web, trophic transfer efficiency is generally low, where large amounts of energy are lost from the system when energy from one trophic level is transferred to the next trophic level through predator-prey interactions. The biomass of ecologically and commercially important top predators is therefore regulated by the amount of primary production fueling the base of the food web, and any changes to primary production can impact the ecosystem as a whole (Pauly & Palomares 2005, Aburto et al. 2012).

In marine ecosystems, food web structure is typically sized-based, where predator-prey relationships are size-specific, and prey mass increases with increasing predator mass (Barnes et al. 2010). Phytoplankton, which are the primary producers in aquatic ecosystems, exhibit a wide-range of sizes, where larger phytoplankton (e.g., diatoms) often dominate in eutrophic conditions, and smaller phytoplankton (e.g., cyanobacteria) may dominate in oligotrophic conditions, as they are better able to utilize resources in nutrient-poor environments (Irwin et al. 2006, Uitz et al. 2010). Differences in the size structure of phytoplankton communities can affect the number of trophic transfers in a food web, where food webs dominated by smaller phytoplankton can have 2-3 additional trophic steps compared with food webs dominated by larger phytoplankton (Dickman et al. 2008, Barnes et al. 2010, Young et al. 2015). Since energy is lost with each trophic transfer, longer food chains reduce the amount of production or biomass that is available to support populations of consumers, compared with shorter food webs (Barnes

et al. 2010, Barnes et al. 2011, Young et al. 2015). Thus, the size structure of phytoplankton and zooplankton can impact the energy flow through ecosystems and ultimately, the amount of production available to populations of higher trophic level species.

Within food webs, top predators and high trophic level species are particularly important, as they serve critical ecosystem functions (e.g., regulating populations of lower trophic level species) and are of incredible conservation and economic interest (Myers and Worm 2003, Baum and Worm 2009, Estes et al. 2011). Environmental variability, escalating climate change, and fishing pressure threaten to destabilize or change predator-prey relationships and overall food web structure in marine ecosystems. To predict the potential for their alteration with continued and future disturbances, it is essential to trace trophic interactions and energy flow through food webs. Environmental fluctuations, such as those associated with low frequency ocean-atmosphere oscillations, can cause significant changes in marine primary production by altering nutrient availability, which propagates through the food web, ultimately affecting top predators (Paul 2002, Stenseth et al. 2002, Polovina et al. 2008, Boyce et al. 2010, Lo-Yat et al. 2011). In addition to natural climatic fluctuations, the effects of rapidly increasing atmospheric carbon dioxide concentrations from anthropogenic climate change have direct physical impacts on marine ecosystems, including: shifts in sea-surface temperature, ocean circulation patterns, biogeochemical cycling, stratification, and increased ocean deoxygenation (Polovina et al. 2008, Doney et al. 2011). These physical changes can have widespread effects on biological processes, marine communities, and species interactions (Polovina et al. 2008, Hoegh-Guldberg & Bruno 2010, Doney et al. 2011, Hazen et al. 2013). Identifying the potential effects of natural environmental variability and anthropogenic climate change on predator-prey relationships is not only significant for our understanding of marine community ecology, but also provides insight

into ecosystem health and function that is essential for the proper management of marine resources.

We know with certainty that populations of protected species like marine mammals, seabirds, and turtles have been severely impacted by a number of anthropogenic threats (Wallace et al. 2011, Davidson et al. 2012) and large pelagic species (e.g., tunas, billfishes, and sharks) have been heavily reduced by large scale fishery removals (Myers & Worm 2003, Sibert et al. 2006, Ferretti et al. 2010). Managing the populations of these top predators poses many challenges, as these species often migrate through multiple jurisdictions, have cryptic life histories, and rely on distinct resources to support their populations.

Effective conservation and management of threatened species requires a firm understanding of their trophic ecology, ecological niches, and their interactions with other members in the food web, particularly as imminent climate change threatens to change their habitats and availability of resources critical to their survival (Hazen et al. 2013, McClatchie et al. 2016). It is therefore essential to understand how both natural and anthropogenic climate variability can alter marine food web structure and the trophic ecology of marine species. My dissertation research addresses questions about variation in trophic dynamics of consumers over time and how environmental variability may affect consumer-resource interactions and food web dynamics. My dissertation largely focuses on these themes with an emphasis on the long-term trends in the foraging ecology of threatened leatherback turtles (*Dermochelys coraciaea*) in two ocean basins, and the potential changes to food web structure during environmental perturbations. The conservation implications of this work provide insight into how variation in food web structure and function affect species of concern, such as leatherback turtles, as well as marine consumers in general.

Leatherback Turtles and Environmental variability

Leatherback turtles merit special consideration for evaluating the effects of environmental variability on trophic and foraging ecology, as previous research has suggested that differences in environmental conditions between leatherback foraging areas may contribute to a population dichotomy between leatherback conspecifics. Leatherback turtles are a single species with several populations throughout the global ocean, however, the International Union for Conservation of Nature (IUCN) conservation status differs among leatherback populations. For example, there are two leatherback populations in the Pacific Ocean, the declining eastern Pacific population, which is in critical danger of extinction (Wallace et al. 2006, Wallace & Saba 2009), and the Western Pacific population, which has exhibited a long-term nesting decline of 5.9% per year since 1984 (Tapilatu et al. 2013). In contrast, the North Atlantic population has been steadily increasing for several decades (Dutton et al. 2005, Stewart et al. 2013).

Nesting beach protection and fisheries regulations (Dutton et al. 2005) have undoubtedly contributed to the increase of North Atlantic leatherbacks. However, previous research suggests that the differences in population status between Pacific and Atlantic leatherbacks are due to a combination of anthropogenic and environmental factors (Wallace & Saba 2009, Wallace et al. 2014). Anthropogenic effects on Pacific leatherbacks are well-documented and include egg poaching, turtle harvesting, plastic pollution, light pollution, coastal development, and fisheries bycatch. Environmental factors, however, have been more difficult to directly link to population declines, although research on loggerhead turtles demonstrates that their population trends are primarily driven by factors such as oceanic productivity and hatchling production, not anthropogenic mortality (Van Houtan & Halley 2011). The extent to which environmental

variability affects leatherbacks is unclear, although differences in oceanic conditions, climate-driven variability, and food supply may contribute to the disparity in leatherback population trajectories between oceans.

Leatherbacks are a highly migratory species, using neritic and pelagic habitats in tropical, subtropical, and temperate ecosystems throughout the global ocean. They migrate from their tropical nesting beaches to distinct temperate foraging regions, presumably to feed on abundant prey (gelatinous zooplankton). Leatherbacks require a specific amount of stored energy to successfully reproduce and the eastern Pacific population requires less energy to reproduce than their North Atlantic counterparts, although they reproduce less frequently (Price et al. 2005, Tapilatu et al. 2013). The average time between nesting years (remigration intervals) for the Eastern Pacific population is longer than that for North Atlantic turtles (3.7 vs. 2 to 3 years), with no resultant increase in reproductive output or greater growth rate for Eastern Pacific turtles (Wallace et al. 2006, Wallace and Saba 2009). It is therefore possible that differences in environmental conditions between leatherback foraging areas affects the abundance and distribution of gelatinous zooplankton prey, which influences leatherback food availability and trophic ecology, and ultimately contributes to their population productivity. Therefore, the Pacific leatherbacks may be more vulnerable to population declines due to higher environmental variability and overall more variability in prey abundances in their foraging areas (Saba et al. 2009).

In the Pacific, the El Niño-Southern Oscillation (ENSO) affects climate variability on an interannual (every 2-7 years) timescale with profound worldwide effects on marine and terrestrial systems. Biological disturbances in the eastern Pacific Ocean associated with the positive phase of ENSO (El Niño) include warmer water and reduced upwelled nutrients which

leads to lower phytoplankton production, and changes in abundance and reproductive success of many fish, seabird, and marine mammal populations (Barber & Chavez 1983, Fiedler et al. 1986, Barber et al. 1996, Chavez et al. 2002, McClatchie & Jahnckenull 2016, Lindegren et al. 2018).

In the Eastern Pacific, a reduction in primary production during ENSO events may affect the food web on a frequent enough basis and reduce prey availability to such a degree that leatherbacks foraging in this region are unable to acquire the energy needed for reproduction, thereby lengthening their remigration intervals and affecting their life history and population dynamics (Saba et al. 2007, Saba et al. 2008, Wallace & Saba 2009). Overall lower primary production in foraging areas of the Pacific compared to the Atlantic coupled with further decreases in primary production associated with El Niño events may limit prey availability and inhibit leatherback reproduction in the Pacific (Wallace et al. 2006, Saba et al. 2008, Wallace and Saba 2009). In addition, North Atlantic foraging areas may provide a more stable prey supply for leatherbacks due to overall higher primary production and less interannual variability in sea-surface temperature compared to the Pacific. Therefore, one hypothesis is that greater stability in prey availability in the North Atlantic Ocean has contributed to leatherback population resilience observed for leatherbacks foraging in the North Atlantic (Saba et al. 2008, Wallace and Saba 2009).

No study has specifically linked a decrease in foraging success or reproductive output of leatherback turtles foraging in the eastern Pacific with ENSO events, but given the well-documented ecological effects of ENSO on marine food webs, it is a potential driver of their population productivity. A better understanding of how ocean-atmosphere phenomena influence marine food web structure, trophic interactions, and population dynamics of higher trophic level predators is essential for developing predictive models of marine ecosystem responses to global

change. My research will specifically focus on two leatherback populations, the North Atlantic and a subset of the Western Pacific that forage in the California Current Large Marine Ecosystem (CCLME). My research also provides insight into how food webs are affected or influenced by environmental perturbations in the Pacific by specifically focusing on a productive marine ecosystem that is used by leatherbacks. I used biogeochemical tracer techniques (stable isotope analyses) to answer questions about leatherback foraging ecology and the effects of environmental perturbations on food web dynamics in a leatherback foraging region in the CCLME.

Stable Isotope Analysis in Food Web Studies

Historically, the characterization of trophic ecology has been based on direct observations and analyses of stomach contents and fecal matter from consumers. These methods are critically important, as they allow for the taxonomical identification of consumers' prey. However, these techniques are also limited, as they provide only a snapshot of a consumer's most recent meal and therefore can be biased (Olson & Boggs 1986, Dellinger & Trillmich 1988). Furthermore, soft-bodied organisms are typically not preserved in stomach contents or scats, making this approach difficult for organisms that consume gelatinous prey, like leatherback turtles. To avoid such shortcomings, and for a non-invasive approach to evaluating trophic ecology, stable nitrogen (δ^{15}N) and carbon (δ^{13}C) isotope ratios from marine organisms have been used for elucidating animal diets and trophic structure (DeNiro & Epstein 1977, Deniro & Epstein 1981, B J Peterson & Fry 1987, Hobson 1999). Specifically, the δ^{15}N values of consumer tissues can be used to estimate the trophic placement of organisms in a food web, as they are predictably higher than δ^{15}N values of its prey (Deniro & Epstein 1981, Hobson et al. 1999). δ^{13}C values are

typically used to distinguish between carbon sources that may be contributing to the diets of consumers. Stable isotope values therefore reflect consumer diets integrated over certain time periods, which are dependent upon the speed of protein turnover specific to different tissue types (Hobson 1999, Kurle 2009).

Although stable isotope analysis of bulk tissue from consumers is a useful tool for evaluating food web structure and interactions, there are several limitations to this method. Most notably, estimating trophic positions (TPs) of consumers using bulk δ^{15}N values requires both the δ^{15}N measurement of a consumer's tissue and the δ^{15}N measurement from the base of the food web (i.e., phytoplankton). This can be problematic, as δ^{15}N values at the base of the food web can exhibit substantial spatial and temporal variability due the relative influences of different nitrogen biogeochemical cycling processes (Cline & Kaplan 1975, Gruber & Sarmiento 1997, Altabet 2001, Deutsch et al. 2001, Voss et al. 2001).

Understanding the relative influences of biogeochemical cycling processes is therefore crucial for the interpretation of δ^{15}N values in animal tissues and for its reliable use in food web ecology. The dominant nitrogen cycling processes in the ocean are nitrogen fixation, which adds nitrogen to the ocean, and denitrification, a sink that removes nitrogen (Cline & Kaplan 1975, Gruber & Sarmiento 1997, Deutsch et al. 2001). These processes, along with the isotopic fractionation associated with phytoplankton utilization of nitrate, influence the δ^{15}N values at the base of food webs, which in turn are reflected in the δ^{15}N values of consumer's tissues (Voss et al. 2001, Montoya et al. 2002, Popp et al. 2007). In regions with high rates of denitrification, preferential retention of ^{14}N and excretion of ^{15}N by microbes leaves the residual nitrate pool enriched in ^{15}N, thereby increasing the δ^{15}N values of the organisms using this pool (Cline and Kaplan 1975, Liu and Kaplan 1989, Voss et al. 2001). Conversely, atmospheric nitrogen has a

$\delta^{15}N$ value of 0 ‰, so the nitrate pool in areas dominated by N_2 fixation have $\delta^{15}N$ values close to zero (Gruber & Sarmiento 1997, Deutsch et al. 2001, Montoya et al. 2002).

One way to circumvent the need to measure the $\delta^{15}N$ values from consumer tissues and measurements of $\delta^{15}N$ values from the base of the food web, is to use a more recently-developed approach, compound specific isotope analysis of amino acids (CSIA-AA). This approach for evaluating food web dynamics and TPs of consumers can overcome limitations of bulk isotope analysis and allow one to differentiate between changes in consumer TP *versus* isotope values that reflect changes in nitrogen cycling at the base of the food web. With CSIA-AA, we can analyze the $\delta^{15}N$ values of specific amino acids in a consumer's tissue, as different amino acids provide more specific information than analyzing the bulk tissue alone. Some amino acids (e.g., alanine, glutamic acid) are isotopically fractionated during transamination and cause a consumer's tissue to become enriched in ^{15}N relative to its prey. These are called 'trophic' amino acids, as they reflect the diet of the consumer (Chikaraishi et al. 2007, Chikaraishi et al. 2009). Conversely, 'source' amino acid (e.g., phenylalanine, lysine) show little isotopic fractionation as their metabolism does not cleave or form nitrogen bonds. Thus, source amino acids reflect the nitrogen isotope composition at the base of the food web (Chikaraishi et al. 2009), which is governed by the dominant nitrogen transformation or cycling processes in the region (e.g. nitrogen fixation, denitrification). Indeed, CSIA-AA has been used to successfully evaluate marine consumer TP and track nitrogen dynamics in marine systems. More recently, CSIA-AA has been used to measure regime shifts or disturbances associated with environmental variability (Sherwood et al. 2011, Décima et al. 2013, McMahon et al. 2015), which underscores its use as a tool for addressing my research objectives.

My dissertation research uses tissue samples from a highly migratory species over two decades, and thus, CSIA-AA is a useful approach for differentiating between sources of variability in bulk δ^{15}N values over time. I use CSIA-AA on leatherback and copepod tissues to quantify consumer TP (estimated, in essence, by calculating the difference in δ^{15}N values between trophic and source amino acids) and evaluate changes in the nitrogen isotope composition at the base of the food web, as reflected by the δ^{15}N values from source amino acids (Chikaraishi et al. 2007, Popp et al. 2007). These data allow me to determine if variation in bulk δ^{15}N values from consumers are due to true trophic changes or if they reflect biogeochemical changes that occurred at the base of the food web.

My research not only uses CSIA-AA to estimate TPs and evaluate changes in food web structure associated with environmental variability, but also contributes to the application of CSIA-AA in ecological studies, as I examine the effects of common chemical preservation methods on bulk and amino acid δ^{15}N values. This is particularly relevant for future studies, as many researchers aim to reconstruct past food webs or evaluate long-term changes in food web dynamics using previously-collected samples or museum specimens that are stored in chemical preservatives.

Chapter summaries

I use isotope analyses and demographic and oceanographic data to evaluate food web responses to environmental variability, understand the long-term trophic and foraging ecology of leatherback turtles, and identify how changes in food web dynamics might affect their populations. My dissertation research builds on previous hypotheses that suggest environmental variability has contributed to the population dichotomy between Pacific and Atlantic leatherback

turtles. Here, I aim to characterize mechanistic drivers of food web changes during environmental perturbations like ENSO events and evaluate long-term changes in the foraging ecology leatherback turtles.

In **Chapter One**, I ask if the foraging ecology of North Atlantic leatherback turtles changes from 1992-2010 and whether this could provide insight into their recent population increase. I addressed this knowledge gap by using nitrogen stable isotope analyses from blood samples collected from nesting leatherback turtles over eighteen years (1992-2010) on St. Croix, U.S. Virgin Islands. I show that although the nitrogen isotope values decreased over time, there were no changes in leatherback TP over my sampling period. Isotope data indicated that the North Atlantic leatherback population use multiple foraging areas prior to nesting, although individuals seem to exhibit foraging area fidelity. There were significant relationships between the North Atlantic Oscillation Index, which is a broad-scale indicator of sea level temperature and sea level pressure in the North Atlantic Ocean, and leatherback nesting parameters (remigration intervals and clutch production), which may suggest that environmental conditions in the North Atlantic were beneficial to leatherbacks and contributed to their population increase over our sampling period.

In **Chapter Two**, I ask about the diet and foraging ecology of Western Pacific leatherbacks and their gelatinous prey in the California Current. I used isotope analyses on tissues collected from leatherbacks over thirteen years (2003-2016) and their potential prey (gelatinous zooplankton) in a known Western Pacific leatherback foraging area. There was no change in leatherback $\delta^{15}N$ or TP over time. However, leatherback $\delta^{13}C$ values were higher in 2005 compared to other years, which indicates a shift in habitat use coinciding with anomalous environmental conditions. I also used isotope mixing models and a Bayesian technique to

estimate TPs of leatherbacks and their prey. My results indicate that leatherbacks are primarily consuming *Chrysaora* scyphozoans but filter-feeding organisms (salps and pyrosomes) may also comprise a substantial portion of their diets. Trophic position estimates were lowest for filter-feeding salps and highest for leatherbacks and *Phacellophora* scyphozoans, while *Aurelia* and *Chrysaora* scyphozoans had intermediate TPs and exhibited trophic/niche overlap.

In **Chapter Three**, I ask how an anomalous, multi-year warming event (i.e. a marine heatwave followed by strong El Niño event) in the North Pacific affected oceanographic conditions, trophic ecology of copepods, and food web structure in the CCLME. To address this question, I evaluated environmental parameters (sea-surface temperature, chlorophyll *a*, upwelling intensity, nutrient concentrations) and nitrogen isotope values of an abundant zooplankton consumer, *Calanus pacificus* to evaluate differences in TP and potential food web length associated with the marine heatwave and strong El Niño event compared to a neutral period. I found differences in certain environmental variables, with increases in temperature and decreases in upwelling intensity, nutrients, and chlorophyll *a* concentrations during both warm water events compared to the neutral period. There was high variability in copepod δ^{15}N values, although they were significantly higher during the warm period, particularly in the spring samples, and source amino acid δ^{15}N values closely mirrored patterns in bulk δ^{15}N values. Trophic positions were relatively consistent over my sampling period, which indicates that food web length was not different during the anomalous warm conditions, and nitrogen cycling biogeochemistry was driving the observed changes in bulk δ^{15}N values.

In **Chapter Four**, I ask how storing tissues in common chemical preservatives (ethanol and formaldehyde) can alter carbon and nitrogen stable isotope values. To address this question, I preserved tuna and squid muscle in ethanol and formaldehyde for up to two years and

compared their isotope values with those from frozen specimens. Additionally, I analyzed copepods that were either frozen or stored in formaldehyde for 25 years. Overall, my findings are promising for δ^{15}N values, as they were minimally altered by preservation. Amino acid δ^{15}N values were also minimally altered by chemical preservation, although valine and phenylalanine values were changed by > 1‰, which warrants future investigation. The results for δ^{13}C suggest some pause for caution, as I found that δ^{13}C values for all species were inconsistently altered by chemical preservation, which is important for future studies that aim to analyze δ^{13}C values of preserved tissues.

Overall my research, a) contributes to our understanding of long-term leatherback foraging ecology for two populations, b) provides insight into pelagic food web responses to low frequency climate fluctuations such as ENSO events, and c) contributes to the further development of CSIA-AA and its reliability for addressing ecological questions. Integrating oceanographic data with isotope analyses and leatherback demographic data allowed me to empirically detect mechanisms driving variability at the base of the food web and determine if this variability affects leatherback turtle foraging dynamics and population trajectories. My work is a highly collaborative effort that uses several long-term, archived tissue sample collections, and provides a more complete oceanographic and ecological picture for better leatherback management and conservation in both ocean basins.

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CHAPTER 1

Long-term trends in the foraging ecology and habitat use of an endangered species: an isotopic perspective

Abstract

Evaluating long-term drivers of foraging ecology and population productivity is crucial for predicting species responses under future climate change scenarios. Here, we examine the trophic ecology and habitat use of North Atlantic leatherback turtles (St. Croix nesting population), and investigate the effects of large-scale oceanographic conditions on leatherback foraging dynamics. We used bulk and compound-specific nitrogen isotope analysis of amino acids (CSIA-AA) to estimate leatherback trophic position (TP) over eighteen years, compare these estimates with TP estimates from a Pacific leatherback population, and elucidate the pre-nesting habitat use patterns of leatherbacks. Our secondary objective was to use oceanographic indices and nesting information from St. Croix leatherbacks to evaluate relationships between trophic ecology, nesting parameters, and regional environmental conditions measured by the North Atlantic Oscillation (NAO). We found no change in leatherback TP over time and no difference in TP between Atlantic and Pacific leatherbacks, indicating that differences in trophic ecology between populations is an unlikely driver of the population dichotomy between Pacific and Atlantic leatherbacks. Isotope data suggested that St. Croix leatherbacks inhabit multiple oceanic regions prior to nesting, although, like their conspecifics in the Pacific, individuals exhibit fidelity to specific foraging regions. Leatherback nesting parameters were weakly related to the NAO, which may suggest that positive NAO phases benefit St. Croix leatherbacks, potentially through increases in resource availability in their foraging areas. Our data contribute to the understanding of leatherback turtle ecology and potential mechanistic drivers of the dichotomy between populations of a protected species.

Introduction

Physical and environmental impacts associated with climate change are altering biological processes and species interactions within marine ecosystems. Additionally, populations of protected species like marine mammals and turtles have been severely impacted by an array of anthropogenic threats (Davidson et al. 2012), and predicting how these species will be further affected by intensifying global climate change requires an understanding of long-term environmental variability and its impact on ecological factors, such as foraging ecology and habitat use. Addressing these types of questions poses a great challenge, particularly in marine systems where species often migrate thousands of miles and use multiple oceanic habitats over the course of their lifetimes.

The leatherback turtle (*Dermochelys coriacea*) is a threatened, highly migratory species that inhabits neritic and pelagic habitats and is divided into several populations throughout the global ocean, some of which differ in life history traits and population trajectories. For example, North Atlantic leatherbacks are larger than their Pacific counterparts (Wallace et al. 2006), have shorter remigration intervals (number of years between nesting events), and an overall higher reproductive output (Wallace et al. 2006). Additionally, the North Atlantic leatherback population is steadily increasing (Wallace et al. 2013) and listed as vulnerable (www.iucnredlist.org), whereas Pacific leatherbacks are precipitously declining and listed as critically endangered.

The population increase of North Atlantic leatherbacks in recent decades can be partially attributed to nesting beach protection and fisheries regulations (Dutton et al. 2005). However, environmental variability and differences in foraging ecology, habitat use, and regional oceanographic conditions may contribute to the dichotomy between Pacific and Atlantic

leatherback population trajectories (Wallace et al. 2006; Saba et al. 2008; Wallace and Saba 2009). Eastern Pacific leatherbacks generally forage in areas with lower primary production than their North Atlantic conspecifics, and periodic El Niño-Southern Oscillation events may further limit the ability of leatherbacks to acquire the nutrients required for reproduction in the eastern Pacific Ocean (Wallace et al. 2006; Saba et al. 2008; Wallace and Saba 2009).

Our study focuses on leatherbacks in the North Atlantic Ocean, where large-scale temporal variability in oceanographic conditions is often explained by patterns associated with the North Atlantic Oscillation (NAO) and the Atlantic Multidecadal Oscillation (AMO), which are ocean-atmosphere phenomena driven by changes in sea level pressure and sea-surface temperature in the North Atlantic Ocean (Hurrell et al. 2001; Otterson et al. 2001; Stensenth et al. 2003). The NAO and AMO fluctuate on decadal or multi-decadal time scales, which may provide more stable interannual foraging conditions for leatherbacks in the North Atlantic compared to Pacific foraging areas. Thus, leatherbacks in the North Atlantic may have access to more stable nutrient supplies compared with their conspecifics in the Pacific, thereby contributing to the greater resilience of their population (Saba et al. 2008; Wallace and Saba 2009).

On the individual level, leatherbacks likely exhibit broad foraging area fidelity and consistently migrate to the same foraging areas between nesting seasons (James et al. 2005; Hays et al. 2006). However, data on habitat use patterns are sparse and largely dependent on a limited number of satellite tracks, incidental fisheries catch data, and direct observations of leatherbacks in their foraging grounds (James et al. 2005; Hays et al. 2006; Fossette et al. 2010). Satellite telemetry data only capture a portion of their time away from nesting beaches, as leatherback remigration intervals are typically 2–3 years but can be longer (Dutton et al. 2005; Wallace et al.

2006). Thus, we have major gaps in our understanding of leatherback habitat use and foraging ecology, and how these factors are influenced by broad-scale oceanographic conditions.

Acquiring information about highly migratory species presents many challenges, and the development of biochemical tracer techniques have provided new approaches to answering essential ecological questions about these species. For example, the analysis of nitrogen stable isotope ratios (i.e., $^{15}N/^{14}N$ expressed as $\delta^{15}N$ values) from animal tissues is frequently used to evaluate species' trophic ecology and more recently has been used to determine important foraging areas of highly migratory species (Madigan et al. 2014; Vander Zanden et al. 2015; Turner Tomaszewicz et al. 2017). The $\delta^{15}N$ values of whole tissues (i.e., bulk isotope analysis; $\delta^{15}N_{bulk}$) provide minimally invasive, time-integrated information about a consumer's diet and location. Thus, $\delta^{15}N$ values from nesting leatherbacks can provide data on diet and habitat use for several months prior and up to sample collection, which may elucidate their pre-nesting habitat use and migration patterns (e.g., Seminoff et al. 2012).

Although $\delta^{15}N$ values from bulk tissues ($\delta^{15}N_{bulk}$) are a useful tool for evaluating food web dynamics and interactions, there are several limitations to this analysis. Most notably, estimating trophic positions (TPs) of consumers using $\delta^{15}N$ values requires, in addition to the $\delta^{15}N$ measurement of a consumer's tissue, a $\delta^{15}N$ measurement from the base of the food web (i.e., phytoplankton). This can be problematic, as $\delta^{15}N$ values at the base of the food web vary spatially and temporally (Somes et al. 2010; McMahon et al. 2013).

A newer approach, compound-specific isotope analysis of amino acids (CSIA-AA) offers potential solutions to limitations of bulk isotope analysis. The CSIA-AA technique relies on the determination of $\delta^{15}N$ values of individual amino acids within a consumer's tissue, as different amino acids can provide more information than analyzing the isotope values from bulk tissue

alone. Certain amino acids (e.g., glutamic acid) exhibit isotopic fractionation during transamination and deamination, thereby causing a consumer's tissue to become enriched in ^{15}N relative to its prey (Popp et al. 2007; Chikaraishi et al. 2009). These are called 'trophic' amino acids and they reflect the diet of the consumer (McClelland and Montoya 2002; Popp et al. 2007, Chikaraishi et al. 2007). Conversely, 'source' amino acid (e.g., phenylalanine) show little isotopic fractionation as their primary metabolic pathways do not cleave or form nitrogen bonds (McClelland and Montoya 2002; Popp et al. 2007; Chikaraishi et al. 2007, 2009). Thus, source amino acids reflect δ^{15}N values at the base of the food web (Chikaraishi et al. 2009), so we can account for baseline δ^{15}N variability and estimate TPs in the tissues of consumer species without additional sampling of the base of the food web.

CSIA-AA has become an increasingly-used approach for quantifying isotope values at the base of the food web and estimating TPs of consumers, although recent studies highlight its limitations. Specifically, the equations used to estimate TP contain a constant for the trophic discrimination factor (TDF), which represents the difference in the δ^{15}N values of specific amino acids between prey and consumer per trophic step. There is growing evidence that TDFs can vary among consumers (McMahon and McCarthy 2016; Fuller and Petzke 2017; O'Connell 2017), and obtaining appropriate TDF estimates is essential for accurately quantifying TPs using this approach. Despite this limitation, CSIA-AA is a useful tool for our study, as our primary objective was not to estimate an "absolute" TP for North Atlantic leatherbacks. Rather, we aimed to evaluate relative changes in TP over time and compare TP estimates between leatherback populations.

The aim of our study was to evaluate potential changes in the trophic ecology and habitat use patterns of North Atlantic leatherback turtles and how these patterns relate to large-scale

oceanographic oscillations. We analyzed the $\delta^{15}N_{bulk}$ and individual amino acid $\delta^{15}N$ values from eighteen years of archived blood samples from the North Atlantic leatherback population. Specifically, for this nesting population of leatherbacks, we evaluated 1) temporal variability in their foraging ecology, 2) potential differences in TP compared to Pacific populations, 3) the utility of isotope analyses to estimate their pre-nesting foraging location and migration patterns, and 4) the relationship between coarse-scale oceanographic conditions and leatherback trophic ecology and nesting history. Our results, therefore, provide insights into differences between Pacific and Atlantic leatherback populations and potential environmental processes that may influence leatherback population trajectories.

Materials and Methods

Sample Collection

North Atlantic leatherbacks are considered one population with several subgroups that nest throughout the wider Caribbean. We used whole blood samples that were collected between 1992-2010 from adult females nesting at Sandy Point National Wildlife Refuge, St. Croix, U.S.V.I. This nesting population has been closely monitored since 1981, represents one group within the North Atlantic and can be used as a proxy for the Northern Caribbean leatherback population, (Dutton et al. 2013). We used whole blood samples, which were collected between 1992-2000 from adult females (See Appendix).

Due to the opportunistic nature of our sampling, the number of leatherback blood samples available for stable nitrogen isotope analysis varied per year (Table S1). This resulted in an unbalanced design, where we analyzed 201 blood samples from 171 leatherbacks over eighteen years, including a two-year (1995-1996) gap where no samples were collected. We included

blood from a subset of 21 turtles that were sampled during multiple nesting seasons, as we were particularly interested in evaluating isotopic variability of individual leatherbacks over time. Of this subset of samples, 19 of 21 turtles were sampled during two nesting years and two turtles were sampled during three nesting years. The number of years between sampling intervals varied.

Compared with bulk tissue analyses, CSIA-AA is a labor intensive and expensive technique, so we selected a subset of 25 samples for this analysis based on variations we observed in $\delta^{15}N_{bulk}$ values. We chose five sampling years (1993, 1999, 2000, 2005, 2010) and analyzed multiple samples (n = 5) from those years that encompassed the range of $\delta^{15}N_{bulk}$ values we observed. We also included samples from 5 additional individuals that were sampled during multiple nesting seasons to evaluate changes in diet and TP of single individuals over time.

The U.S. Fish and Wildlife Service provided nesting histories for turtles, when data were available. Nesting information was then used to calculate the number of clutches laid (clutch productivity) and the remigration intervals for individuals from which we collected blood for isotope analyses (see Appendix).

Isotopic Analyses

We freeze-dried, homogenized, and weighed whole blood into tin capsules for bulk isotope analysis. For quality control, we analyzed a set of reference materials with known $\delta^{15}N$ values and all reference materials were within ± 0.1 ‰ of their calibrated values. We analyzed the subset of samples selected for CSIA-AA at the University of Hawaii’s Stable Isotope Biogeochemistry Laboratories. The CSIA-AA samples were analyzed in triplicate, corrected the

δ^{15}N values to internal reference compounds. The analytical errors for amino acid δ^{15}N values were largely under 1.0 ‰, but ranged from 0.03 – 1.46 ‰, and averaged 0.38 ‰ (see Appendix).

Trophic Position Estimates

We estimated TP using several approaches (see Appendix), as recent studies highlight the uncertainties associated with TPs derived from amino acid δ^{15}N values. We used three variations of the following equation:

$$TP_{Trophic-Source} = \frac{(\delta^{15}N_{Trophic} - \delta^{15}N_{Source}) - \beta}{TDF} + 1 \quad (1)$$

where $TP_{Trophic\text{-}Source}$ is the TP based on the difference in mean δ^{15}N values from the trophic and source amino acids, TDF is the trophic discrimination factor (the ^{15}N enrichment of trophic relative to source amino acids per trophic step), and β represents $\delta^{15}N_{Trophic}$- $\delta^{15}N_{Source}$ in primary producers. Since there are no TDF estimates for leatherback turtles, we used values vetted in the literature and derived from meta-analyses (see Appendix).

Additionally, we applied a novel Bayesian approach to estimate TP, using the ‘tRophicposition’ package in the statistical software R (Quezada-Romegialli et al. 2018). This approach couples Markov Chain Monte Carlo Simulations with stable isotope data to estimate TP, and we adapted the model to estimate TPs using amino acid δ^{15}N values (see Appendix).

Ocean-atmosphere indices

In the North Atlantic Ocean, the NAO and AMO affect the strength of trade winds, mixed-layer depth, and nutrient supply to the euphotic zone (Hurrell et al. 2001; Stensenth et al. 2003). Therefore, indices of the NAO and AMO can be used as indicators of broad-scale oceanographic conditions, reflecting the supply and nitrogen isotopic composition of nutrients.

We obtained standardized and unsmoothed monthly AMO values from NOAA's Earth Systems Research Laboratory (http://www.esrl.noaa.gov). NAO values were from NOAA's climate prediction Center (http://www.cpc.ncep.noaa.gov/), where values were unsmoothed, and standardized by the 1981-2010 climatology. Monthly AMO and NAO values were averaged to obtain annual values for each year, since $\delta^{15}N$ values represent data integrated over several months and we were interested in annual changes in trophic ecology, nesting parameters, and habitat use.

Data Analyses

For all statistical analyses, we considered p values < 0.05 statistically significant. We used univariate linear regression analyses to evaluate relationships between $\delta^{15}N_{bulk}$ values and time, and the relationships between $\delta^{15}N_{bulk}$ and source amino acid $\delta^{15}N$ (phenylalanine and lysine) values. Linear mixed effects (LME) models were built using the R package 'nlme' to detect potential changes in the $\delta^{15}N$ values at the base of the food web (i.e., in source amino acid $\delta^{15}N$ values) during our sampling period. LMEs are useful for analyzing CSIA-AA data, as samples are typically analyzed in triplicate, and thus the analysis provides replicate $\delta^{15}N$ measurements accounting for within-sample variability. We included year as a linear term (fixed effect) and sample number as a random effect.

We were also interested in the variation in $\delta^{15}N_{phe}$ values from individual turtles over time, as the isotope values from source amino acids may provide geographic information about foraging area fidelity and pre-nesting habitat use patterns of leatherbacks. We first tested for an interaction between the individual turtle and the sampling year using the following model: $\delta^{15}N_{phe}$ ~ Individual * Year, as blood was collected during different years due to the

opportunistic nature of our study. Since there was no interaction, we removed year and used an ANOVA to test for differences in amino acid $\delta^{15}N$ values among individuals.

To assess changes in North Atlantic leatherback TPs over time, we used univariate linear regression analysis. We also used an LME model to evaluate changes in a proxy for TP ($\delta^{15}N_{glu}$-$\delta^{15}N_{phe}$, e.g., see Decima et al. 2013) from 1992-2010, where we included year as a linear term (fixed effect), and sample number as a random effect. Although using a proxy for TP does not provide an TP estimate, or account for variability in TDFs, it circumvents the issue of a dependence on β and TDF values and allowed us to evaluate relative changes in TP over time.

We then compared the TP estimates of St. Croix leatherbacks with those from two foraging groups of western Pacific leatherbacks from Seminoff et al. (2012) using Appendix Eqn 1, as Pacific leatherback TPs were also estimated using this approach. We then tested the differences in TP between populations using an ANOVA. The $\delta^{15}N$ values from certain amino acids (e.g., lysine) were not detected in the Pacific leatherback samples from Seminoff et al. (2012), and therefore we were unable to compare TP estimates using equations that rely on several trophic and source amino acid $\delta^{15}N$ values (Appendix Eqns 2 and 3).

We evaluated relationships between $\delta^{15}N_{bulk}$ values, the annual NAO Index and AMO Index, and two leatherback nesting parameters using linear regression analyses. We also used LMEs to evaluate the relationships between source amino acid $\delta^{15}N$ values (a proxy for the base of the food web) and environmental conditions, where the NAO and AMO Indices were included as linear terms (fixed effects) and the sample number was included as a random effect.

Results

Trends in $\delta^{15}N$ values

The $\delta^{15}N_{bulk}$ values from leatherback blood collected from 1992 to 2010 on St. Croix ranged from 4.2 to 12.6 ‰, with a mean ± SD of 8.9 ± 1.6 ‰ (Appendix Table 1S.1). There was high intra-annual variability in $\delta^{15}N_{bulk}$ values, but a statistically significant decrease in leatherback $\delta^{15}N_{bulk}$ values over time (Adj. $R^2 = 0.14$, $F_{(1,197)} = 32.7$, $p < 0.0001$; Figure 1.1).

We determined the $\delta^{15}N$ values of eighteen amino acids from 25 leatherback blood samples, but twelve amino acids were consistently detected on chromatograms and $\delta^{15}N$ values from six amino acids were used to estimate TP (Table 1.1). The $\delta^{15}N$ values from the source amino acids lysine ($\delta^{15}N_{lys}$), and phenylalanine ($\delta^{15}N_{phe}$) were used to evaluate fluctuations in the nitrogen isotope composition at the base of the food web, which influence bulk isotope values of consumers. The $\delta^{15}N_{phe}$ values were variable and ranged from 2.0 to 7.0 ‰. We found positive relationships between the $\delta^{15}N_{bulk}$ and source amino acid $\delta^{15}N$ values: $\delta^{15}N_{phe}$ (Adj. $R^2 = 0.43$, $F_{(1,23)} = 18.1$, $p < 0.001$) and $\delta^{15}N_{lys}$ (Adj. $R^2 = 0.59$, $F_{(1,23)} = 35.7$, $p < 0.00001$; Figure 1.2a). We found a weak, although statistically significant, relationship between $\delta^{15}N_{phe}$ values and year using both linear regression analyses (Figure 1.2b) and an LME model (Table 1.2), where $\delta^{15}N_{phe}$ values decreased from 1992-2010.

We analyzed a subset of samples from five individual leatherbacks during multiple nesting years (Table 1.3) and the $\delta^{15}N_{phe}$ values ranged from 2.0 – 6.2 ‰. We found weak, detectable differences in the source amino acid $\delta^{15}N$ values among individuals, for $\delta^{15}N_{phe}$ (Adj. $R^2 = 0.32$, $F_{(1,9)} = 5.77$, $p = 0.04$) and $\delta^{15}N_{lys}$ (Adj. $R^2 = 0.27$, $F_{(1,9)} = 4.64$, $p = 0.06$). However, the variability in the $\delta^{15}N_{phe}$ values within individuals sampled across time periods was low, generally within 1-2 ‰ between sampling events (Appendix Figure 4).

Trophic Position Estimates

We used several approaches to estimate TPs from amino acid δ^{15}N values. TP estimates from three variations of Eqn 1 yielded similar results with means ± SD of 2.4 ± 0.2 (Appendix Eqn 1), 2.6 ± 0.3 (Appendix Eqn 2), and 2.6 ± 0.3 (Appendix Eqn 3). Using a Bayesian approach, we found the TP ranged from 2.4-3.2 with a mean and median of 2.8. There was no significant change in North Atlantic leatherback TP over time (Adj. R^2 = -0.04, $F_{(1,23)} = 0.08$, $p > 0.5$), and similarly no change in the proxy for North Atlantic leatherback TP over time ($\delta^{15}N_{glu}$ - $\delta^{15}N_{phe}$; Table 1.2), using an LME model. Additionally, there was no differences in the mean ±SD TP estimates between the North Atlantic leatherbacks (2.4 ± 0.2) and the eastern Pacific-foraging (2.4 ± 0.01) or western Pacific-foraging group (2.4 ± 0.01; one-way ANOVA, 95 % confidence, $F_{(1,29)} = 0.08$, $p > 0.5$; Figure 1.3).

Links between δ^{15}N values, *Oceanography, and Nesting Parameters*

Both oceanographic indices were in positive phases throughout our sampling period; however, the AMO Index increased from 1992–2010, whereas the NAO Index decreased, and our sampling period ended with a large negative NAO event in 2010. We found detectable positive relationships between $\delta^{15}N_{bulk}$ values and the NAO Index (Adj. R^2 = 0.06, $F_{(1,197)} = 17.1$, $p < 0.00001$; Figure 1.4 and Appendix Figure 1A.1), and a weak, although detectable negative relationship with the AMO Index (Adj. R^2 = 0.08, $F_{(1,197)} = 18.7$, $p < 0.00001$; Appendix Figure 1A.2). Using an LME model, we found a weak relationship between the $\delta^{15}N_{phe}$ values from 1992–2010 and the winter NAO Index (Table 1.2), but no relationship with the AMO Index.

Overall, we found weak relationships between δ^{15}N values, oceanographic indices, and nesting parameters. There was a detectable, negative relationship between the $\delta^{15}N_{bulk}$ values and the length of leatherback remigration intervals prior to our sample collection (Adj. R^2 = 0.08,

$F_{(1,50)} = 4.8$, $p < 0.05$; Appendix Figure 1A.3B), and a weak positive linear relationship between $\delta^{15}N_{bulk}$ values and the number of clutches laid by individuals during the corresponding sampling year (Adj. $R^2 = 0.03$, $F_{(1,114)} = 4.4$, $p < 0.05$; Appendix Figure 1A.3A).

The NAO Index was weakly related to demographic parameters, where clutch frequency increased with increasing NAO ($R^2 = 0.042$, $F_{(1,114)} = 6.0$, $p = 0.01$) and the remigration intervals were negatively related to NAO ($R^2 = 0.15$, $F_{(1,48)} = 9.8$, $p < 0.01$). We found a weak negative relationship between the remigration interval and the AMO Index ($R^2 = 0.05$, $F_{(1,114)} = 5.7$, $p = 0.02$), but no relationship between clutch frequency and the AMO Index ($R^2 = 0.02$, $F_{(1,48)} = 2.1$, $p > 0.1$).

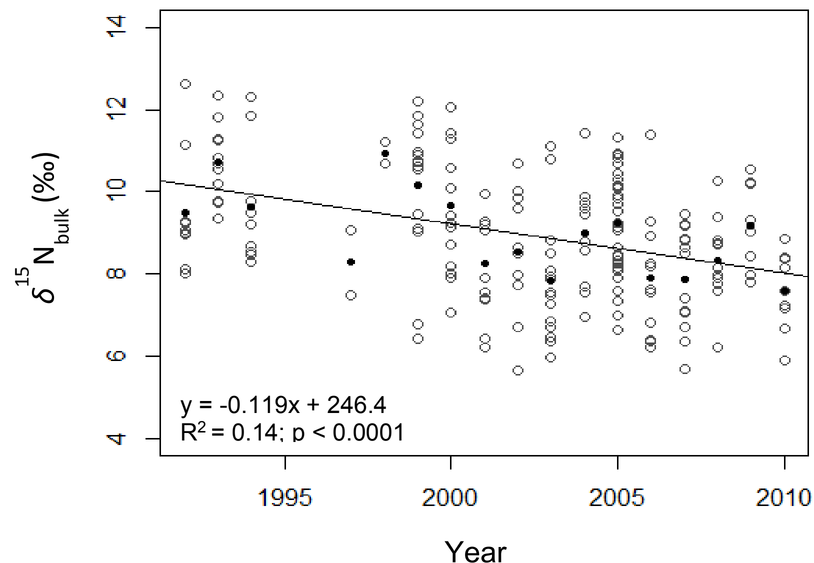

Figure 1.1. Linear relationship between the $\delta^{15}N_{bulk}$ values from leatherback turtle blood and year of sample collection. Filled circles indicate the mean $\delta^{15}N_{bulk}$ values for each year and open circles are the $\delta^{15}N_{bulk}$ values from each sample.

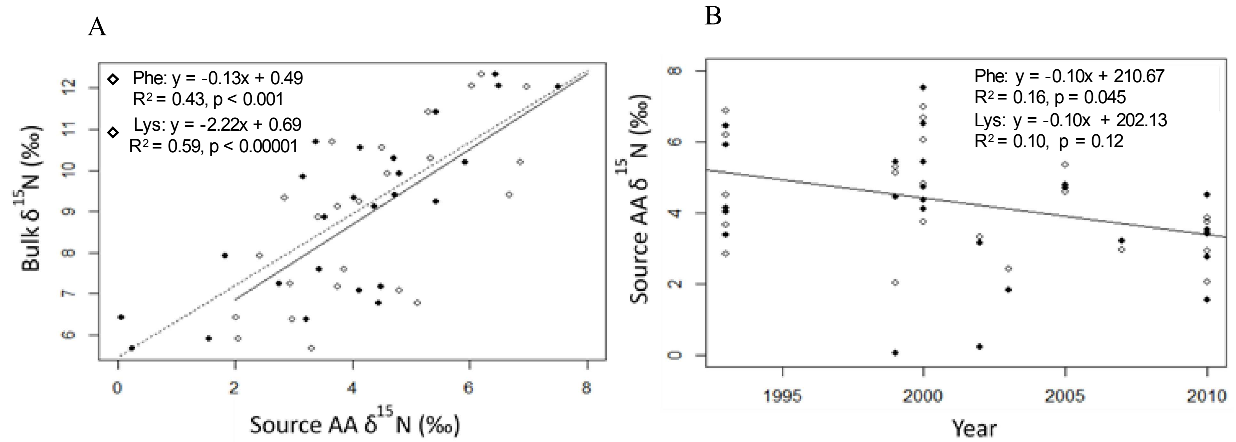

Figure 1.2. Linear relationships between A) the $\delta^{15}N_{bulk}$ values and the $\delta^{15}N$ values of the source amino acids phenylalanine ($\delta^{15}N_{phe}$) and lysine ($\delta^{15}N_{lys}$) from leatherback blood, and B) the $\delta^{15}N_{phe}$ and $\delta^{15}N_{lys}$ values and year of sample collection.

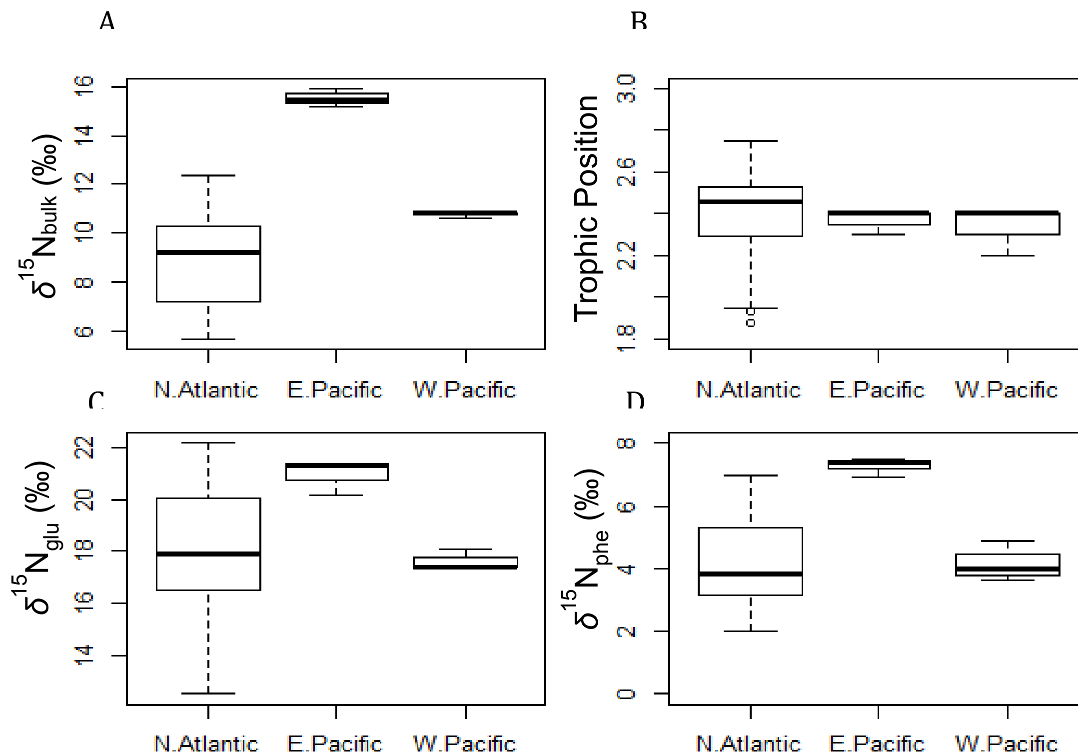

Figure 1.3. A comparison of the $\delta^{15}N$ values between leatherbacks nesting on St. Croix in the North Atlantic (N. Atlantic), and two groups of Pacific leatherbacks: eastern (E. Pacific) and western Pacific foragers from Seminoff et al. (2012), where A) are the $\delta^{15}N_{bulk}$ values, B) are the trophic position estimates which were calculated using Eqn 1, C) are the $\delta^{15}N$ values of glutamic acid ($\delta^{15}N_{glu}$), a trophic amino acid, and D) are the $\delta^{15}N$ values of phenylalanine acid ($\delta^{15}N_{phe}$), a source amino acid.

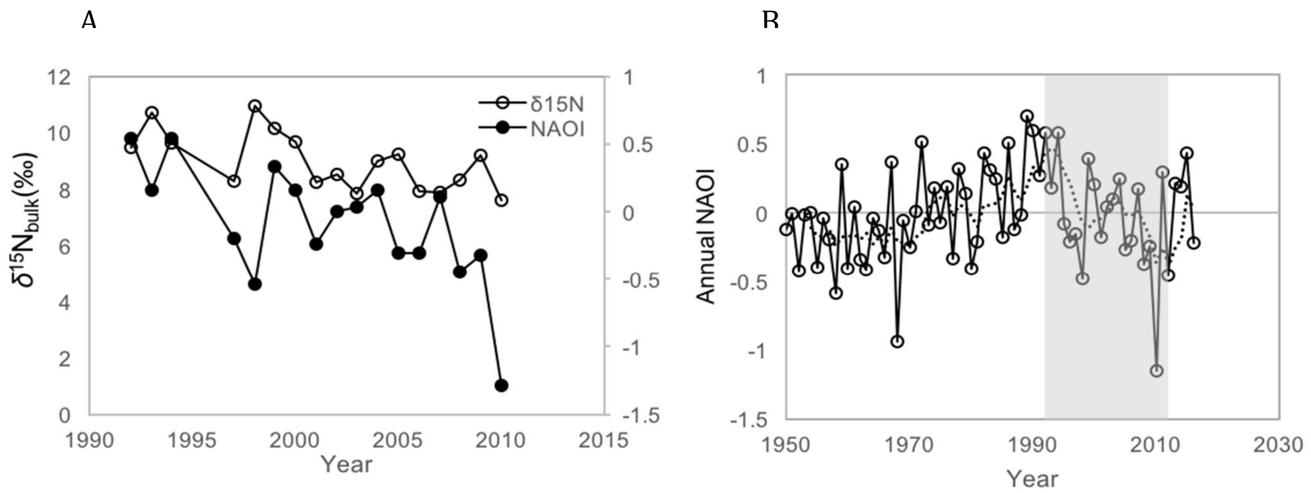

Figure 1.4. A) The mean annual North Atlantic Oscillation Index (NAOI) and mean $\delta^{15}N_{bulk}$ values of blood collected from leatherback turtles between 1992 and 2010, and B) a historical context of mean annual NAOI values from NOAA's Climate Prediction Center (http://www.cpc.ncep.noaa.gov/), where the dotted black line represents a 5-year running average, and the solid line represents unsmoothed, annual winter average NAOI values, where both lines were standardized using the 1980-2010 base period. The gray box indicates our sampling period.

Table 1.1. Stable isotope data from the subset of blood samples from leatherback turtles we analyzed for compound-specific isotope analysis of amino acids (CSIA-AA) including the year sampled, the $\delta^{15}N_{bulk}$ values, the trophic position (TP) estimate for each turtle, and the $\delta^{15}N$ values of the selected trophic and source amino acids used to estimate TP: Alanine (Ala), Leucine (Leu), Glutamic acid (Glu), Phenylalanine (Phe), Lysine (Lys), and Glycine (Gly).

				Trophic AAs (‰)			**Source AAs (‰)**		
Year	**$\delta^{15}N_{bulk}$ (‰)**	**TP**	**Glu-Phe (‰)**	**Ala**	**Leu**	**Glu**	**Phe**	**Lys**	**Gly**
1993	9.3	2.5	15.0	21.5	18.5	17.9	2.8	4.0	10.7
1993	10.6	2.7	16.7	24.5	22.1	21.2	4.5	4.1	12.8
1993	10.2	2.0	10.7	20.4	18.1	17.6	6.9	5.9	11.8
1993	10.7	2.5	14.5	19.3	19.0	18.2	3.7	3.4	9.5
1999	6.4	1.9	10.5	17.6	14.4	12.5	2.0	0.1	8.6
2000	7.1	2.0	10.6	19.0	14.3	15.4	4.8	4.1	9.9
2000	12.0	2.3	13.6	24.3	21.1	20.6	7.0	7.5	14.9
2000	9.2	2.5	14.5	18.8	18.0	18.7	4.1	5.4	9.8
2000	9.4	2.5	15.1	24.7	22.1	21.7	6.7	4.7	10.8
2000	9.1	2.3	13.2	18.6	18.1	17.0	3.7	4.4	12.2
2002	5.7	1.9	10.1	nd	14.8	13.4	3.3	0.2	8.2
2003	7.9	2.3	13.5	19.6	17.4	15.9	2.4	1.8	8.4
2007	6.4	2.2	12.7	16.7	13.9	15.6	3.0	3.2	9.0
2010	5.9	2.5	14.6	19.4	16.0	16.6	2.1	1.6	8.0
2010	7.2	2.2	12.4	20.6	16.5	16.1	3.7	4.5	11.3
2010	7.6	2.3	13.6	21.6	17.1	17.4	3.9	3.4	10.6
2010	8.9	2.7	16.0	23.2	18.9	19.4	3.4	3.5	9.8
2010	7.2	2.3	13.6	19.6	16.9	16.5	2.9	2.8	9.9
1993	12.3	2.7	16.0	23.4	22.7	22.2	6.2	6.4	13.3
1999	11.4	2.4	14.1	22.8	21.4	19.4	5.3	5.4	10.7
1999	6.8	2.5	14.9	21.8	16.0	20.1	5.1	4.4	8.4
2000	12.1	2.5	15.0	24.4	22.5	21.0	6.0	6.5	10.2
2002	9.8	2.5	14.7	20.2	18.7	17.9	3.2	3.2	8.7
2005	10.3	2.6	15.3	23.4	21.1	20.6	5.3	4.7	8.8
2005	9.9	2.6	15.3	21.9	20.8	19.9	4.6	4.8	10.6

Table 1.2. Estimated parameters from the linear mixed effects models for the $\delta^{15}N$ values of phenylalanine ($\delta^{15}N_{phe}$) from leatherback blood samples *versus* year, $\delta^{15}N_{phe}$ vs. the North Atlantic Oscillation Index, and a proxy for trophic position ($\delta^{15}N_{glu}$ - $\delta^{15}N_{phe}$) *versus* year. Shown for the fixed effect component of each LME are the estimated coefficients, with the standard errors and p-values in parentheses. Shown for the random effect component of each LME are the estimated standard deviations of the random effect distributions and the approximate 95% confidence intervals in parentheses.

Parameter	$\delta^{15}N_{phe}$ ~ Year	$\delta^{15}N_{phe}$ ~ NAO	$\delta^{15}N_{glu}$ - $\delta^{15}N_{phe}$ ~Year
Fixed Effects			
Intercept	210.2 (97.46; $p < 0.05$)	4.06 (0.29, $p < 0.001$)	59.01 (167.70, $p > 0.1$)
Year	-0.10 (0.05; $p < 0.05$)	0.71 (0.31, $p < 0.05$)	-0.02 (0.08, $p > 0.1$)
Random Effects			
SD_{sample}	1.36 (1.0, 1.83)	1.34 (0.99, 1.82)	1.63 (1.34, 1.99)

Table 1.3. Stable isotope data from five individual leatherbacks that were sampled during multiple nesting seasons, with years sampled, mean source amino acid ($\delta^{15}N_{phe}$) values, and the differences between sampling events in: phenylalanine values ($\Delta\ \delta^{15}N_{phe}$), trophic position ($\Delta$ TP), and a proxy for trophic position ($\Delta\ \delta^{15}N_{glu} - \delta^{15}N_{phe}$).

Individual	**Year**	**$\delta^{15}N_{bulk}$ (‰)**	**$\delta^{15}N_{glu} - \delta^{15}N_{phe}$ (‰)**	**$\delta^{15}N_{glu}$ (‰)**	**$\delta^{15}N_{phe}$ (‰)**
1	2000	12.1	15.0	21.0	6.0
1	2005	10.3	15.3	20.6	5.3
Δ		**1.8**	**-0.3**	**0.4**	**0.7**
2	1993	12.3	16.0	22.2	6.2
2	1999	6.8	14.9	20.1	5.1
2	2005	9.9	15.3	19.9	4.6
Δ 93-99		**5.6**	**1.1**	**2.1**	**1.1**
Δ 93-05		**2.4**	**0.7**	**2.3**	**1.6**
Δ 99-05		**-6.0**	**-3.1**	**-0.4**	**0.2**
3	1999	6.4	10.5	12.5	2.0
3	2003	7.9	13.5	15.9	2.4
Δ		**-1.5**	**-3.0**	**-3.4**	**-0.4**
4	1999	11.4	14.1	19.4	5.3
4	2002	9.8	14.7	17.9	3.2
Δ		**1.6**	**-0.7**	**1.5**	**2.1**
5	2002	5.7	10.1	13.4	3.3
5	2007	6.4	12.7	15.6	3.0
Δ		**-0.7**	**-2.6**	**-2.3**	**0.3**

Discussion

Our study underscores the utility of nitrogen isotope data to evaluate long-term trends in the foraging ecology and habitat use of migratory marine species. Using the information provided by CSIA-AA, we found no changes in leatherback TP over 18 years, despite detectable decreases in the $\delta^{15}N_{bulk}$ and $\delta^{15}N_{phe}$ values from 1992–2010. Our results indicate that Pacific and Atlantic leatherback populations occupy nearly identical TPs so differences in their population trajectories cannot be explained by a major trophic dichotomy. Source amino acid $\delta^{15}N$ values decreased over time, suggesting that trends in $\delta^{15}N_{bulk}$ values can be somewhat attributed to changes in the $\delta^{15}N$ values at the base of the food web, which are likely driven by regional

oceanography and nitrogen biogeochemistry, not changes in TP. Our results also provide insight into leatherback habitat use during the cryptic, pre-nesting portion of their breeding migrations, which supports previous hypotheses that the North Atlantic leatherback population uses multiple oceanic areas prior to nesting. However, at the individual level, turtles seem to exhibit fidelity to specific foraging areas, as evidenced by the similarity in isotope values from individuals over multiple sampling seasons.

We found weak relationships between leatherback nesting parameters, North Atlantic oceanography, and nitrogen isotope values, where higher δ^{15}N values corresponded with positive NAO, lower remigration intervals, and a higher clutch frequency. This may suggest that positive modes of the NAO, which occurred during our sampling period and coincided with an increase in the North Atlantic leatherback population, affects sea-surface temperature and nutrient dynamics in a way that is beneficial to leatherbacks foraging in the North Atlantic Ocean, thereby facilitating their population increase. However, future studies focused on fluctuations in the abundances of leatherback prey in relation to oceanographic conditions would be useful to evaluate this hypothesis and the mechanistic links between large-scale oceanography and leatherback population productivity.

Estimating Trophic Position with CSIA-AA

Although CSIA-AA is an increasingly-used tool to estimate TPs of consumers, recent studies highlight its limitations, particularly regarding variability in the TDF of consumers (McMahon and McCarthy 2016). There is increasing evidence that TDFs can vary widely across taxa and the often used TDF of 7.6 ‰ from a seminal study (Chikaraishi et al. 2009) is not appropriate for certain taxa (Hetherington et al. 2016; McMahon and McCarthy 2016). It is

somewhat unclear why TDFs vary, although they may be affected by the diet quality of the consumer, whereby if the consumer's amino acid composition is similar to that of its prey, the TDF is lower than consumers whose amino acid composition is quite different than their prey (McMahon and McCarthy 2016; Fuller and Petzke 2017; O'Connell 2017). Additionally, the consumer's method of nitrogen excretion may also affect its TDF value, wherein organisms that produce urea or uric acid, like turtles, have lower TDFs than ammonia-excreting organisms (Germain et al. 2013; McMahon and McCarthy 2016).

Ideally, we would estimate TP using a species-specific TDF derived from a leatherback feeding experiment, however, no published TDF estimates are available for marine turtles. Therefore, we used TDFs from previous studies (Chikaraishi et al. 2009; Bradley et al. 2015; Nielsen et al. 2015) to estimate TP. Alternative methods for estimating TDFs are needed, as highly migratory species are difficult or impossible to maintain in laboratory settings for controlled feeding experiments. One novel, promising technique, Stable Isotope Discrimination Estimation in R (SIDER) relies on using phylogenetic relatedness approaches to estimate TDFs (Healy et al. 2017). Unfortunately, we were unable to use SIDER in our study, as it currently can only be applied to bird and mammal data, but it may provide useful for future studies.

Due to the aforementioned uncertainties, we used multiple approaches to estimating leatherback TPs. The Chikaraishi et al. (2009) approach, which relies on the difference in δ^{15}N values of one trophic and one source amino acid, produced the lowest TP estimates (mean = 2.4; Appendix Eqn 1). More recent meta-analyses (Bradley et al. 2015; Nielsen et al. 2015) suggest that using multiple trophic and source amino acids, in addition to a lower TDF produces more biologically realistic TPs for certain taxa. These approaches yielded identical TP estimates, which were marginally higher (mean = 2.6) than estimates using the Chikaraishi et al. (2009)

approach. Trophic position estimates from the Bayesian approach were more variable and the mean (2.8) was slightly higher. The larger range of TP estimates from this approach can likely be attributed to incorporating variability in TDF values.

Leatherback TPs from CSIA-AA may be slight underestimates, particularly regarding estimates from the Chikaraishi et al. (2009) approach, which uses a higher TDF than the other equations. Our results bolster a growing body of literature (Germain et al. 2013; Bradley et al. 2015; McMahon et al. 2015; Hetherington et al. 2016) demonstrating consistent underestimation of TP for higher trophic level marine species using this approach. Leatherbacks are specialist consumers that prey on gelatinous zooplankton, including carnivorous scyphozoans and filter-feeding organisms like salps and pyrosomes. Thus, TPs of ~3 are reasonable if leatherbacks are feeding on a mixture of carnivorous jellyfish and filter-feeding tunicates. Based on our understanding of leatherback feeding ecology, the Bayesian approach provided the most realistic TP estimates. However, it is possible that leatherbacks consume a higher proportion of filter-feeding organisms (e.g., salps and pyrosomes) than is currently recognized, which would result in lower $\delta^{15}N$ values and TPs than expected if they were primarily consuming carnivorous scyphozoans.

Our work and other recent studies highlight the critical need for more experimental studies on TDF variability, turnover rates for amino acids, and the metabolic mechanisms driving variability. Recently, studies have focused on understanding the biochemical underpinnings that influence patterns in source and trophic amino acid $\delta^{15}N$ values and suggest that the source and trophic groupings have metabolic origins, specifically the cycling of amino-nitrogen between amino acids (O'Connell 2017). Ultimately, understanding the mechanisms driving variability in TDFs and amino acid $\delta^{15}N$ values will be critical for the continued development and application

of CSIA-AA in ecological studies. Regardless of the method used to calculate leatherback TP from amino acid δ^{15}N values, none changed as a function of time.

Trends in Trophic Position

CSIA-AA was useful for evaluating relative changes in TP from 1992–2010 and comparing North Atlantic leatherback TP estimates with those from western Pacific populations, which was our primary objective. Although we found a detectable long-term decline in the $\delta^{15}N_{bulk}$ values from 1992–2010, results from CSIA-AA indicated there were no changes in St. Croix leatherback TP over time regardless of the method used to calculate TP. In addition, we found no differences in TP between St. Croix leatherbacks and those from two Pacific foraging groups that were estimated by Seminoff et al. (2012) using the same CSIA-AA approach.

The mean TP estimates for North Atlantic leatherbacks were nearly identical to those from two foraging groups from the western Pacific population, indicating that western Pacific and North Atlantic leatherbacks occupy the same TP. Our sample size was larger, which may explain the larger range in TP values from North Atlantic leatherbacks compared with those from Pacific leatherbacks in Seminoff et al. (2012). Additionally, Pacific leatherback δ^{15}N values were coupled with satellite telemetry data where the individuals selected for stable isotope analysis migrated from distinct foraging areas (either the western or eastern Pacific Ocean) prior to nesting in the western Pacific, which contrasts with our study where satellite telemetry data were not available, and leatherbacks likely migrated from several or more foraging areas.

Our results support the hypothesis that, globally, leatherbacks occupy the same trophic level, and population-level differences in feeding ecology cannot explain the diverse population trends between Pacific and Atlantic leatherback populations. However, the δ^{15}N values do not

provide information about food quality or prey abundance. Thus, it is possible that leatherbacks have access to greater quantities of gelatinous prey in the North Atlantic, which could contribute to their population growth potential, length of remigration intervals, and overall population productivity, but not change their TP.

Linking Oceanography to Nesting Parameters

We paired demographic information for individual leatherbacks with their $\delta^{15}N$ values and ocean indices. Overall, we found weak relationships, where lower $\delta^{15}N_{bulk}$ values were associated with higher remigration intervals, lower clutch productivity, and lower NAO values. These results may indicate that broad-scale oceanographic conditions influenced leatherback trophic ecology, and nesting parameters, whereby positive NAO phases create oceanographic conditions that are beneficial for leatherbacks. Although the underlying mechanisms by which the NAO influences leatherback demography is somewhat unclear, changes in the NAO and AMO can influence the abundance and distribution of phytoplankton, zooplankton, and higher trophic level species (Beaugrand et al. 2009; Nye et al. 2014).

During positive NAO phases, certain regions of the northwestern Atlantic where leatherbacks forage are associated with higher SSTs (Marshall et al. 2001), and there may be an increased abundance of gelatinous zooplankton associated with higher SSTs in certain regions (e.g., Lucas et al. 2014). Positive NAO indices have also been linked to increases in gelatinous zooplankton abundance (Attrill et al. 2007), so leatherbacks may have a more abundant food supply leading to potentially shorter reproductive intervals during positive NAO periods. Therefore, the oceanographic conditions in the 1990s and early 2000s may have contributed to North Atlantic leatherback population increases and positive phases of the NAO and AMO may

benefit leatherback foraging in the North Atlantic. However, our low R^2 values indicate that, although these relationships were statistically significant, they do not explain much of the variability in our data. Thus, future studies are needed to explicitly evaluate fluctuations in gelatinous zooplankton abundances and to evaluate the linkages between large-scale environmental conditions and leatherback foraging.

Biogeochemistry and N Cycling

Since TP did not change from 1992–2010, we evaluated potential mechanisms driving the decrease we observed in the $\delta^{15}N_{bulk}$ values from leatherback blood. The concurrent decrease in $\delta^{15}N_{phe}$ values over time indicates that patterns in $\delta^{15}N_{bulk}$ values could be attributed to changes in nitrogen cycling and its effect on the nitrogen isotopic composition at the base of the food web. Nitrogen is supplied to the food chain via transport from subsurface waters with high nutrient concentrations, N_2 fixation by diazaotrophs, and atmospheric N deposition and nitrogen is removed from the system via denitrification (Gruber and Sarmiento 1992; Montoya et al. 2002). The relative influences of these processes drive spatial and temporal patterns in the $\delta^{15}N$ values at the base of the food web, which then propagate up to consumers (Somes et al. 2010).

In addition to natural variability in N cycling, anthropogenic influences can alter the N cycle in marine systems (e.g., Duce et al. 2008). For example, Ren et al. (2017) found long-term decreases in $\delta^{15}N$ values of corals, which they attributed to an increase in anthropogenic nitrogen deposition in the western Pacific. Alternatively, Polovina et al. (2008) suggested that the subtropical gyres are expanding with ongoing climate change and, consequently, N_2 fixation is becoming more widespread, which would lead to a temporal decrease in $\delta^{15}N$ values. Our long-term decrease in $\delta^{15}N$ values could also reflect of an expansion of the subtropical gyres or

increased anthropogenic nitrogen deposition, although future studies are needed to test these hypotheses.

Leatherback habitat use

There are spatial gradients in $\delta^{15}N$ values in marine environments (Montoya et al. 2002, Somes et al. 2010), which we can use to interpret leatherback $\delta^{15}N$ values and gain insight into their pre-nesting foraging locations. For the subset of samples that we analyzed for CSIA-AA, we found a gradient of amino acid isotope values ($\delta^{15}N_{phe}$ range: 2.0–7.0 ‰), suggesting that the St. Croix leatherbacks used multiple oceanic areas, perhaps with differing biogeochemical cycling regimes, prior to nesting on St. Croix. The $\delta^{15}N_{phe}$ values for more than half of our samples were very low (< 4‰), suggesting that this portion of NA leatherbacks were in tropical or subtropical areas of the northwestern Atlantic with documented low $\delta^{15}N$ values (Somes et al. 2010; McMahon et al. 2013; Mompean et al. 2016). It is possible that low $\delta^{15}N_{phe}$ values indicate leatherbacks were in the greater Caribbean for several months before nesting on St. Croix. However, previous data indicate that leatherbacks typically arrive at their breeding grounds and begin nesting within a few weeks (Plotkin 2003), reducing the plausibility of this scenario.

Although we attributed the decrease in $\delta^{15}N_{bulk}$ and $\delta^{15}N_{phe}$ values over time to changes in N biogeochemistry, this trend could also suggest a shift in North Atlantic leatherback foraging areas over our study period. It is possible that leatherbacks shifted their pre-nesting foraging region to an area with higher rates of N_2 fixation and subsequently lower $\delta^{15}N$ values. However, we found high inter- and intra-annual variability through our 18 years of sampling, which suggests that leatherbacks were consistently migrating to St. Croix from multiple foraging locations, rather than converging on one foraging region over time. Additionally, we sampled a

subset of individual turtles over multiple nesting years and found that variability in $\delta^{15}N_{phe}$ values of individual turtles was low between years, but the $\delta^{15}N_{phe}$ variation among individuals was higher. If leatherbacks exhibited a major shift in foraging area during our sampling period, we would expect changes in the $\delta^{15}N_{phe}$ values of individual turtles between nesting years.

Although our sample size was limited, our results support previous hypotheses that North Atlantic leatherbacks have flexible foraging tactics and inhabit multiple regions of the North Atlantic Ocean (Hays et al. 2006; Fossette et al. 2010 a,b), but individuals appear to have regional foraging area fidelity. Our isotope data provide inferences about recently occupied leatherback foraging areas prior to nesting on St. Croix. However, individuals likely transition among other habitats during earlier periods that are no longer reflected in their blood, as leatherback remigration intervals last for several years.

Future studies evaluating spatial isotopic differences between specific leatherback foraging areas and pairing turtle telemetry data with $\delta^{15}N$ values would further enhance our understanding of habitat use and residency duration different leatherback foraging areas. Our results have implications for management and conservation. Since leatherbacks are using several oceanic areas, our results urge for holistic management practices that account for multiple jurisdictions and future studies that investigate habitat duration in each foraging area to best protect leatherbacks during different stages of their remigration intervals.

Acknowledgements

We would like to thank the Dr. Kelly Stewart, Dr. Jeanne Garner, Claudia Lombard, Mike Evans, the U.S. Fish and Wildlife Service, The US Virgin Islands Department of Planning and Natural Resources, and all employees and volunteers who assisted in field work at Sandy Point

National Wildlife Refuge. All samples were collected under federal and state permit guidelines. We would also like to thank Natalie Wallsgrove, Joel Schumacher, and Andrea Liu for their assistance in sample preparation and isotope analyses. This research was funded by the Inter-university training in continental-scale ecology award from the National Science Foundation (award #1137336), the Boyd Lyon Sea Turtle Fund, and the University of California San Diego. This is School of Ocean Science and Technology contribution number ####.

Chapter 1, in full, has been submitted and accepted for publication of the material as it may appear in Oecologia, 2018, Hetherington, ED, Seminoff, JA, Robison, L, Dutton, P, Popp, BN, and Kurle, CM. The dissertation author is the primary investigator and author of this paper.

Appendix

I. Sample Collection

Blood was drawn from the dorsal cervical sinus or a rear flipper using standard serum collection tubes that contain anticoagulants to prevent clotting (Dutton 1996). Samples were frozen at -20°C at the field station and stored long-term at -80°C until further analysis. The bulk δ^{15}N from whole blood from turtles reflect data integrated over ~six months before the time of tissue collection (Seminoff et al. 2007), and thus adequately reflect a time frame indicative of leatherback re-migration from their foraging grounds to St. Croix. We limited our analyses to samples collected from recently arrived leatherbacks at the start of the nesting season, as their δ^{15}N values would best reflect those from their pre-nesting locations.

II. Isotope Analyses

We determined bulk δ^{15}N ($\delta^{15}N_{bulk}$) values using a CE1108 Elemental analyzer interfaced via a CONFLO III device to a Thermo-Electron Delta Plus XP mass spectrometer at the University of California, Santa Cruz. We report stable nitrogen isotope values in δ notation relative to atmospheric N_2.

For CSIA-AA we prepared the samples by acid hydrolysis followed by derivatization of amino acids (see Popp et al. [2007] and Hannides et al. [2013] for details). Samples were hydrolyzed (6N HCl, 150 °C for 70 minutes), esterified (4:1 isopropanol:acetyl chloride), derivatized (3:1 methylene chloride:trifluoroacetyl anhydride), and analyzed using a Trace GC gas chromatograph and a Thermo Delta XP mass spectrometer through a GC-C III combustion furnace (980°C), reduction furnace (680°C), and a liquid nitrogen cold trap. Samples were

injected (split/splitless, 5:1 split ratio) with a 180℃ injector temperature and a constant helium flow rate of 1.4 mL min^{-1}.

We analyzed the samples in triplicate, and corrected the $\delta^{15}N$ values to internal reference compounds norleucine and aminoadipic acid as these have known nitrogen isotope compositions and were co-injected with each sample. We confirmed quality control by analyzing an amino acid suite, with known $\delta^{15}N$ values of 12 amino acids, before and after each triplicate sample run. We analyzed the $\delta^{15}N$ values of 18 amino acids, although, some were not abundant enough for detection on all chromatographs. We grouped amino acids into standard 'trophic' and 'source' categories, based on previous studies (McClelland and Montoya 2002, Popp et al. 2007, Chikaraishi et al. 2009).

III. Estimating Trophic Position (TP) using variations of Eqn 1

We estimated TP using three variations of Eqn 1 in the main text of the manuscript, which rely on somewhat different approaches. First, we used one source ($\delta^{15}N_{phe}$) and one trophic ($\delta^{15}N_{glu}$) amino acid, an approach from Chikaraishi et al. 2009:

$$TP_{Glu-Phe} = \frac{(\delta^{15}N_{Glu} - \delta^{15}N_{Phe}) - 3.4}{7.6} + 1 \qquad \text{(SI 1)}$$

where $TP_{Glu\text{-}Phe}$ is the TP based on mean $\delta^{15}N$ values from the trophic and source amino acids glutamic acid ($\delta^{15}N_{Glu}$) and phenylalanine ($\delta^{15}N_{Phe}$), respectively, the trophic discrimination factor (TDF) is 7.6 ‰ and represents the ^{15}N enrichment of $\delta^{15}N_{Glu}$ with respect to $\delta^{15}N_{Phe}$ per trophic step, and β represents $\delta^{15}N_{Glu}$- $\delta^{15}N_{Phe}$ in primary producers (3.4 ± 0.9 ‰).

More recent studies have indicated that using multiple source and trophic amino acids, along with a lower TDF to estimate TP, may provide more realistic TP estimates for certain taxa.

Thus, we also estimated TP using approaches from Bradley et al. (2015), which was derived from a meta-analysis of teleosts:

$$TP_{Trp-Src} = \frac{(\delta^{15}N_{Trp} - \delta^{15}N_{Src}) - 3.6}{5.7} + 1 \quad \text{(SI 2)}$$

where $TP_{Trp-Src}$ is the TP based on the weighted mean δ^{15}N values of the trophic (Trp) amino acids alanine, glutamic acid, and leucine, and the source (Src) amino acids glycine, lysine, and phenylalanine, 3.6 ‰ is the ß value, and the TDF is 5.7 ‰.

Similar to Bradley et al. (2015), we also estimated TP using an equation derived from a recent meta-analysis of fish from Nielsen et al. 2015:

$$TP_{Trp-Src} = \frac{(\delta^{15}N_{Trp} - \delta^{15}N_{Src}) - 2.9}{5.7} + 1 \quad \text{(SI 3)}$$

where $TP_{Trp-Src}$ is the same as SI Eqn 2 above, but the ß value is 2.9 ‰, and the TDF is 6.6 ‰.

IV. Estimating Trophic Position using a Bayesian Approach

Lastly, we used a novel Bayesian approach, where we can estimate the TPs of consumers relative to baseline isotope data. Here, we used the R package 'tRophicposition' (Quezada-Romegialli et al. 2018), which combines an interface to JAGS (Just Another Gibbs Sampler) in R, using the library "rjags". JAGS uses Markov Chain Monte Carlo (MCMC) simulations to create Bayesian models, and 'rjags' couples JAGS with R, which allows us to sample posterior density distributions from the MCMC simulations. The MCMC simulations are coupled with stable isotope data to create Bayesian models to estimate TP. See Quezada-Romegialli et al. (2018) for more details on this approach.

There are several advantages to this approach. We can include variability in two key parameters that are used to estimate TP: the baseline isotope value of primary producers and the

trophic discrimination factors. Thus, our TP estimates are likely more robust than relying on the aforementioned approaches to estimating TP. The equation used to in ‘tRophicposition’ is:

$$\delta^{15}N_{consumer} = \delta^{15}N_{baseline} + \mathrm{TDF}(\mathrm{TP} - \lambda) \quad \text{(SI 4)}$$

where λ represents the TP of the baseline isotope data. The ‘tRophicposition’ package was built for bulk isotope data. However, we used a novel approach to apply this package to amino acid isotope data, where we used $\delta^{15}N_{phe}$ as our baseline, $\delta^{15}N_{glu}$- ß as our consumer value, a TDF of 5.7 ± 0.9 ‰ from Bradley et al. (2015) and set λ = 1, since $\delta^{15}N_{phe}$ represents primary consumer values.

We defined a Bayesian model using the function jagsOneBaseline, where we defined our priors as a normal distribution of an *a priori* TP and standard deviation (3 ± 0.5). Then, we fed the Bayesian model with isotope data using the function TPmodel, and defined the number of parallel chains (2) and adaptive iterations (10000). We ran the model to generate large numbers of posterior density distributions of TP and then sampled these posterior trophic position estimates to obtain summary statistics for the posterior estimates.

V. Calculating Remigration Intervals

Since the remigration interval is the number of years between nesting events, calculating this value required nesting information for turtles over multiple nesting seasons. We calculated the remigration intervals for individuals in which blood samples and nesting data were available for the same years. Therefore, the remigration interval reflected the number of years, prior to the year of our sample collection, since the turtle was documented at Sandy Point National Wildlife Refuge (SPNWR). Blood samples were often taken on the first sighting of turtles at SPNWR, so nesting information prior to our sample collection was not always available, and thus, we could

not calculate remigration intervals for all individuals. Based on previous knowledge about leatherback remigration intervals (Dutton et al. 2005, Price et al. 2005), we excluded remigration intervals > 6 years because those individuals likely nested elsewhere in the years between documentation at SPNWR.

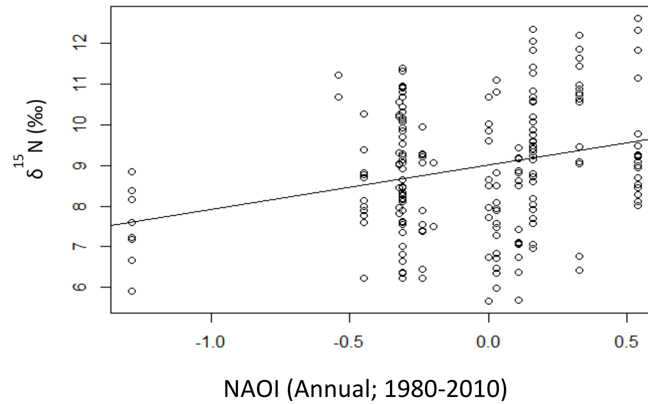

Figure 1A.1 Relationship between $\delta^{15}N_{bulk}$ values and the North Atlantic Oscillation Index., Adj. $R^2 = 0.06$, $F_{(1,197)} = 17.1$, $p < 0.00001$.

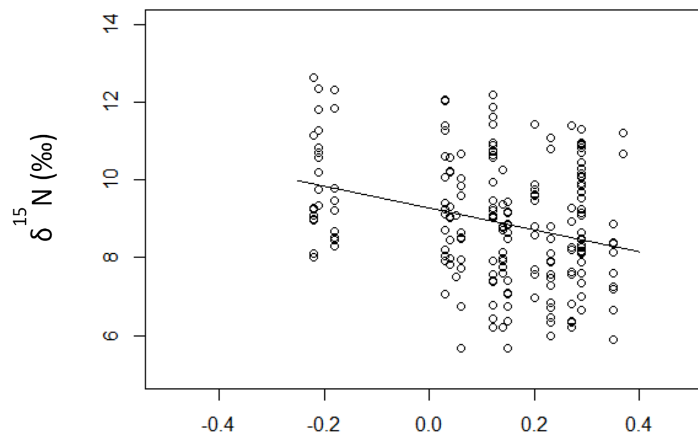

Figure 1A.2. Relationship between the $\delta^{15}N_{bulk}$ values and the Atlantic Multidecadal Oscillation Index, Adj. $R^2 = 0.08$, $F_{(1,197)} = 18.7$, $p < 0.00001$.

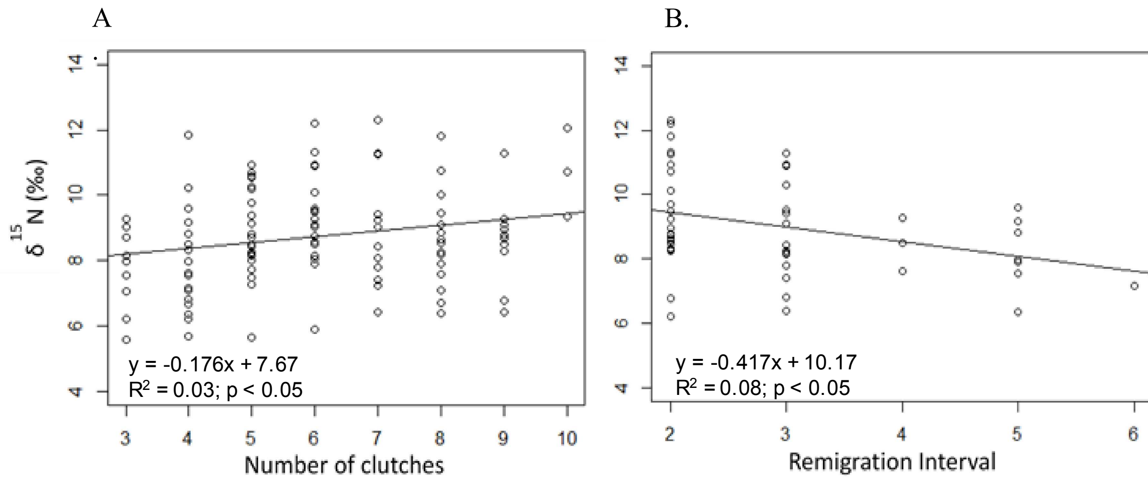

Figure 1A.3. Linear relationships between the $\delta^{15}N_{bulk}$ values from leatherback turtle blood and A) the number of clutches laid by individual leatherback turtles (i.e. clutch productivity) during the nesting season in which we collected blood samples for stable isotope analysis, and B) leatherback remigration intervals (years) prior to blood sample collection.

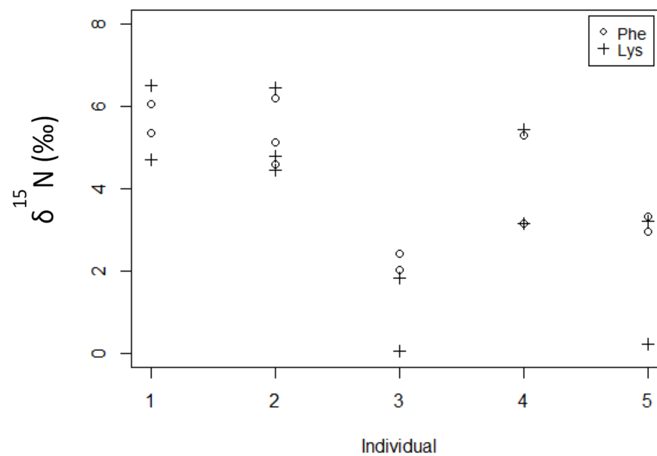

Figure 1A.4. The $\delta^{15}N$ values for the source amino acids phenylalanine (Phe) and lysine (Lys) for five individual turtles sampled during multiple nesting years.

Table 1A.1. The mean (±SD) $\delta^{15}N_{bulk}$ values from leatherback turtles for each year of sample collection at Sandy Point National Wildlife Refuge, St. Croix, USVI.

Year	**n**	**δ^{15}N (‰) ± SD**
1992	9	9.5 ± 1.5
1993	11	10.7 ± 0.9
1994	9	9.6 ± 1.5
1997	2	8.3 ± 1.1
1998	3	9.2 ± 3.1
1999	15	9.7 ± 2.3
2000	14	9.7 ± 1.6
2001	12	8.2 ± 1.2
2002	13	8.5 ± 1.4
2003	15	7.9 ± 1.5
2004	12	9.0 ± 1.2
2005	31	9.2 ± 1.3
2006	11	7.9 ± 1.6
2007	14	7.9 ± 1.2
2008	11	8.3 ± 1.1
2009	9	9.2 ± 1.0
2010	10	7.4 ± 1.1

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CHAPTER 2

Re-examining trophic dead ends: stable isotope values link gelatinous zooplankton to leatherback turtles in the California Current

Abstract

Predator-prey interactions provide essential information for tracing energy flow through food webs and evaluating the structure and function of ecosystems. In pelagic environments, these interactions can be difficult to discern, which is problematic for identifying energy pathways that support populations of species and for managing species under an ecosystem-based framework. Here, we examine the gelatinous energy pathways that support an endangered population of leatherback sea turtles (*Dermochelys coriacea*) in the California Current-Large Marine Ecosystem (CCLME). To examine food web structure and trophic ecology in relation to leatherbacks, we used carbon and nitrogen bulk stable isotope analysis and compound-specific isotope analysis of amino acids (CSIA-AA) on leatherback and gelatinous zooplankton tissues (scyphozoans and thaliaceans) collected in the CCLME. This is a critical foraging region for leatherbacks, and understanding leatherback trophic ecology, habitat use, and potential temporal changes in these ecological parameters is relevant for management of this population. Our objectives were to evaluate 1) temporal changes in leatherback trophic position, 2) the contribution of different gelatinous prey to leatherback diets, and 3) trophic structure of gelatinous zooplankton by estimating their trophic positions and isotopic niches using multiple approaches, and 4) leatherback seasonal residency in the northeastern Pacific Ocean. Leatherback trophic positions did not change over time, although carbon isotope values suggest a potential shift in leatherback habitat use coinciding with anomalous upwelling conditions. Leatherbacks mainly consumed carnivorous sea nettles (*Chrysaora fuscescens*), but filter-feeding thaliaceans also contributed to their diets. Isotope analyses used to evaluate trophic structure indicated that trophic positions were lowest for thaliaceans, intermediate for scyphozoans, and highest for leatherbacks, with niche and trophic overlap among scyphozoan species. Isotope

values were unimodally distributed, which may suggest that leatherbacks resided in the northeastern Pacific for several months prior to our sampling. Overall, our findings provide information on leatherback foraging ecology over thirteen years and the trophic structure of gelatinous zooplankton which support their population in a productive upwelling ecosystem.

Introduction

Predator-prey interactions are the foundations of food web architecture, providing fundamental information on how energy flows through ecosystems, which ultimately informs our understanding of ecosystem structure and function (Paine 1980, Polis and Strong 1996). Within food webs, top predators can perform critical functions, such as regulating populations of prey through top-down processes (Estes & Duggins 1995, Pace et al. 1999, Baum & Worm 2009). Many top predators are threatened and face unprecedented challenges due to an array of anthropogenic pressures (e.g., fishing pressure, pollution, climate change; Myers & Worm 2003, Sibert et al. 2006, Wallace et al. 2011, Davidson et al. 2012, Hazen et al. 2013) and are therefore of great conservation concern. To protect and manage these species, we first must identify the energy flow pathways and distinct prey resources that support predator populations. However, in pelagic ecosystems, predator-prey (i.e., trophic) interactions are often difficult to directly observe, which leads to significant gaps in our comprehension of food web structure and the trophic pathways that support top predators. This lack of dietary data identifying specific resources used by predators can therefore obscure our understanding of potential drivers of predator trophic ecology or habitat use patterns.

Our study focuses on leatherback sea turtles (*Dermochelys coriacea*), which illustrate this challenge, as leatherbacks are highly migratory, spending the majority of their lives in

inaccessible pelagic habitats (Hays et al. 2004, Bailey et al. 2012). They meet their energetic demands by consuming high abundances of gelatinous zooplankton, an understudied component of marine ecosystem, including scyphozoan jellies and thaliaceans (Jones and Seminoff 2015). Specifically, we focus on the foraging ecology of leatherback turtles and their gelatinous prey in the California Current Large Marine Ecosystem (CCLME), a productive eastern boundary upwelling system and critical foraging area for leatherbacks (Dutton et al. 2007, Benson et al. 2011, Seminoff et al. 2012). Leatherbacks that forage in the CCLME are a subgroup of the western Pacific nesting population, which is critically endangered, declining by 5.5% each year, and is of particular conservation concern (Tapilatu et al. 2013). Western Pacific leatherbacks nest in Indonesia, and a subset of the population that nests in the boreal summer migrates to the northeastern Pacific Ocean. This subset of leatherbacks has a particularly high energetic demand (Lontoh 2014) due to their trans-oceanic migration, which they must meet by foraging on seasonally abundant prey in the CCLME.

The availability and distribution of gelatinous prey is likely an important driver of leatherback foraging ecology and habitat use, yet we lack essential information about leatherback prey selectivity, and the abundances and ecological roles of gelatinous organisms in leatherback foraging areas. This is important, as previous studies that categorize leatherback prey as 'gelatinous zooplankton' oversimplify the potential variation in nutritional quality of different gelatinous organisms that may contribute to their diets. Indeed, ecological studies on marine food webs and ecosystems have given less consideration to gelatinous organisms compared to other mid-trophic level species. Traditionally, gelatinous organisms have been viewed as non-important ecosystem members and "trophic dead ends" that do not transfer energy to many higher trophic level species (Ruzicka et al. 2012, Purcell 2016). Recent studies have begun to

challenge the trophic dead-end paradigm by illustrating the important contributions of gelatinous zooplankton to food web dynamics, and highlighting the need for more research on these organisms (Henschke et al. 2016, Pascual et al. 2016, Choy et al. 2017).

The term gelatinous zooplankton is often used to group together a suite of organisms that share a gelatinous body plan, but otherwise represent a diverse collection of species with differing population dynamics, ecology, morphology, evolutionary and life history (Madinand and Harbison 2001). Gelatinous organisms therefore occupy different trophic positions (TPs), ecological niches, and serve different ecosystem functions within the CCLME food web (Figure 2.1). It is important to understand the contribution of different energy pathways and gelatinous prey groups to leatherback diets, as energy is lost with each trophic transfer in a food web, and the number of transfers therefore affects the amount of energy that reaches apex consumers (Polis 1991, Post 2002, Barnes et al. 2010). However, this information regarding leatherback diet is largely unknown for individuals foraging in the CCLME. The few observation and animal-borne camera data that exist indicate leatherbacks consume large scyphozoan jellyfish (e.g., *Chrysaora spp.*) but also consume small scyphozoans and other gelatinous prey (e.g., thaliaceans) when they are densely-aggregated (Starbird 1993, Benson et al. 2007a, Fossette et al. 2012, Heaslip et al. 2012). Here, we aim to identify the specific gelatinous energy pathways that support the population of leatherback turtles foraging in the CCLME.

In addition to the relative contribution of different gelatinous prey to leatherback diets, the overall abundances of prey in the CCLME may also impact leatherback foraging ecology and energetics. Annual aerial surveys conducted between mid-August to November have shown that leatherback abundance in the CCLME varies interannually (Benson et al. 2007b). This variation may be driven by environmental variability that affects leatherback prey abundances. The

CCLME is a highly dynamic region which experiences considerable variability in environmental conditions on seasonal, interannual, decadal, and multidecadal time scales (Lynn & Simpson 1987, Bograd & Lynn 2003, Jacox et al. 2016). The extent to which environmental variability affects leatherbacks is unclear, although previous research (Saba et al. 2008, Wallace and Saba 2009) has suggested that differences in oceanic conditions, climate-driven variability, and food supply in leatherback foraging areas may contribute to the disparity in their reproductive output and overall leatherback population trajectories. Specifically, decreases in primary production caused by periodic El Niño events likely limit prey availability to leatherbacks in the eastern Pacific (Wallace et al. 2006, Saba et al. 2008, Wallace & Saba 2009).

During years of favorable conditions, the energetic benefit of foraging in the CCLME may outweigh the cost of migration. However, during environmental perturbations that cause a reduction in upwelling, leatherbacks in the CCLME may be nutrient limited, which could impact the frequency of their reproduction and their overall population trajectory (Wallace et al. 2006, Wallace & Saba 2009). Extrinsic factors (e.g., fisheries bycatch, egg harvest, mortality on nesting beaches) undoubtedly contribute to leatherback population declines in the Pacific, however, differences in environmental conditions and leatherback foraging ecology between oceanic regions may also influence the population trajectory of western Pacific leatherbacks.

Although we have fine scale information on leatherback habitat use and diets from aerial surveys, we lack a comprehensive understanding of leatherback prey preferences, residency duration in the CCLME, or temporal trends in trophic ecology, which are necessary for effective management and conservation efforts. Historically, the characterization of trophic ecology has been based on analyses of stomach contents and fecal matter from consumers (Hyslop 1980). However, these methods are often ineffective for examining gelatinous trophic pathways, as

gelatinous organisms are quickly digested and show little trace in the feces of consumers. Additionally, opportunities for stomach contents analysis on leatherbacks is limited only to bycatch from fisheries and rare strandings of dead individuals. To avoid such shortcomings, we stable isotope analyses to answer fundamental questions about the trophic ecology of leatherback turtles and their often-understudied gelatinous prey. Stable isotope analyses provide a method by which we can evaluate predator-prey connections in the often-cryptic gelatinous food web pathway. Specifically, bulk nitrogen isotope ratios ($\delta^{15}N$) from marine organisms have been used extensively for elucidating animal diets and trophic structure and bulk carbon isotope ratios ($\delta^{15}C$) have been used to delineate carbon sources in food webs (DeNiro & Epstein 1981, Hobson 1999, Fry 2006).

Estimating TPs of species and interpreting spatial or temporal changes bulk $\delta^{15}N$ values of consumers in a food web can be challenging, as these values are ultimately dependent on the $\delta^{15}N$ values of primary producers at the base of the food web. These baseline $\delta^{15}N$ values can vary substantially across spatial and temporal scales in marine ecosystems and are governed by the dominant nitrogen transformation or cycling processes in the region (i.e. nitrogen recycling, N_2-fixation, or denitrification;(Cline & Kaplan 1975, Altabet 2001, Voss et al. 2001, Montoya et al. 2002, Hannides et al. 2009). Thus, to estimate TPs of species using bulk isotope data, it is necessary to evaluate $\delta^{15}N$ values at the base of the food web, which is often not possible.

One way to account for this baseline variability in $\delta^{15}N$ values is to use compound-specific isotope analysis of amino acids (CSIA-AA), which is becoming increasingly-used in ecological studies. With CSIA-AA, we analyze the $\delta^{15}N$ values of specific amino acids in a consumer's tissue. Certain 'trophic' amino acids (e.g., alanine, glutamic acid) are isotopically enriched in a consumer's tissue, relative to its prey, and thus they reflect the diet of the consumer

(Popp et al. 2007, Chikaraishi et al. 2009). Conversely, 'source' amino acids (e.g., phenylalanine, lysine) show little isotopic fractionation during trophic interactions and they reflect the nitrogen isotope composition at the base of the food web (Chikaraishi et al. 2009). We can then therefore use these amino acid δ^{15}N values to estimate TPs of consumers while accounting for variability in baseline δ^{15}N values (Popp et al. 2007, Chikaraishi et al. 2009) and answer questions about temporal changes in leatherback foraging ecology over time. Our approach was to use multiple methods for estimating TPs of species, relying on both bulk and CSIA-AA and compare these estimates to obtain robust estimates of leatherback and gelatinous zooplankton TPs.

Here, we used carbon and nitrogen stable isotope analyses on leatherback turtles and gelatinous organisms, upwelling data, and abundances of certain gelatinous organisms in the CCLME to examine: 1) potential changes in leatherback trophic ecology over 13 years, 2) the contribution of different gelatinous organisms to leatherback diets, 3) trophic structure and ecological niches of gelatinous zooplankton that support leatherback turtles in the CCLME and 4) whether isotope values support the hypothesis that leatherbacks seasonally reside in the CCLME. Many studies on leatherbacks are limited to their nesting beaches when turtles are more easily accessible, whereas our research disentangles their foraging ecology in an important habitat, the CCLME. We offer insight into trophic ecology and ecological niches of abundant gelatinous zooplankton in the CCLME and trace energy flow pathways from these organisms to an endangered consumer of great conservation concern.

Materials and Methods

Sample Collection and Isotope Analyses

Leatherback sampling locations and dates were based on knowledge of occurrence of foraging leatherback turtles from previous aerial surveys (Benson et al. 2007). Capture and sampling activities occurred in neritic waters (<100-m depth) between Monterey Bay and San Francisco, California, USA (approximately 36.65°N - 37.80°N). Turtles were located with the aid of a spotter aircraft and captured from a boat using a specially designed break-away hoop net. We obtained a skin sample (<5 mm diameter, approximately 1 mm deep) from each captured turtle (n = 41) using a forceps and razor. A new razor was used for each sample. The samples were taken from the epidermis layer at the margin of the carapace and the rear flipper. Samples were stored on ice and frozen and/or in a saturated salt solution for isotope analysis. Skin tissue sampling was conducted under Endangered Species Act permit numbers 1227, 1596, and 15634, using approved animal handling protocols and in conformance with all applicable laws.

Samples were stored in a freezer at -20 °C until further analysis. Samples from leatherbacks and gelatinous zooplankton were rinsed with deioninzed water, lyophilized, homogenized into a fine powder, weighed in tin capsules and shipped to the stable isotope laboratory at the University of Florida (Supplementary Material). Stable isotope ratios are expressed in standard delta (δ) notation in parts per thousand (‰).

We analyzed a small subset of gelatinous zooplankton samples selected for CSIA-AA at the University of Hawaii's Stable Isotope Biogeochemistry Laboratories. For CSIA-AA we prepared the samples by acid hydrolysis followed by derivatization of amino acids. Amino acid isotope values for leatherbacks were previously published in Seminoff et al. (2012) and were analyzed at the same facility, using the same protocol as this study (see Appendix).

Environmental Data

To infer upwelling intensity, we used a mean, monthly index, generated from NOAA's Pacific Fisheries Environmental Laboratory (https://www.pfeg.noaa.gov/). These values represent the wind strength forcing on the ocean, which is measured in metric tons per second per 100 m of coastline. These units therefore represent the mean amount of water that is upwelled through the bottom of the Ekman layer each second, along a defined region of coastline. Data were available for 26 positions in the Northeastern Pacific and we used data from 119°W, 33°N, as it is closest to our sampling locations and known habitat for leatherbacks. We used these upwelling data to create time series, which we decomposed to remove the seasonal component, and tested for outliers to identify potentially anomalous upwelling conditions during our sampling period.

We obtained abundances (number per m^2) of thaliaceans (salps and pyrosomes) from spring sampling efforts from the California Cooperative Oceanic Fisheries Investigations (CalCOFI) and the Scripps Institution of Oceanography's open-access Zooplankton Database (https://oceaninformatics.ucsd.edu/zooplankton/). Abundance estimates represent the "Central California" region of the CalCOFI sampling grid, which encompasses lines 60 to 70, stations out to and including 90. Zooplankton samples were collected using bongo tows (see calcofi.org for details on sample collection). Samples from multiple bongo tows were pooled within a region to obtain estimates of spring abundance for certain taxa. We used these pooled estimates of salp and pyrosome abundances to evaluate changes in thaliacean abundances over our sampling period, which may be relevant for interpreting trends in leatherback diet and habitat use.

Leatherback isotope values, Mixing Models, and Isotopic Niches

To test for potential temporal changes in leatherback trophic ecology or diet, we used univariate linear models to evaluate changes in δ^{13}C and δ^{15}N from 2003–2016. We also tested for differences in both isotope values and size (curved carapace length (CCL)) between male and female leatherbacks using Welch's two sample t-tests.

Prior to estimating the contributions of different gelatinous prey to leatherback diets, we used an ANOVA and Tukey's *post-hoc* analysis to evaluate whether prey values from prey multiple species were distinct a) from one another and b) from leatherbacks. Then, to evaluate the contributions of these potential prey to leatherback diet, we used the package SIMMR (Stable Isotope Mixing Models in R) in the statistical software R (Team 2016), which relies on a Bayesian framework to solve mixing equations with stable isotope data (Parnell et al. 2013). The functions in this package were used to run Markov Chain Monte Carlo (MCMC) simulations (See Appendix for details), which repeatedly estimated the proportions of different prey items in leatherback diets. Over many simulations, we estimated the values (proportions) which represent the best fit based on our inputted isotope data. Our model included 10,000 iterations with a burn-in period of 1,000 iterations. We then sampled those iterations to calculate the probability distributions of the following prey groups to leatherback diets: Thaliacea (salps), *Chrysaora spp.* (sea nettles), *Phacellophora spp.* (egg-yolk jellies), and *Aurelia spp.* (moon jellies), which are hereafter referred to by their class (Thaliacea) or genera (Chrysaora, Phacellophora, Aurelia).

Stable isotope values can provide information about resource use and have been used to estimate components of organisms' niche space, referred to as an 'isotopic niche' (Newsome et al. 2007, Jackson et al. 2011, Rossman et al. 2016). We aimed to compare the isotopic niche widths of leatherbacks and gelatinous zooplankton, using the package SIBER (Stable Isotope Bayesian Ellipses in R). Using SIBER functions, we calculated the isotopic ranges for each

species or group. This provided univariate measures of δ^{13}C and δ^{15}N ranges, which are comparable to standard deviations around univariate data and these were used to calculate the bivariate ellipses (Jackson et al. 2011), Here, the standard ellipse area (SEAc) represents the isotopic niche width of a species, corrected for sample size. This metric is advantageous over other metrics (e.g., calculating convex hulls), as it captures the majority of the data but is not skewed by extreme values (see Jackson et al. [2011] for detailed methodology on SIBER metrics and functions). Within SIBER, we also used Bayesian approaches (MCMC simulations) to estimate isotopic niche width. This approach provided a metric by which to measure uncertainty (i.e., credible intervals) around our SEAc estimates. To compare SEAc estimates among groups, we used a maximum likelihood approach to compare pairs of MCMC simulations of posterior distributions of different groups to test for differences in isotopic niche width among species.

Estimating Trophic Positions (TPs)

Estimating TPs of species allows for the trophic placement of organisms within a food web. However, TP estimates from different approaches can produce conflicting results. We estimated TPs of leatherbacks and gelatinous organisms using multiple approaches, which rely on either bulk or amino acid isotope values. First, we used bulk δ^{15}N values to estimate TPs of species using a novel Bayesian approach in the package 'tRophicposition' in R (Quezada-Romegialli et al. 2018; Supplementary Material), which relies on the following equation:

$$\delta^{15}N_{consumer} = \delta^{15}N_{baseline} + \mathrm{TDF}(\mathrm{TP} - \lambda) \quad (1)$$

where λ represents the TP of the baseline isotope data, which we set to 1 for primary producers, and TDF is the trophic discrimination factor. The advantages of this Bayesian approach are that we can include variability in two key parameters that are used to estimate TP: the baseline

isotope values of primary producers and the TDF. Thus, our TP estimates are likely more robust than relying on traditional approaches for estimating TP. We used a range of baseline δ^{15}N values, from particulate organic matter in the California Current from Kurle & McWhorter (2017). For TDFs, we used a value for leatherbacks from Seminoff et al. (2009) and TDFs from McCutchen et al. (2003) for gelatinous zooplankton. See Supplementary Material for more details.

We also estimated TPs using amino acid δ^{15}N values from CSIA-AA. The sample size was limited due to the high cost and labor associated with this analysis. We used the following equation from Chikaraishi et al. (2009) to estimate TPs of three scyphozoan species:

$$TP_{Trophic-Source} = \frac{(\delta^{15}N_{Trophic} - \delta^{15}N_{Source}) - \beta}{TDF} + 1 \qquad (2)$$

where $TP_{Trophic\text{-}Source}$ is the TP based on the difference in mean δ^{15}N values from the trophic (glutamic acid) and source (phenylalanine) amino acids, TDF is 7.6 ‰ and represents the ^{15}N enrichment of trophic relative to source amino acids per trophic step, and β represents $\delta^{15}N_{Trophic}$-$\delta^{15}N_{Source}$ in primary producers (3.4 ‰). Since our sample size for CSIA-AA was limited, we could not test for statistical differences between TP estimates derived from CSIA-AA. Rather, we used bulk isotope values to compare TPs across groups. We were unable to use more-recently developed approaches for estimating TP with amino acid δ^{15}N values (e.g., Bradley et al. 2015, Nielsen et al. 2015) because certain amino acids required for those equations were not detected on chromatograms of our samples.

Results

Leatherback Isotope values

Leatherback turtle $\delta^{15}N$ or $\delta^{13}C$ values were unimodally distributed and we found no overall trend in leatherback $\delta^{15}N$ values over time. Although there was no difference in $\delta^{15}N$ across years from 2003–2016 ($F_{(1,51)} = 1.0$, $R^2 = 0.001$, $p = 0.30$), we found a weak significant difference in $\delta^{13}C$ values ($F_{(1,51)} = 4.95$, $R^2 = 0.07$, $p = 0.03$; Figure 2.2a and b). A Tukey's *post-hoc* test indicated that this difference was driven by the high $\delta^{13}C$ values in 2005. $\delta^{13}C$ values in 2005 were significantly higher than 2003 ($p = 0.01$), 2004 ($p < 0.001$), 2007 ($p = < 0.001$), and 2016 ($p < 0.0001$). $\delta^{15}N$ values were positively related to leatherback size, measured by curved carapace length ($F_{(1,36)} = 13.02$, $R^2 = 0.24$, $p < 0.001$) but we found no relationship between $\delta^{13}C$ and curved carapace length ($F_{(1,36)} = 0.11$, $R^2 = –0.24$, $p > 0.1$; Figure 2.2 c and d).

Female turtles were larger than males ($t = 3.06$, $df = 43.62$, $p = 0.004$), where means ± SD for females and males were 158.9 ± 7.7 cm and 154.1 ± 5.1 cm, respectively (Appendix Figure 2S.2). There were no differences in isotope values between male and female leatherback turtles ($\delta^{15}N$: $t = 1.29$, $df = 37.7$, $p = 0.20$; $\delta^{13}C$: $t = –0.38$, $df = 34.92$, $p = 0.70$; Appendix Figure 2S.2). The mean $\delta^{15}N$ and $\delta^{13}C$ values for females were 13.6 ± 1.0 ‰ and –16.2 ± 0.8 ‰, respectively, and for males were 13.2 ± 0.8 ‰ and –16.1 ± 0.8 ‰, respectively.

Gelatinous Energy Pathway/Food Web Analyses

We found significant differences in $\delta^{15}N$ among species of gelatinous zooplankton and leatherback turtles ($F_{(5,286)} = 198.1$, $R^2 = 0.77$, $p < 0.00001$; Figure 2.3). A Tukey's pairwise comparison indicated that twelve of fifteen comparisons were significantly different ($p < 0.05$). There were no significant differences ($p > 0.05$) in $\delta^{15}N$ values between *Chrysaora* and *Aurelia*, or *Phacellophora* and *Aurelia*. $\delta^{13}C$ values were also significantly different among species

($F_{(5,286)} = 175.4$, $R^2 = 0.75$, $p < 0.00001$; Figure 2.3), but Tukey's pairwise comparisons indicated significant differences ($p < 0.05$) only between thaliaceans and leatherback tissues.

We used an isotope mixing model approach for estimating the contributions of different gelatinous organisms to leatherbacks' diet. Based on the SIMMR model, leatherbacks in the CCLME are primarily foraging on *Chrysaora* and thaliaceans (Figure 2.4, Table 2.1). We found the highest probability (0.59) for the following order of prey, from largest to smallest contributors of leatherback diet: *Chrysaora*, thaliaceans, *Aurelia*, *Phacellophora*. The second highest probability was 0.30 for the following order: *Chrysaora*, thaliaceans, *Phacellophora*, and *Aurelia*.

To estimate the isotopic niche widths of species, we calculated the standard ellipse areas, corrected for sample size (SEAc) of leatherbacks and four gelatinous prey groups: thaliaceans, *Phacellophora*, *Aurelia*, and *Chrysaora*. Isotopic niche widths varied among taxa (Figure 2.5a). The ellipse areas for gelatinous zooplankton were, in order from smallest to largest area: *Aurelia spp.* (1.02 $‰^2$), thaliaceans (1.42 $‰^2$), *Chrysaora* (1.62 $‰^2$) and *Phacellophora* (1.91 $‰^2$). The SEAc was the largest for leatherbacks (3.08 $‰^2$). MCMC simulations provided credible intervals, or uncertainty estimates, around our SEAc (Figure 2.5, Table 2.2).

Trophic position estimates

Using a bulk $\delta^{15}N$ data and a Bayesian approach for estimating TPs, we found median TPs from posterior distributions in the following order, from highest to lowest; leatherbacks (3.4), *Phacellophora* (3.0), *Aurelia* (2.6), *Chrysaora* (2.1), and thaliaceans (2.6; Table 2.3). Pairwise comparisons showed that there was a high probability that leatherback TPs were higher than all prey items (> 95 %), and thaliacean TPs were lower than all other species (100%).

Among the scyphozoans, the TP estimate for *Chrysaora* was less than *Aurelia* and *Phacellophora*, which had the highest TP of all gelatinous zooplankton (Appendix).

We also used amino acid δ^{15}N values to estimate TP, although the small sample size obscured our ability to statistically test differences in TPs among species. The gelatinous prey TPs were similar among species, where TP for *Phacellophora* was 2.9 (n = 2), *Chrysaora* ranged from 2.2-2.7 (n = 2) and *Aurelia* (n = 2) ranged from 2.8-3.0, respectively. Leatherback TPs from CSIA-AA from Seminoff et al. (2012) were 2.4 (n = 3; Table 2.4).

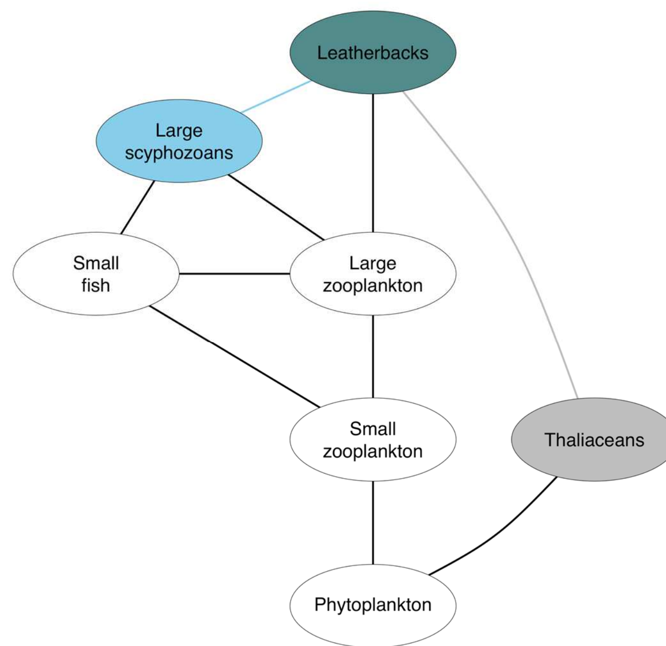

Figure 2.1. Conceptual diagram of gelatinous food web pathways in the California Current Large Marine Ecosystem (CCLME) that support a subpopulation of endangered leatherback turtles, where lines indicate trophic interactions (predator-prey relationships).

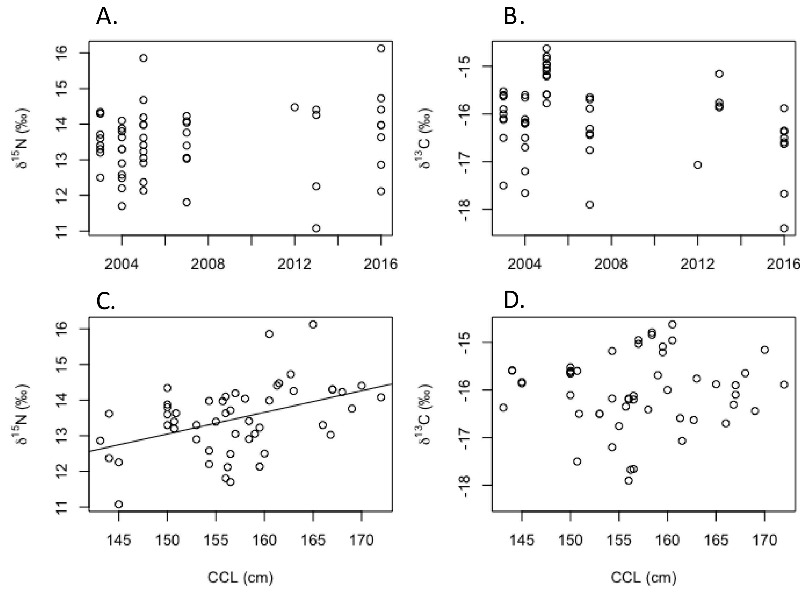

Figure 2.2. The A) δ^{15}N values from leatherback skin samples (n = 41) did not change over time from 2003 to 2016 B) δ^{13}C values from leatherbacks in 2005 were higher compared to other years. There was a significant positive linear relationship between the C) δ^{15}N values from leatherbacks and their size (curved carapace length or CCL), but not for their D) δ^{13}C values and size.

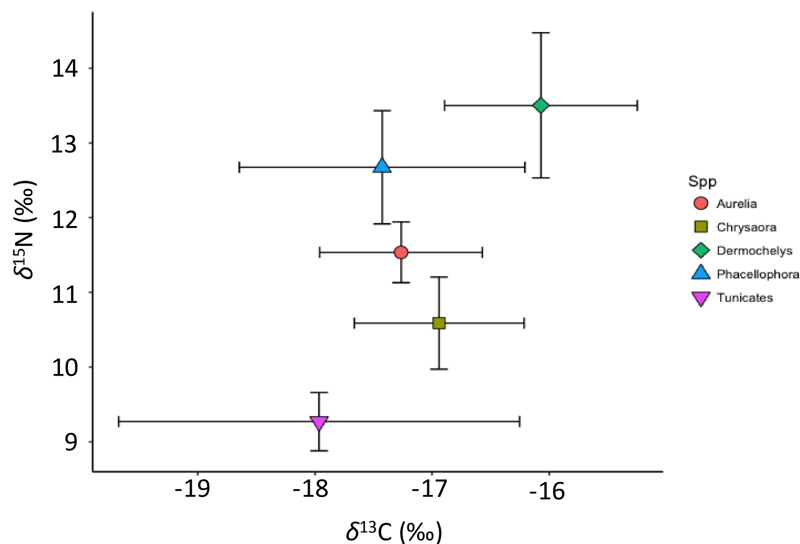

Figure 2.3. The mean δ^{15}N and δ^{13}C values (uncorrected for trophic discrimination factors) for leatherback turtles (*Dermochelys*) and four potential gelatinous prey groups: *Aurelia*, *Chrysaora*, *Phacellophora*, and thaliaceans (tunicates), where error bars represent 1 standard deviation from the mean.

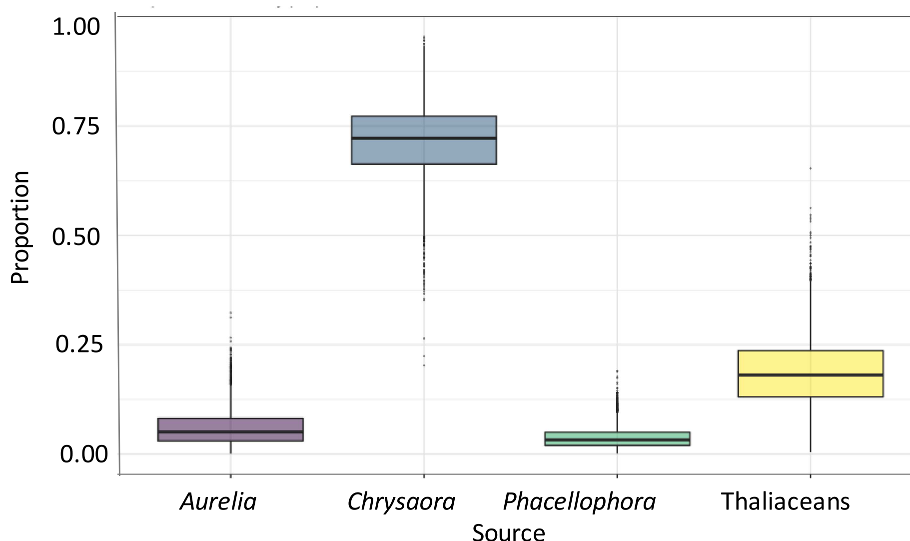

Figure 2.4. Estimates of the mean contribution (proportion) of four gelatinous prey groups to the diets of leatherbacks in the California Current Large Marine Ecosystem. Estimates were derived from 10,000 Markov Chain Monte Carlo simulations in the R package SIMMR, where error bars indicate 95 % credible intervals.

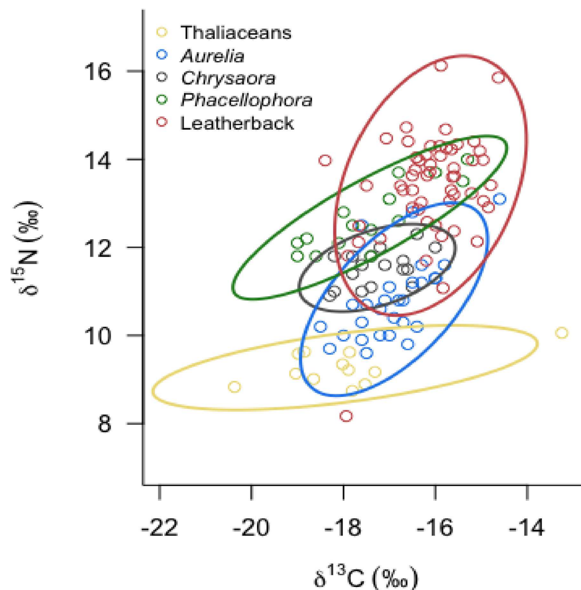

Figure 2.5. The δ^{15}N and δ^{13}C values of leatherbacks and their potential gelatinous prey (uncorrected for trophic discrimination factors), where colors are indicative of different species and prey groups (Tha = Thaliaceans, Aur = *Aurelia*, Chry = *Chrysaora*, Phac = *Phacellophora*, Dc = leatherbacks. The ellipses represent the maximum likelihood standard ellipse areas (SEA), See Table 2.2 for SEA values calculated from Monte Carlo Markov Chain simulations for leatherbacks and their prey.

Table 2.1. Estimated probabilities of distinct prey contributions to the diets of leatherback turtles foraging in the California Current. Values were calculated using Bayesian inference in the package 'SIMMR' (see methods and supplementary material for details) and they represent probabilities of the proportion of each prey contribution. The frequency of probability estimates are grouped into the following quantiles: 2.5, 25, 50 (median), 75, and 97.5 %.

	2.5%	**25%**	**50%**	**75%**	**97.5%**
Aurelia	0.01	0.03	0.05	0.08	0.16
Chyrsaora	0.51	0.66	0.72	0.77	0.87
Phacellophora	0.01	0.02	0.03	0.05	0.10
Thaliaceans	0.05	0.13	0.18	0.24	0.77

Table 2.2. Estimated Standard Ellipse Area sizes ($‰^2$), which represent isotopic niche width. Values were estimated using a Bayesian approach that relies on Markov Chain Monte Carlo simulations in the 'SIBER' package in the statistical software R.

	Min	**1st Quartile**	**Median**	**Mean**	**3rd Quartile**	**Max**
Leatherbacks	2.14	2.87	**3.14**	3.18	3.45	6.55
Aurelia	0.56	0.98	**1.13**	1.18	1.35	2.87
Chrysaora	0.48	0.73	**0.82**	0.84	0.94	2.02
Phacellophora	0.85	1.53	**1.18**	1.91	2.19	5.08
Thaliaceans	0.69	1.13	**1.59**	1.68	1.95	5.61

Table 2.3. Trophic Position (TP) estimates from bulk δ^{15}N values (SI Equation 1) using a Bayesian approach in the package tRophicposition in R. From repeated sampling and MCMC simulations, we present the range of TP estimates (minimum and maximum), along with the median, mean, and interquartile range (1st and 3rd quantiles), which represent the proportion of the data that contained each TP estimate.

Species	**Min**	**1st Quantile**	**Median**	**Mean**	**3rd Quantile**	**Max**
Leatherback	2.8	3.3	**3.4**	3.4	3.5	4.4
Aurelia	2.2	2.5	**2.6**	2.6	2.7	3.3
Chrysaora	1.8	2.1	**2.1**	2.1	2.2	2.7
Phacellophora	2.4	2.9	**3**	3	3.2	3.9
Thaliaceans	1.2	1.5	**1.6**	1.6	1.6	2

Table 2.4. Bulk stable isotope values, source ($\delta^{15}N_{phe}$) and trophic ($\delta^{15}N_{glu}$) amino acid values (‰), and TP estimates of leatherback turtles and gelatinous organisms. Trophic positions were estimated using the Chikaraishi et al. (2009) approach (TP_{AA}).

Species	Sample	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N_{glu}$	$\delta^{15}N_{phe}$	TP_{AA}
Leatherback	1	14.7	-17.4	21.3 (0.6)	7.4 (0.4)	2.4 (0.2)
	2	15.9	-17.5	20.2 (0.7)	6.9 (0.3)	2.3 (0.2)
	3	15.2	-17.1	21.4 (0.3)	7.5 (0.4)	2.4 (0.2)
Aurelia	1	12.2	-16.6	22.4 (0.2)	5.3 (0.7)	2.8 (0.2)
	2	12.1	-17.3	22.9 (0.1)	4.6 (0.4)	3 (0.2)
Chrysaora	1	10.1	-17.6	20.3 (0.6)	4.2 (0.6)	2.7 (0.2)
	2	10.6	-17.7	19.6 (0.2)	7.2 (0.7)	2.2 (0.2)
Phacellophora camstachatica	1	12.4	-16.9	21.9 (0.3)	4.1 (0.4)	2.9 (0.2)
	2	12.6	-16.1	24.0 (0.5)	5.9 (0.5)	2.9 (0.2)

Discussion

Pelagic marine food webs can be difficult to disentangle due to complex predator-prey relationships, cryptic life histories and sampling difficulties of many species (e.g., endangered or highly migratory species, gelatinous or fragile organisms). Our study aimed to examine the trophic relationships in an often-understudied component of marine food webs: gelatinous pathways that support several higher trophic level species, including endangered leatherback turtles. This study highlights the utility of applying Bayesian techniques to stable isotope data to answer questions about food web dynamics and the trophic ecology of species. Here, we estimated trophic positions and isotopic niche widths for leatherbacks and a several common gelatinous organisms in the CCLME.

We found no trends in leatherback diet or trophic position over 13 years (2003-2016), indicating relative trophic stability over our sampling period. However, $\delta^{13}C$ values were significantly higher during 2005, which may suggest differences in leatherback habitat use during a period that coincided with anomalous upwelling and thaliacaean abundances. Overall,

the isotope mixing model revealed that leatherbacks are primarily feeding on *Chrysaora* (sea nettles), although thaliaceans (salps) comprised a substantial portion of their diet, which has previously not been documented for this foraging group of leatherbacks. Additionally, these results suggest that leatherbacks are selective, as they are not consuming *Aurelia* (moon jellies) or *Phacellophora* (egg yolk jellies) despite their common occurrence in leatherback their foraging areas. Using isotope analyses, the trophic structure of this gelatinous pathway showed that leatherback TPs were highest, followed by *Phacellophora*, *Aurelia*, *Chyrsaora*, and thaliaceans, although there were some discrepancies between our two analytical approaches for estimating TP. There was niche, or trophic overlap between the scyphozoan jellies while leatherbacks had a substantially higher niche width compared with its prey groups.

Trends in leatherback foraging ecology

In this study leatherbacks were sampled from foraging animals, which provided a rare opportunity to gain information about male turtles, as they do not go onto the beaches during nesting season, and are thus sampled less frequently. We found no difference in $\delta^{15}N$ or $\delta^{13}C$ values between males and females, which suggests that male and female leatherbacks exhibit similar habitat use and trophic patterns, which supports data from foraging leatherbacks in the North Atlantic (Dodge et al. 2011). Satellite telemetry data have also indicated that that males and females mix at temperate foraging grounds in the western North Atlantic (James et al. 2007), which is consistent with our findings and provides promise for future isotopic studies which only have access to females on nesting beaches.

$\delta^{15}N$ values can provide insights into broad-scale habitat use patterns of migratory animals, as there are natural isotopic gradients in marine systems due to spatial differences in

nitrogen biogeochemical cycling processes, which are well-documented in the Pacific Ocean (Gruber & Sarmiento 1997, Voss et al. 2001, Somes et al. 2010). Isotope values from leatherback skin is integrated over several months, likely on the order of 4-6 months for adults (Wallace et al. 2006, Seminoff et al. 2007), and therefore they may provide insight into leatherback habitat use. A previous study reported regional differences in the δ^{15}N values of Pacific leatherbacks, where eastern Pacific-foragers had characteristically higher δ^{15}N values compared to western or central Pacific foragers (Seminoff et al. 2012). In this study, both δ^{15}N and δ^{13}C values were unimodal and normally distributed (Appendix Figure 2A.1), which suggests that leatherbacks were residing in the eastern Pacific for several months prior to sample collection. If leatherbacks inhabited the central or western Pacific prior to our sample collection, we would expect a bimodal distribution (Seminoff et al. 2012, Tomaszewicz et al. 2017) or a wider range of δ^{15}N values. Previous work suggests that between nesting intervals in Indonesia, the eastern Pacific foragers migrate to the CCLME during the summer and fall but move to lower-latitude areas during winter months and then return to the CCLME the following year (Benson et al. 2011). Our results provide support for this hypothesis and suggest that the leatherbacks sampled in our study seasonally resided in the CCLME.

We were particularly interested in using isotope values to assess changes in foraging ecology over time. We found no long-term trend or changes in leatherback δ^{15}N values from 2003-2016, which indicates that leatherback diet or TP did not shift over our sampling period. However, δ^{13}C values, which can reflect habitat use, were higher in 2005 compared with other years. Higher δ^{13}C values can be associated with nearshore systems compared with offshore, pelagic systems with lower δ^{13}C values (Clementz & Koch 2001). This corroborates data from 2005 aerial surveys, which noted that leatherbacks exhibited a restricted range and were only

encountered around San Francisco Bay (Peterson et al. 2006). In Monterey Bay, where leatherbacks are typically encountered but were not observed during that year, *Phacellophora* and *Aurelia* were the dominant scyphozoan species, but around San Francisco Bay, *Chrysaora* were densely aggregated in locations where leatherbacks were encountered (Peterson et al. 2006). Our isotope data therefore support the hypothesis that leatherbacks selectively prey on *Chrysaora*, which may explain their change in distribution during 2005.

Interestingly, in 2005, salp and pyrosome abundances were higher than most other years in our time series (Appendix Figure 2A.4). It is possible that environmental conditions were unfavorable for scyphozoans but favorable for filter-feeding thaliaceans. Thaliacean abundances were also high in 2012, when a wide-spread salp bloom occurred in the southern CCLME (Smith Jr et al. 2014), which is apparent in our thaliacean time series. Unfortunately, we could not directly compare leatherback foraging ecology between 2005 and 2012 because we were only able to collect one leatherback skin sample in 2012.

We hypothesize that anomalous conditions in the CCLME in 2005 impacted the distribution of gelatinous prey and subsequently, leatherbacks. Benson et al. (2007a) suggested that hydrographic features on the California coast create localized areas where upwelled-water is retained in nearshore habitats (i.e., upwelling shadows, Graham & Largier 1997), which produces favorable conditions for gelatinous organisms that leatherbacks consume. In 2005, upwelling was delayed and there were fewer periods of wind relaxation events during the summer (Peterson et al. 2006). We evaluated the temporal trends in upwelling strength around Monterey Bay and found that upwelling was low in 2005 compared to other years, which is in agreement with Peterson et al. 2006 (Figure 2A.3). These anomalous conditions may have impacted the abundance and distribution of gelatinous zooplankton and therefore affected

leatherback habitat use during 2005, where their range was more restricted to areas where *Chrysaora* were still abundant.

Isotopic Niches

Our objective was not only to evaluate the trophic ecology of leatherback turtles, but also to examine the trophic ecology of their potential gelatinous prey. Many gelatinous organisms are understudied components of marine food webs, as they have been viewed as trophic dead-ends that do not directly transfer energy to higher-trophic level species (Ruzicka et al. 2012). Furthermore, gelatinous organisms have a fragile body plan and are destroyed by certain sampling techniques (e.g., net tows), which has further limited our ability to examine their ecology and biology. However, recent studies (e.g., Henschke et al. 2016, Choy et al. 2017) show that gelatinous organisms can compete with forage fish, prey on fish eggs and larvae, export large amounts of carbon to the sea deep ocean, and can quickly respond to pulses in nutrient concentrations (Smith et al. 2014, Choy et al. 2017). These organisms can often undergo considerable blooms in coastal areas, contribute substantially to the biomass of zooplankton, and thus are important components of marine food webs.

To examine the ecological roles of our species of interest, we used $\delta^{15}N$ and $\delta^{13}C$ data to estimate their 'isotopic niches', which can provide information about resource use and diet ($\delta^{15}N$), and primary production and environment ($\delta^{13}C$; Newsome et al. 2007, Rossman et al. 2016). We estimated the isotopic niche widths of leatherbacks and gelatinous zooplankton by comparing standard ellipse areas (SEAc) between our species. SEAc values indicated niche overlap among gelatinous zooplankton, particularly between *Chrysaora* and *Aurelia*. This is in general agreement with a previous study found a 75% diet overlap between *Chrysaora* and

Aurelia in the Northern California Current, where euphausiid eggs and nauplii, and copepods comprised much of their diets, although *Aurelia* consumed fewer copepods and more pteropods and larvaceans than *Chrysaora* (Suchman & Brodeur 2005). Of the gelatinous zooplankton, *Phacellophora* had the highest SEAc, which is reflective of its carnivorous and opportunist feeding strategies. *Phacellophora* are carnivorous predators that consume copepods, fish larvae, and chaetognaths (Suchman and Broeduer 2005). They also eat other medusa, including *Aurelia*, and we would expect higher TPs and isotopic niche widths for *Phacellophora* compared with other scyphozoans.

Our results indicate that leatherbacks had a larger niche than their gelatinous prey. It is possible that the higher SEAc values in leatherbacks may also be attributed to their highly migratory behavior. Since isotope values are integrated over time and space and our leatherback values were collected over thirteen years, these values incorporate intrinsic variability in δ^{15}N and δ^{13}C values in within the CCLME. However, the δ^{15}N of leatherbacks did not change over time, and previous studies (Rau et al. 2003, Ohman et al. 2012) show relative temporal stability in δ^{15}N in the CCLME. Therefore, it seems unlikely that baseline δ^{15}N variability contributed to the high SEAc for leatherbacks. We hypothesize that the high SEAc values of leatherbacks reflect their ability to consume different types of gelatinous prey that span multiple trophic levels (e.g., salps, carnivorous scyphozoans).

Trophic structure

We estimated the TPs of leatherbacks and their gelatinous prey in the CCLME food web using two approaches. As isotope analyses becomes increasingly-used in studies on food web dynamics, there have been novel developments in how to best estimate TPs of organisms (Popp

et al. 2007, Chikaraishi et al. 2009, Quezada-Romegialli et al. 2018). In this study, we relied on both bulk and amino acid δ^{15}N values to estimate TP, as these approaches have different advantages and limitations.

The Bayesian approach for estimating TP with bulk δ^{15}N values incorporates variability both in δ^{15}N values at the base of the food web and in the trophic discrimination factor (TDF). This approach therefore overcomes previous limitations of bulk isotope analysis and provides a more robust method for estimating TPs. Estimates from this approach indicated differentiation between species, where leatherback TPs were higher than all potential prey, which is ecologically realistic considering that leatherbacks consume gelatinous zooplankton and thus should have higher TPs than their prey. Thaliaceans had lower median TPs (1.6) compared to other taxa, which is what we anticipated for filter-feeding organisms. There was trophic overlap between *Aurelia* (median TP = 2.6) and *Chrysaora* (median TP = 2.1), but *Phacellophora* TP estimates were higher than other scyphozoans (median TP = 3.0) and almost as high as leatherback TPs, which supports the hypothesis that they are carnivorous predators and likely feed a variety of prey across multiple trophic levels (see above).

Our second approach was to estimate TPs of species using amino acid δ^{15}N values. CSIA-AA can overcome limitations of previous bulk isotope approaches, as it does not require sampling the base of the food web to account for baseline variability in δ^{15}N (Popp et al. 2007, McMahon and McCarthy 2016). However, this approach also has limitations, which have been highlighted by recent studies (McMahon & McCarthy 2016). Most notably, amino acid TDFs can vary widely among taxa (Bradley et al. 2015, McMahon and McCarthy 2016, Hetherington et al. 2017), which can introduce errors in TP estimates. Without controlled feeding experiments, it is often difficult to discern the TDF of consumers.

The TP estimates for scyphozoan jellies using CSIA-AA were ecologically realistic, ranging from 2.2–3.0. Overall, these TP estimates indicated trophic overlap between *Phacellophora*, *Aurelia*, and *Chrysaora*, which suggests niche overlap between these species and is consistent with both our isotopic niche estimates and TP estimates using bulk isotope values. For *Chrysaora*, we found intraspecific variation in TP, where estimates were 2.2 and 2.7. These results were somewhat unsurprising, as scyphozoan medusa have variable diets and this difference could be a relic of our small sample size and the variability in diet of these carnivorous predators.

TP estimates from leatherbacks were lower than anticipated (TP = 2.3–2.4), which is not higher than their known prey, indicating that these values are ecologically unrealistic. This supports previous hypotheses that CSIA-AA produces unrealistic TPs for leatherback turtles (Hetherington et al. under review), which is likely due to the high TDF (7.6 ‰) value that we used to estimate TP. Although there are no published TDFs for leatherbacks, 7.6 ‰ is likely too high for marine turtles, given their mode of nitrogen excretion (i.e. uric acid excretion) and trophic level. When we applied a TDF for captive green turtles (5.03 ‰ from Lemons 2018), leatherback TP estimates were more realistic (3.1).

We aimed to compare the results from these two approaches and found the largest discrepancy in TPs of leatherbacks, where TPs derived from the CSIA-AA approach were lower than we anticipated. We attribute this difference to the TDF that was used to estimate TP using the CSIA-AA approach (see above). For the scyphozoans, *Aurelia* and *Phacellophora* TP estimates were comparable between approaches, with an indication that *Phacellophora* may have a slightly higher trophic position than *Aurelia*. This is consistent with current knowledge on their diets, where *Phacellophora* prey on a wide variety of prey items, including other scyphozoans

and fish. Among the scyphozoans, *Chrysaora* had the highest departure between one CSIA-AA sample and bulk isotope estimates, which may indicate that *Chrysaora* predominately feeds at a relatively lower TP, but is capable of consuming higher trophic level prey.

Through estimating TPs using multiple approaches, we gained greater insight into the trophic placement of gelatinous energy pathways that support leatherbacks in the CCLME. Although CSIA-AA can undoubtedly overcome some limitations of bulk isotope analysis by accounting for the variability in isotope values at the base of the food web, it is limited by reliance on TDF values, which vary widely among taxa. For many species, including megafauna like leatherbacks, and delicate organisms like gelatinous zooplankton, determining TDFs through laboratory feeding experiments is unrealistic. Recent work provides promise for using phylogenic relationships to estimate TDFs of species (Healy et al. 2017), which may be useful for future studies that aim to evaluate TPs of consumers with unknown TDFs. To overcome this limitation, we used an additional method to estimate TP that relied on bulk isotope values and incorporates variability in the TDF and may provide more robust TP estimates.

Conclusions

Overall, our isotope data provided a metric to evaluate trophic structure and energy pathways to leatherbacks in the CCLME. Our results illustrate that leatherback TPs did not change over our sampling period from 2003-2016 but anomalous upwelling conditions may affect their habitat use. Results from an isotope mixing model suggest that leatherbacks selectively feed on *Chrysaora* and thaliaceans, and unimodally distributed isotope values suggest that leatherbacks inhabited the eastern Pacific for several months prior to our sample collection. As leatherback turtles continue to face an array of threats on both their nesting beaches (e.g., egg

poaching) and in their foraging regions (e.g., bycatch in longline fisheries, pollution), intensifying climate change may concomitantly affect leatherbacks through changes in prey abundances in their foraging areas. Prey availability is often a driver of predator habitat use and migration, and therefore identifying resources that support populations of threatened species and understanding energy pathways that lead to these predators in important for proper conservation and management.

Our findings can be applied to models used to predict leatherback foraging ecology and habitat use in the CCLME and how these parameters may change under future environmental conditions. Isotope values from gelatinous zooplankton and leatherback tissues can also be incorporated into future ecosystem models of the CCLME, as many ecosystems do not include data on gelatinous trophic pathways. Furthermore, our research highlights the utility of long-term sample collections, isotope analysis of leatherbacks and their prey as a complementary approach for examining trophic ecology and habitat use, which can be applied to studies on other leatherback populations or highly migratory predators.

Acknowledgements

We would like to thank all employees and volunteers, especially T. Todd Jones, who assisted in field work to collect leatherback samples off Central California. All samples were collected under federal and state permit guidelines. We would also like to thank Karen Arthur and the University of Hawaii's Isotope Geochemistry Laboratory for assistance in sample preparation and isotope analyses. This research was funded by the National Marine Fisheries Services. This is School of Ocean Science and Technology contribution number ####. Salp and pyrosome abundance data were obtained from Scripps Institution of Oceanography (SIO), where

plankton sample analysis was supported by NSF grants to M.D. Ohman, Scripps Institution of Oceanography, and the SIO Pelagic Invertebrates Collection.

Chapter 2, in full, is being prepared for submission in Marine Ecology Progress Series, 2018, Hetherington, ED, Kurle, CM, Benson, S, and Seminoff, JA. The dissertation author is the primary investigator and author of this paper.

Appendix

Stable isotope analysis

For bulk stable isotope analysis, we used a costech ecS 4010 elemental combustion system interfaced via a conFlo III device (Finnigan Mat, Bremen, Germany) to a Deltaplus gas isotope-ratio mass spectrometer (Finnigan Mat, Bremen, Germany). Stable isotope values were measured against laboratory standards: ^{13}C was Baker Acetanilide calibrated monthly against the Peedee Belemnite (PDB) limestone formation international standard. $R_{standard}$ for ^{15}N was IAEA N1 Ammonium Sulfate calibrated monthly against atmospheric N_2 and USGS Nitrogen standards. We analyzed standard materials every 6 to 7 samples to ensure analytical precision.

For compound-specific isotope analysis of amino acids, samples were hydrolyzed (6N HCl, 150 °C for 70 minutes), esterified (4:1 isopropanol:acetyl chloride), derivatized (3:1 methylene chloride:trifluoroacetyl anhydride), and analyzed using a Trace GC gas chromatograph and a Thermo Delta XP mass spectrometer through a GC-C III combustion furnace (980℃), reduction furnace (680℃), and a liquid nitrogen cold trap. Samples were injected (split/splitless, 5:1 split ratio) with a 180℃ injector temperature and a constant helium flow rate of 1.4 mL min^{-1}. The CSIA-AA samples were analyzed in triplicate, corrected the $\delta^{15}N$ values to internal reference compounds. See Popp et al. 2007, Hannides et al. 2013 for more details on CSIA-AA sample preparation and analysis.

Bayesian Approaches

The packages 'Stable Isotope Mixing Models in R' (SIMMR), 'Stable Isotope Bayesian Ellipses in R' (SIBER), and 'tRophicPosition' all rely on a Bayesian framework to either solve mixing equations with stable isotope data, estimate the isotopic niche widths of species using by calculating ellipses, or estimating trophic positions. These package uses Markov chain Monte Carlo (MCMC) simulations through JAGS (Just Another Gibbs Sampler). Using the package 'rjags', we can combine an interface to JAGS in R, which ultimately allows us to sample the posterior density distributions from the MCMC simulations. Here, the MCMC simulations are used to repeatedly estimate a value, and over many simulations, finds values which represent the best fit based on the inputted data. At the beginning of the model run, the MCMC simulations are usually poor, called the 'burn in periods', and these values are discarded. Iterations following the burn in period are then used to create a posterior distribution. The equation used to estimate TP in the 'tRophicPosition' package was:

$$\delta^{15}N_{consumer} = \delta^{15}N_{baseline} + \mathrm{TDF}(\mathrm{TP} - \lambda) \quad (1)$$

where λ represents the TP of the baseline isotope data, which we set to 1, and TDF is the trophic discrimination factor. For our analyses, we defined a Bayesian model using the function *jagsOneBaseline*, where we defined our priors as a normal distribution of an *a priori* TP and standard deviation (3 ± 0.5). Then, we fed the Bayesian model with isotope data using the function *TPmodel*, and defined the number of parallel chains (2) and adaptive iterations (10000). We ran the model to generate large numbers of posterior density distributions of TP and then sampled these posterior trophic position estimates to obtain summary statistics for the posterior estimates.

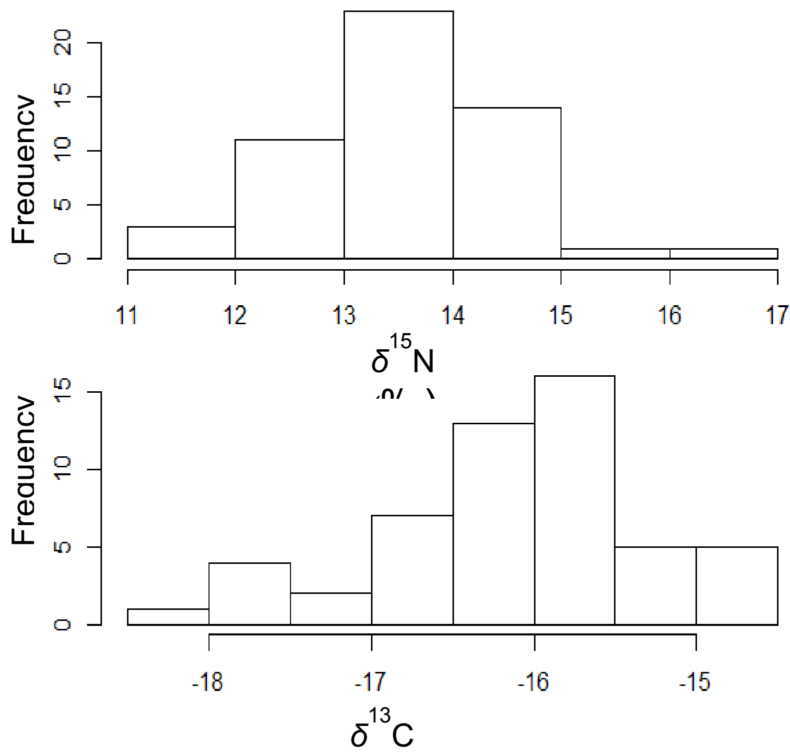

Figure 2A.1. Histograms of A) $\delta^{15}N$ and B) $\delta^{13}C$ values of leatherback turtle skin samples in the CC-LME collected over thirteen years (2003-2016).

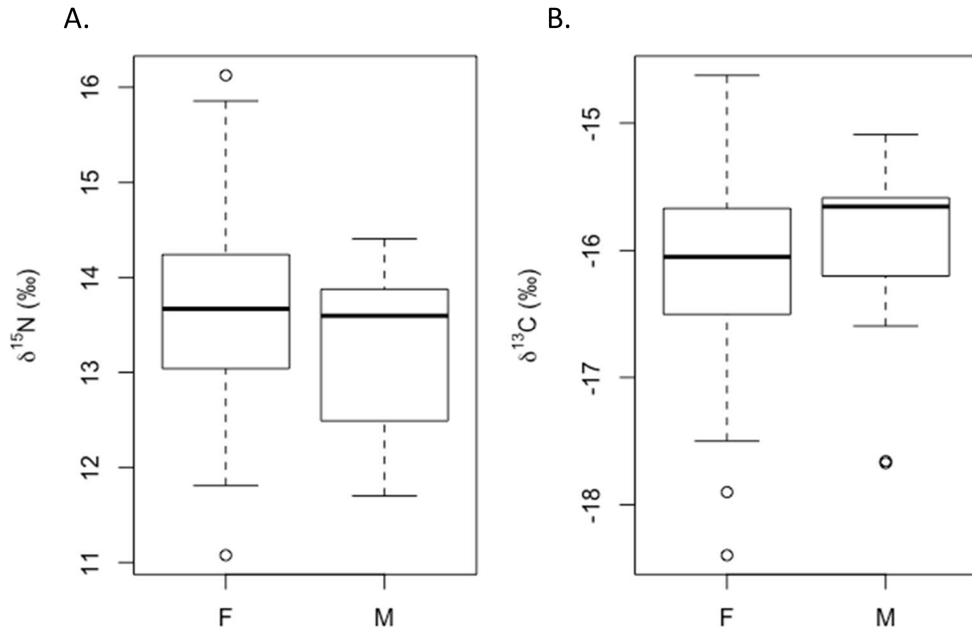

Figure 2A.2. Differences in A) δ^{15}N and B) δ^{13}C values between male and female leatherback turtles in the CCLME.

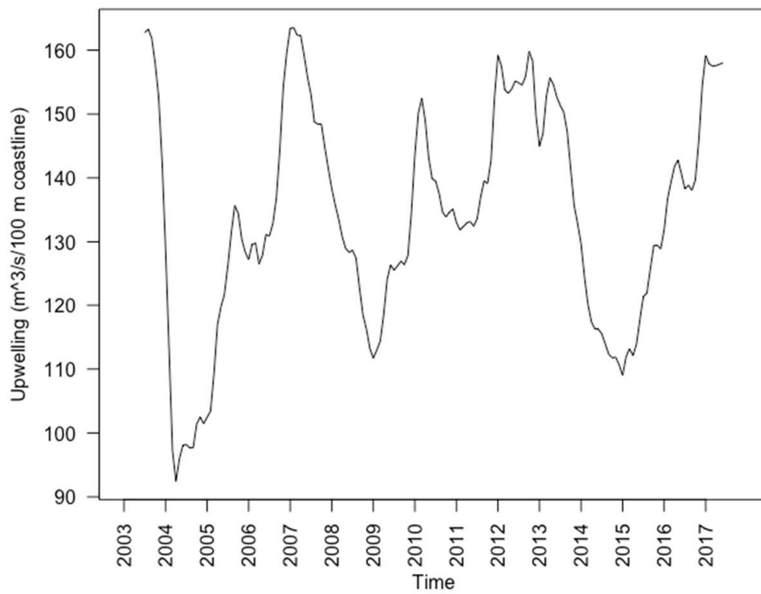

Figure 2A.3. Time series of upwelling index from Monterey Bay over our sampling period. The time series was decomposed and the seasonal component was removed.

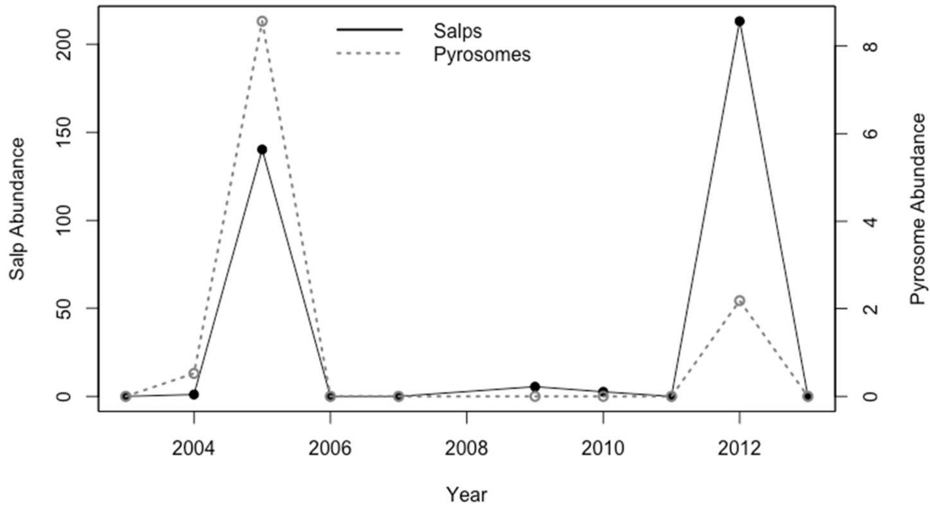

Figure 2A.4. Salp and pyrosome abundances (number per m^2) from CalCOFI samples from 2003-2016. The reported value is an average over multiple pooled samples from central-southern California.

Table 2A1. Isotope values and sample collection information for leatherback samples.

d15N	d13C	%N	%C	Year	Month	Day	Latitude	Longitude	Sex	CCL	CCW (cm)
13.2	-15.6	13.1	42.5	2003	8	29	36.78	-121.8	F	150.7 (	153.3
13.4	-17.5	13.6	44.4	2003	8	29	36.78	-121.8	F	150.7	153.3
13.6	-15.63	13.29	41.94	2003	8	30	36.24	-121.67	M	150	107
14.34	-15.53	13.69	41.29	2003	8	30	36.24	-121.67	M	150	107
14.3	-15.9	13.7	42.4	2003	9	18	36.31	-121.47	F	167	118.5
14.3	-16.1	14.1	44.4	2003	9	18	36.31	-121.47	F	167	118.5
12.5	-16	13.9	43.4	2003	9	19	36.13	-121.69	F	160	115.5
13.3	-16.5	11.7	38.9	2003	9	20	36.9	-121.4	F	153	108.5
13.71	-16.12	15.11	46.86	2003	9	21	36.35	-121.3	F	156.5	107
13.3	-16.7	7.5	25.8	2004	9	6	36.8	-121.9	F	166	121.4
11.7	-16.2	9.4	26.9	2004	9	8	36.56	-121.61	M	156.5	120
12.49	-17.66	7.33	26.13	2004	9	8	36.56	-121.61	M	156.5	120
12.2	-17.2	14.7	40.9	2004	9	22	37.01	-122.65	F	154.3	111
12.58	-16.18	12.11	38.9	2004	9	22	37.01	-122.65	F	154.3	111
13.3	-15.66	11.58	39.16	2004	9	22	37.36	-122.19	M	150	115.5
13.63	-16.18	13.6	41.93	2004	9	22	37.68	-122.4	M	156	105
13.8	-15.6	12.9	38.8	2004	9	22	37.36	-122.19	M	150	115.5
14.1	-16.2	12.8	39.5	2004	9	22	37.68	-122.4	M	156	105
12.9	-16.5	11.3	38.1	2004	9	24	37.41	-122.86	M	153	110.5
12.91	-14.84	11.2	35.72	2005	9	12	37.4	-122.8	F	158.4	117.5
13.41	-14.79	11.27	39.01	2005	9	12	37.4	-122.8	F	158.4	117.5
13.98	-15.18	9.68	31.04	2005	9	13	37.08	-122.43	M	154.3	132
13.99	-14.96	11.81	38.84	2005	9	13	37.52	-122.21	F	160.5	155.5
14.68	-15.78	14.67	46.76	2005	9	13	37.62	-122.6	F		
15.86	-14.63	8.15	37.31	2005	9	13	37.52	-122.21	F	160.5	155.5
12.37	-15.59	12.21	40.4	2005	9	14	37.68	-122.97	M	144	100
13.62	-15.59	11.65	38.31	2005	9	14	37.68	-122.97	M	144	100
12.13	-15.09	10.32	34.98	2005	9	15	37.77	-122.01	M	159.5	111.5
13.23	-15.21	10.72	34.62	2005	9	15	37.77	-122.01	M	159.5	111.5
13.05	-14.95	11.9	39.72	2005	9	21	37.04	-122.12	F	157	118
14.19	-15.03	11.94	38.38	2005	9	21	37.04	-122.12	F	157	118
				2005	9	21	37.06	-122.05	F		
13.06	-15.69	14.6	46.46	2007	9	11	37.64	-122.55	F	159	118.5
13.76	-16.44	15.03	46.15	2007	9	13	37.41	-122.19	F	169	130
14.04	-16.41	15.1	45.79	2007	9	13	37.12	-122.71	F	158	116
14.08	-15.89	15	45.86	2007	9	14	37.99	-122.66	F	172	123.5
13.4	-16.76	15.24	47.1	2007	9	15	37.64	-122.21	F	155	110
11.81	-17.9	14.62	47.42	2007	9	23	37.72	-122.88	F	156	110.5
13.03	-16.31	14.78	46.09	2007	9	25	37.63	-122.58	F	166.8	119.7
14.23	-15.65	14.63	46.07	2007	9	26			F	168	119
14.48	-17.07	14.98	46.27	2012	7	28			F	161.5	121.5
14.26	-15.76	14.89	46.63	2013	9	7	36.8		F	163	119
12.26	-15.86	15.47	47.08	2013	9	27	37		F	145	103
11.08	-15.83	14.82	46.24	2013	9	28			F	145	108
14.41	-15.16	15.06	46.11	2013	9	29			F	170	113
13.98	-18.4	7.88	25.81	2016	8	10			F		
16.13	-15.88	9.01	28.21	2016	9	18	37.1		F	165	120.7
13.97	-16.35	8.55	28.2	2016	9	19	37		F	155.7	118.2
14.41	-16.59	7.73	23.98	2016	9	25			M	161.3	116
14.73	-16.63	9.33	28.75	2016	9	25			F	162.7	133
12.12	-17.67	8.91	27.55	2016	9	26			M	156.2	112.9
12.86	-16.37	8.84	28.25	2016	9	26			F	143.1	108.4
13.63	-16.5	8.47	26.91	2016	9	26			F	150.9	105.7

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CHAPTER 3

Investigating the effects of warm water anomalies on zooplankton trophic ecology in the California current and the implications for food web dynamics

Abstract

In pelagic marine ecosystems, unfavorable environmental conditions can have a profound effect on primary and secondary production, which ultimately affect food web dynamics and energy flow to economically important species and top predators. The recent multi-year warm water anomalies in the Northeastern Pacific (marine heatwave followed by a strong El Niño event) provide an excellent opportunity to evaluate the ecological impacts of warming and environmental variability on the California Current Large Marine Ecosystem (CCLME). Here, we coupled nitrogen stable isotope analyses (bulk and compound-specific isotope analysis of amino acids) of the abundant copepod, *Calanus pacificus*, with environmental data to evaluate differences in trophic ecology, food web dynamics, and environmental conditions associated with the anomalous warming events. To evaluate ecosystem responses to these anomalies, we analyzed δ^{15}N values of quarterly-collected zooplankton samples in the CCLME from 2012-2016. With the onset of warm conditions in the CCLME, we found decreases in an upwelling index, chlorophyll *a* concentrations, nitrate concentrations, and zooplankton biomass. The δ^{15}N values of *C. pacificus* also increased with the warm water anomalies, which was most evident in spring samples, both in bulk tissue and source amino acids, which we used as a proxy for δ^{15}N values at the base of the food web. There was no difference in the trophic position of *C. pacificus* between the neutral and warm period, which suggests that food web elongation did not occur and there were no changes trophic structure over our sampling period. Furthermore, δ^{15}N values were correlated with nitrate concentrations and temperature, indicating that patterns in *C. pacificus* δ^{15}N values were driven by nitrogen cycling dynamics rather than trophic difference. We hypothesize that the increase in δ^{15}N values of *C. pacificus* can be attributed to an increase in nitrate utilization by phytoplankton, relative to supply, during a period of nutrient limitation.

Although we did not find trophic differences with the warm water anomalies, our study demonstrates the effects of warm conditions on a pelagic ecosystem and the trophic ecology of an abundant zooplankton consumer, which may be useful for predicting ecosystem responses to future warming events in the CCLME.

Introduction

Evaluating the configuration of food webs and tracing energy flow through ecosystems is essential for understanding ecosystem structure and function (Paine 1980, Polis & Strong 1996). Food web structure can be regulated by bottom-up (Ware & Thomson 2005, Frederiksen et al. 2006), top-down (Boris & A. 2003, Baum & Worm 2009), or wasp-waist (Griffiths et al. 2013) control, and the relative influences of these processes can change through time and space (Hunt & McKinnell 2006, Lindegren et al. 2018). These processes therefore regulate how energy flows through an ecosystem, which ultimately influences the amount of energy available to species within that ecosystem. It is imperative to understand how environmental variability, increased fishing pressure, and projected climate change may alter food web structure and dynamics, particularly in productive upwelling systems that support a high biomass of commercially and ecologically important species.

The California Current Large Marine Ecosystem (CCLME) is a productive eastern boundary upwelling system, primarily regulated through bottom-up forcing (Lindegren et al. 2018). However, the CCLME is also a highly dynamic ecosystem that is affected by seasonal variability, interannual variability (El Niño-Southern Oscillation (ENSO)), and low-frequency decadal to multidecadal oscillations (e.g., the Pacific Decadal Oscillation and the North Pacific Gyre Oscillation), which can affect ecosystem and food web dynamics on different time scales.

In the CCLME, strong El Niño events (positive mode of ENSO) are associated with increases in sea-surface temperature, the weakening of upwelling-favorable winds, a decrease in upwelling intensity, and subsequent decreases in primary and secondary production, which can have widespread, short-term effects on biology (Barber & Chavez 1983, Barber et al. 1996, Chavez et al. 2002).

ENSO has an average periodicity of 2-7 years (Zhang & Levitus 1997), but both the frequency and intensity of ENSO events are highly variable, where strong events that have more profound effects on biology are much less frequent. Due to the infrequency of strong ENSO events and the difficulties of long-term monitoring, there have been few opportunities to evaluate the change in ecosystem dynamics associated with strong ENSO events. Furthermore, understanding the ecological effects of ENSO events is of particular importance, as some scientists predict an increase in the frequency and intensity of ENSO events with escalating global climate change (Cai et al. 2014), although further research is needed. It is therefore critical to evaluate the effects of warm anomalies as each event can provide insight for predicting species and ecosystem responses to future warm periods.

Recent conditions in the Northeastern Pacific Ocean provide an excellent opportunity to assess the effects of anomalous warming on physical and biological properties in the CCLME. In late 2013, changes in atmospheric pressure led to the development positive temperatures anomalies in the northeastern Pacific (Bond et al. 2015, Di Lorenzo & Mantua 2016). This large area of warm water, termed a 'marine heatwave' (Di Lorenzo & Mantua 2016) continued to develop and persist throughout the northeastern Pacific, where SST rose up to 7°C in certain regions (Di Lorenzo & Mantua 2016, Zaba & Rudnick 2016). Concomitantly, in 2015, strong El Niño conditions developed in the equatorial Pacific, and became the strongest documented El

Niño event in recent history. The marine heatwave and 2015-2016 ENSO event is therefore unique not only in its intensity, but also in its length, as warm conditions persisted for multiple years (Di Lorenzo & Mantua 2016, Jacox et al. 2016, Jacox et al. 2018).

Many ecological effects on marine species were documented during these warm water anomalies (WWA) from 2013-2016, including: the intrusion of tropical and zooplankton and fish species, increased mortality of sea birds, California sea lions, and several whale species (McClatchie & Jahnckenull 2016, Peterson et al. 2016). Numerous changes in trophic ecology and food web dynamics have been associated with previous strong El Niño events, including marine mammal mortality events associated with starvation, declines in commercial fisheries landings, and overall reduction of biomass in the Peru and California Currents (Barber & Chavez 1983, Barber et al. 1996, Chavez et al. 2002). There are several mechanisms through which unfavorable conditions associated with ENSO can propagate through the food web and affect energy flow to higher trophic level species. During these periods of unfavorable conditions, the CCLME food web can shift from a system dominated by bottom-up control to one analogous to a wasp-waist system, where there are interactive effects of both bottom-up and top-down control (Lindegren et al. 2018), which have implications for how energy flows through the ecosystem.

Our study aims to evaluate environmental conditions, trophic ecology of an abundant marine zooplankton species, and pelagic food web length in a region off Central California from 2012-2016, which encompasses the WWA and the preceding neutral period. In marine ecosystems, food web structure is typically sized-based, where predator-prey relationships are size-specific, and prey mass increases with increasing predator mass (Barnes et al. 2010). Phytoplankton exhibit a wide-range of sizes and larger phytoplankton (e.g., diatoms) often dominate in eutrophic conditions, and smaller phytoplankton (e.g., cyanobacteria) may dominate

in oligotrophic conditions (Irwin et al. 2006, Uitz et al. 2010). Food web length, or the number of trophic transfers in a food web, can therefore vary with differing phytoplankton community composition (Dickman et al. 2008, Young et al. 2015). Areas dominated by smaller phytoplankton can have food webs with 2-3 additional trophic steps compared with areas dominated by larger phytoplankton (Barnes et al. 2010, Barnes et al. 2011, Young et al. 2015). Since trophic transfer efficiency at each step in the food chain is relatively constant, longer food chains reduce the amount of production that is available to populations of consumers compared to shorter food webs (Barnes et al. 2010, Young et al. 2015).

The size structure of phytoplankton and zooplankton can therefore impact the energy flow through ecosystems and ultimately, the amount of production available to higher trophic level species. For example, in the eastern Pacific Ocean, the decrease in nutrient supply associated with warm El Niño -Southern Oscillation (ENSO) events not only causes an overall reduction in primary production, but may also influence phytoplankton size structure, which ultimately impacts the amount of energy available to the remainder of the food web.

One approach for measuring potential food web elongation associated with environmental anomalies is through stable isotope analyses. Nitrogen (δ^{15}N) stable isotope analyses have been used to evaluate changes in primary production and trophic ecology or diets of organisms. Recently, compound-specific isotope analysis of amino acids (CSIA-AA) has been used to overcome a critical limitation of bulk isotope analysis, as δ^{15}N values of 'source' amino acid (e.g., phenylalanine, lysine, alanine) are minimally fractionated and can be used as a proxy for δ^{15}N values at the base of the food web (e.g. particulate organic matter; (Popp et al. 2007, Yoshito et al. 2007, Chikaraishi et al. 2009). Other 'trophic' amino acids are fractionated to a larger extent and are therefore represent the consumer's diet. Using the CSIA-AA approach, we

can use amino acid δ^{15}N values to estimate trophic position of species (Popp et al. 2007, Yoshito et al. 2007, Chikaraishi et al. 2009), evaluate temporal changes in nitrogen cycling patterns (Sherwood et al. 2011), and test for fluctuations in food web length across spatial and temporal scales (Hetherington et al. 2017, Ruiz-Cooley et al. 2017).

In the CCLME, previous studies (Rau et al. 2003, Ohman et al. 2012, Décima et al. 2013) reported increases in the bulk δ^{15}N values of zooplankton during El Niño years, which were primarily attributed to increased phytoplankton utilization of nitrate relative to supply (Waser et al. 1998) and the effects of Rayleigh distillation (Wu et al. 1997). Décima et al. (2013) used amino acid δ^{15}N values to evaluate changes in euphausiid and copepod trophic positions during the transition from El Niño to La Niña conditions in 1998-1999. Although Décima et al. (2013) reported an increase in euphausiid trophic position during El Niño compared with La Niña conditions, they did not find significant differences in *Calanus pacificus* trophic positions between the different ENSO phases. However, they used samples collected during one season (spring), and their annual sampling may have failed to fully capture the trophic differences of zooplankton due to time-lags of consumer tissues reaching isotopic equilibrium. Furthermore, the recent WWA represent a more prolonged period of warming compared with previous ENSO events.

Our objectives were to assess the impact of a of persistent, multi-year warming event in a region that is used by many species of great commercial, economic, or conservation interest. We evaluated oceanographic variables, biological parameters, and the nitrogen isotope values of the copepod *C. pacificus*, to evaluate the effects of a prolonged warming event and to specifically test whether there were differences in food web length between warm and neutral periods. *C. pacificus* is an abundant marine zooplankton species that is critical for transferring energy from

primary producers to higher trophic level species. Depending on the availability of phytoplankton prey, *C. pacificus* can switch between passive suspension feeding on large phytoplankton (e.g., diatoms) and predatory feeding on microzooplankton (Landry 1981). Therefore, we expect that changes in *C. pacificus* diet and trophic ecology may occur during environmental perturbations that cause variations in the overall phytoplankton community composition and the abundances of different-sized phytoplankton. Our study provides insight into ecological effects of an unprecedented warm anomaly, which is essential for predicting the impacts of future environmental variability on pelagic food web structure in the CCLME.

Methods

Sample and Data Collection

Samples were collected by the California Cooperative Oceanic Fisheries Investigations (CalCOFI) program, which has collected hydrographic, zooplankton, and ichthyoplankton samples in the California Current since 1949 (Figure 3S.1). The CalCOFI program samples biological and environmental data on a quarterly basis, which provided the unique opportunity to evaluate patterns in environmental and ecological variables with the onset of the marine heatwave, and through 2015-2016 El Niño event. Zooplankton samples used for this study were collected using a bridle-free, cylindrical-conical bongo net with 0.505 mm mesh net and a cod end with 0.333 mm mesh net. The ratio of mesh aperture area to mouth area was 4:8:1. Each tow was conducted to approximately 210 m, and ship speed was adjusted to maintain a vertical angle of approximately 45 degrees.

We selected an abundant and ecologically important copepod species, *Calanus pacificus* to use for isotope analyses. Our sampling locations were limited by the abundance of *C.*

pacificus in the bongo samples, where stations farthest offshore did not contain enough *C. pacificus* to utilize for isotope analysis. We selected two sampling stations on Line 80, one nearshore (80.55) and one offshore (80.80; Figure 3S.1). *Calanus pacificus* was selected because preliminary analyses indicated that *C. pacificus* was present in all four seasons, and is abundant enough to utilize for stable isotope analysis at stations 80.55 and 80.80. To account for potential variation in δ^{15}N values of different life history stages of *C. pacificus*, we limited our study to adult females. We sorted 50 *C. pacificus* individuals from quarterly bongo samples from 2012-2016 to analyze for bulk and CSIA-AA.

We used physical and biological data collected by the CalCOFI program at the same sampling locations as our *C. pacificus* samples. The biological data include chlorophyll *a* concentrations and total zooplankton biomass (cm^3 /$1000m^3$ strained), which was calculated as wet displacement volume after the removal of organisms > 5 ml (http://calcofi.org/data/). Details about biomass calculations and procedures can be found in Kramer et al. (1972). We also used temperature, nutrient concentration, salinity data collected onboard CalCOFI cruises in conjunction with our copepod isotope data to provide a better understanding of oceanographic conditions, and mechanisms driving variability in δ^{15}N and δ^{13}C values during our sampling period.

In addition to in situ sampling on CalCOFI cruises, we also obtained temperature anomaly data from the California Underwater Glider Network dataset (see Rudnick et al. 2017 for more details), which includes data from Line 80 of the CalCOFI sampling grid, where our samples were collected. We obtained monthly values of multiple climate indices, the Oceanic Nino Index and the Pacific Decadal Oscillation (PDO) Index, from NOAA's National Centers for Environmental Information. To infer upwelling intensity, we used a mean, monthly index,

generated from NOAA's Pacific Fisheries Environmental Laboratory (https://www.pfeg.noaa.gov/). These values represent the wind strength forcing on the ocean, which is measured in metric tons per second per 100 m of coastline. These units therefore are used as a proxy for upwelling intensity, to represent the mean amount of water that is upwelled through the bottom of the Ekman layer each second, along a defined region of coastline. Data were available for 26 positions in the Northeastern Pacific and we used data from the closest station to our sampling locations.

Isotope Analyses

We freeze-dried, homogenized, and weighed copepods into tin capsules for bulk isotope analysis. We determined bulk $\delta^{15}N$ values using a CE1108 Elemental analyzer interfaced via a CONFLO III device to a Thermo-Electron Delta Plus XP mass spectrometer at the University of California, Santa Cruz. We report stable nitrogen isotope values in δ notation relative to atmospheric N_2. For quality control, we analyzed a set of reference materials with known $\delta^{15}N$ values and all reference materials were within ± 0.1 ‰ of their calibrated values.

A subset of samples was selected for CSIA-AA due to the high cost and labor associated with this analytical approach. These samples were analyzed at the University of Hawaii's Stable Isotope Biogeochemistry Laboratories. Specifically, we selected spring samples for CSIA-AA, as we could compare these data to those from a previous ENSO event (Decima et al. 2013), and they coincide with the spring bloom, which is important for secondary production in the CCLME. Our bulk $\delta^{15}N$ values from the fall of 2012 were anomalously higher and higher than we anticipated, so we also analyzed amino acid $\delta^{15}N$ values from those samples to investigate the potential drivers of high bulk $\delta^{15}N$ values.

For CSIA-AA we prepared the samples by acid hydrolysis followed by derivatization of amino acids. Samples were hydrolyzed (6N HCl, 150 °C for 70 minutes), esterified (4:1 isopropanol:acetyl chloride), derivatized (3:1 methylene chloride:trifluoroacetyl anhydride), and analyzed using a Trace GC gas chromatograph and a Thermo Delta XP mass spectrometer through a GC-C III combustion furnace (980℃), reduction furnace (680℃), and a liquid nitrogen cold trap. Samples were injected (split/splitless, 5:1 split ratio) with a 180℃ injector temperature and a constant helium flow rate of 1.4 mL min^{-1}. See Popp et al. (2007) and Hannides et al. (2013) for details on CSIA-AA sample preparation and analysis.

We analyzed the samples in triplicate, and corrected the $\delta^{15}N$ values to internal reference compounds norleucine and aminoadipic acid as these have known nitrogen isotope compositions and were co-injected with each sample. We confirmed quality control by analyzing an amino acid suite, with known $\delta^{15}N$ values of 12 amino acids, before and after each triplicate sample run. We grouped amino acids into standard 'trophic' and 'source' categories, based on previous studies (McClelland and Montoya 2002, Popp et al. 2007, Chikaraishi et al. 2009). CSIA-AA samples were analyzed in triplicate, corrected the $\delta^{15}N$ values to internal reference compounds.

Calculations and Statistical Analyses

There are several approaches for estimating TPs using amino acid $\delta^{15}N$ values. Although many studies focus on the source and trophic amino acids phenylalanine and glutamic acid, respectively (Chikaraishi et al. 2009, McMahon and McCarthy 2016), we were unable to use phenylalanine $\delta^{15}N$ values in this study. In some of our samples, unknown compounds co-eluded with phenylalanine and obscured our ability to accurately measure $\delta^{15}N$ of phenylalanine.

Therefore, we used an alternative source amino acid, lysine, as our source amino acid, and estimated TP using the following equation

$$TP_{Trophic-Source} = \frac{(\delta^{15}N_{Trophic} - \delta^{15}N_{Source}) - \beta}{TDF} + 1 \quad (1)$$

where $\delta^{15}N_{Trophic}$ is glutamic acid, $\delta^{15}N_{Source}$ is lysine, β is the difference between glutamic acid and lysine at the base of the food web (3.9 ‰), and the TDF is the trophic discrimination factor, is used to represent ^{15}N enrichment of $\delta^{15}N_{Glu}$ with respect to $\delta^{15}N_{Phe}$ per trophic step (5.2 ‰).

We used univariate linear models to detect differences in bulk and amino acid $\delta^{15}N$ values and TPs during the WWA compared to a neutral period. To test for relationships between $\delta^{15}N$ values and environmental parameters at each station, we calculated the Pearson product correlation coefficients and tested the significance of each correlation. We included the following variables: year, temperature, chlorophyll *a*, nitrate, upwelling, and zooplankton biomass. To determine which variable best explained trends in bulk $\delta^{15}N$ values, we used a stepwise (forward and backwards) selection process based on the Akaike Information Criterion (AIC) to determine the best model and used a linear model to test the significance of the final model selected by the AIC procedure.

Results

We analyzed copepod samples and oceanographic data at two stations over 5 years from 2012-2016. Temperature data from the underwater glider network clearly indicate warm anomalies between 2014 and 2016 where our samples were collected on line 80 of the CalCOFI sampling grid. Temperatures were anomalously warm throughout the sampling region from the coast to 350 miles off shore in 2014-2016, both near the surface (10m) and at a depth of 50m (Figure 3.2).

We found differences in isotope values and environmental parameters between the warm water anomaly and neutral conditions at our sampling locations. We combined data from both stations to test for differences the warm water anomalies and neutral conditions (Figure 3.3) and found significant differences in temperature, $\delta^{15}N$ values, the upwelling index, and zooplankton biomass ($p < 05$; Figure 3.3, Table 3.1). There were no differences between the mean values of chlorophyll *a* ($p = 0.09$), or nitrate concentrations ($p = 0.08$), however, the ranges were different, where the maximum values for both chlorophyll a and nitrate were substantially higher in during neutral conditions compared to the warm water anomaly (Figure 3.3, Table 3.1).

Overall, there was variability in bulk $\delta^{15}N$ values from *C. pacificus*, but there was not a significant increasing or decreasing trend over our sampling period. Bulk $\delta^{15}N$ values were highest in the fall of 2012 (Figure 3.4), which was not associated with the warm anomalies. With fall 2012 included, there was no trend over time ($F_{(1,29)} = 3.4$, $p = 0.08$), however with this outlier removed, we found higher $\delta^{15}N$ values during the WWA ($F_{(1,29)} = 11.4$, $p = 0.002$). We were particularly interested in the $\delta^{15}N$ values of spring samples and when we evaluated this subset of samples, we found higher bulk $\delta^{15}N$ values during the warm water anomaly ($F_{(1,8)} = 7.76$, $p = 0.02$) compared to the neutral period.

For CSIA-AA, we analyzed a subset of samples that included all spring samples from both stations and the fall of 2012. For the samples that we analyzed for CSIA-AA, we found a positive relationship between $\delta^{15}N$ bulk and $\delta^{15}N$ lysine ($F_{(1,9)} = 10.25$, $R^2 = 0.48$ $p = 0.01$). Similarly to bulk $\delta^{15}N$ values from spring samples, the lysine $\delta^{15}N$ values increased over time ($F_{(1,7)} = 10.58$, $R^2 = 0.54$ $p = 0.01$; Figure 3.5). Overall, the TP estimates for *C. pacificus* ranged from 2.1-2.8 and the mean ± standard deviation was 2.4 ± 0.2. There was no difference in TP among years when both stations were analyzed together ($F_{(1,9)} = 2.29$, $R^2 = 0.11$ $p = 0.16$),

however, the TP at station 80.80 was higher during 2012 (2.8) and decreased to 2.2-2.4 for the remainder of our sampling period (Figure 3.5).

To evaluate the relationships between δ^{15}N values and environmental parameters, we used a Pearson-product correlation matrix (Figure 3.6) and tested the significance of each correlation. We found significant correlations among most environmental variables, particularly for upwelling, where the strength of upwelling was correlated with zooplankton biomass, year, temperature, and chlorophyll *a* concentrations and weakly correlated with δ^{15}N at both stations (Figure 3.6, Table 3.2). At Station 80.55, δ^{15}N values were most strongly correlated with nitrate concentrations (Pearson product correlation coefficient = -60, $p = 0.01$) but was also significantly correlated with temperature (Pearson product correlation coefficient = 0.36, $p = 0.04$). At Station 80.80, δ^{15}N values were correlated with more variables than at Station 80.55, and the strongest correlation was between δ^{15}N values and temperature (Pearson product correlation coefficient = 0.48, $p = 0.02$) and was only weakly correlated with nitrate (Figure 3.6, Table 3.2). Using AIC stepwise selection procedure, the best fit models were nitrate for Station 80.55 and temperature for Station 80.80. Both univariate models were significant: δ^{15}N ~ nitrate at Station 80.55 ($F_{(1,13)} = 7.39$, $R^2 = 0.31$ $p = 0.01$), and δ^{15}N ~ temperature at Station 80.80 ($F_{(1,13)} = 6.0$, $R^2 = 0.26$, $p = 0.03$).

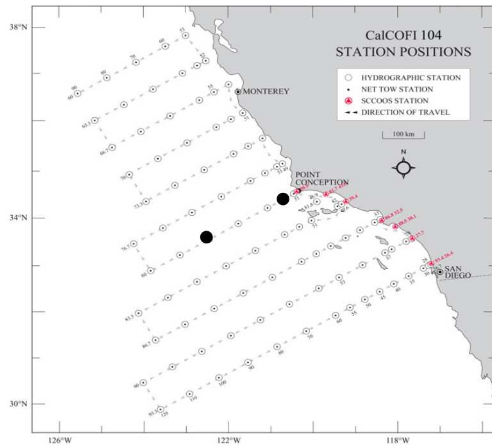

Figure 3.1. The California Cooperative Oceanic Fisheries Investigations (CalCOFI) sampling grid, where filled in black circles indicate our two sample locations.

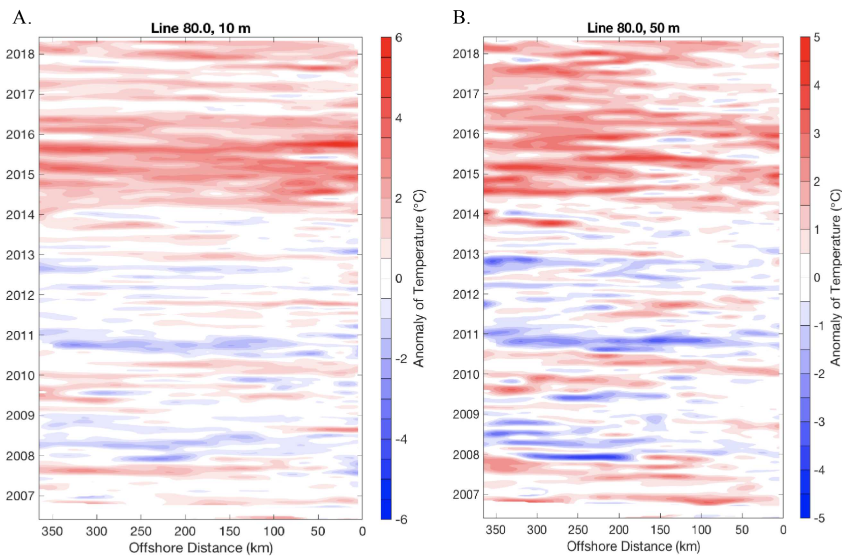

Figure 3.2. Temperature anomalies at A) 10 m and B) 50 m at Line 80 from 2007-2018, where large warm anomalies were evident from 2014-2016, reflecting the marine heatwave and El Niño event. Temperature anomaly data were obtained from the California Underwater Glider Network dataset (doi: 10.21238/S8SPRAY7292; see Rudnick et al. 2017 for more details).

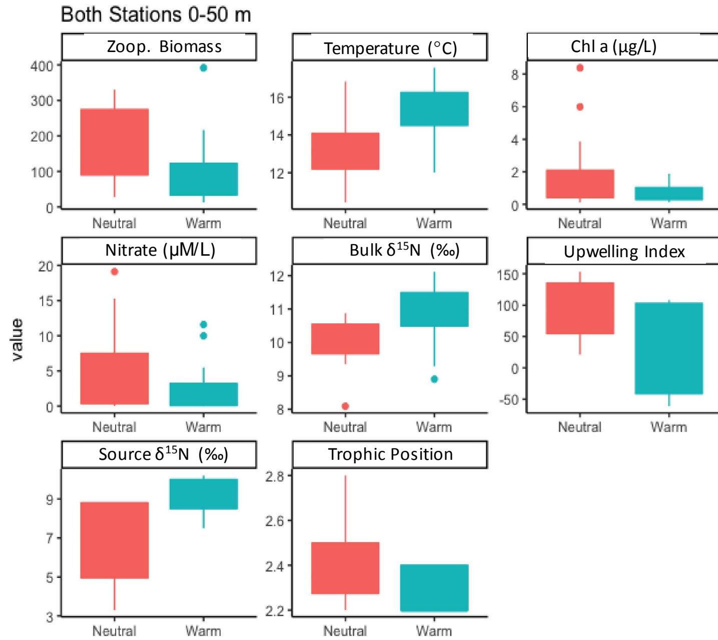

Figure 3.3. Comparison of environmental data and $\delta^{15}N$ values before and during the marine heatwave and El Niño event, where sampling events were divided into warm (green) and neutral (pink) periods based on when water anomalies began in our sampling region. Zoop. Biomass represents wet displacement volume after the removal of large (> 5 ml) organisms. See table 3.1 for test statistics.

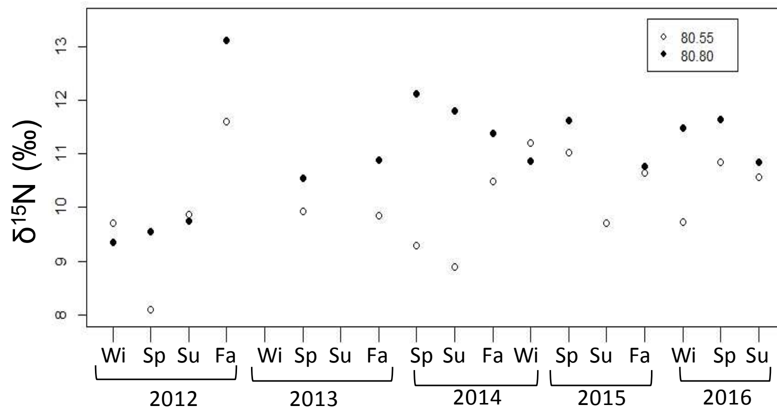

Figure 3.4. Bulk $\delta^{15}N$ values of *Calanus pacificus* adult females from 2012-2016, where there was no significant trend in $\delta^{15}N$ over time and the highest $\delta^{15}N$ values at both stations were in Fall 2012. Gray bars indicate spring samples.

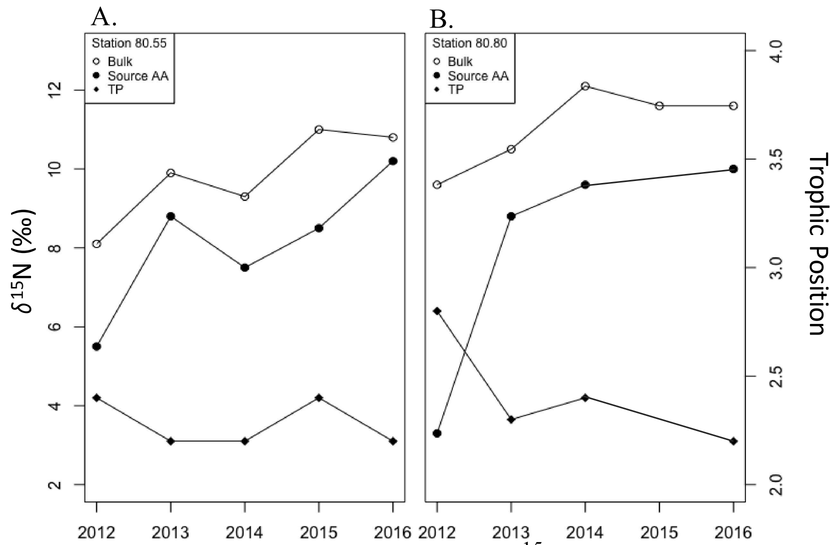

Figure 3.5. Bulk and source amino acid $\delta^{15}N$ values and trophic position estimates of *Calanus pacificus* from spring samples at A) Station 80.55 (nearshore) and B) Station 80.80 (offshore).

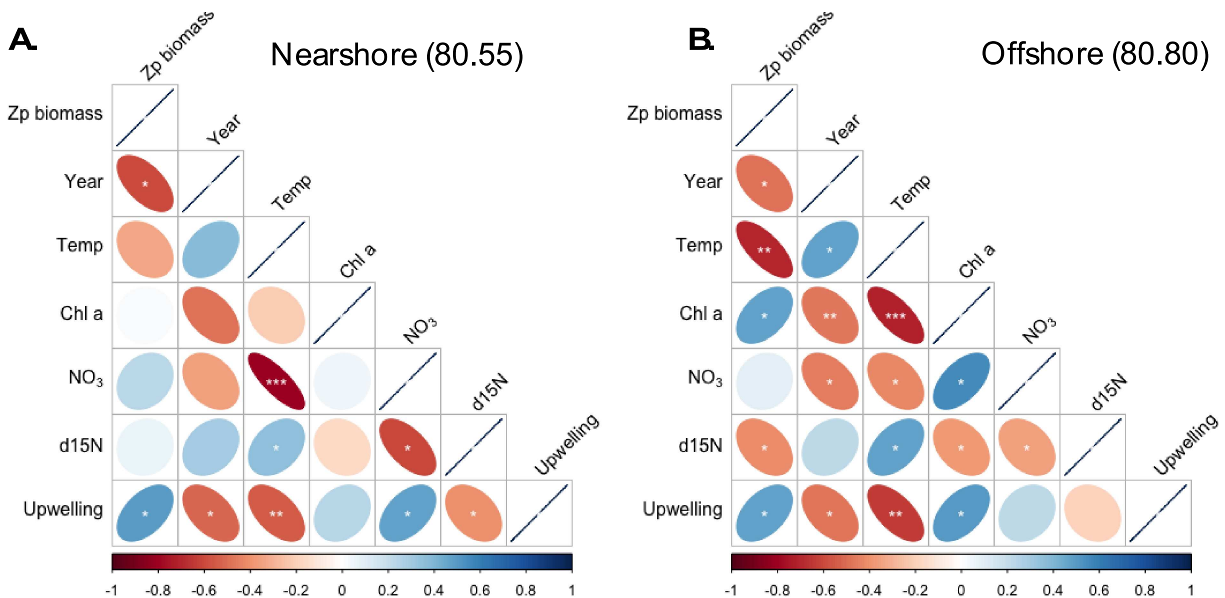

Figure 3.6. Pearson's product correlation matrices for nitrogen isotope values ($\delta^{15}N$) and environmental variables at stations 80.80 and 80.55, where color indicates the strength of the correlation. Temperature, salinity, chlorophyll a, and nitrate concentrations were averaged from 0-50 m. ONI represents monthly values of the Oceanic Niño Index, which were obtained from NOAA's Climate Prediction Center (www.cpc.ncep.noaa.gov). The upwelling index is from NOAA's Pacific Fisheries Environmental Laboratory (www.pfeg.noaa.gov). Significant correlations are indicated by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

Table 3.1. Comparison of multiple parameters between the warm water anomaly (WWA) and the neutral period prior to the warming events, with the range and median for both time periods, and the test statistics from t-tests for each parameter.

* denotes the unit of measurement for an upwelling index, which a proxy for upwelling, as it represents the amount (cubic meters) of water upwelled per second per 100 meters of coastline (m^3/s/100 m of coastline).

	WWA		Neutral		t-test statistics	
	Range	**Median**	**Range**	**Median**	**t**	**p**
Temperature (°C)	12.0–17.6	15.5	10.4–16.8	13.1	3.9	< 0.001
Chlorophyll *a* (µg L^{-1})	0.1–1.9	0.6	0.1–8.4	0.6	-1.8	0.09
NO_3 (µM L^{-1})	0.0–11.6	0.5	0.02–19.2	3.2	-1.8	0.08
$\delta^{15}N$ (‰)	8.9–12.1	10.9	8.09–10.9	9.9	2.8	0.01
Upwelling Index*	-61.1–108.0	23.9	21.1–153.4	93.3	-3.35	< 0.01
Source $\delta^{15}N$ (‰)	7.5–10.2	9.6	3.3–8.8	7.2	1.8	0.15
Trophic Position	2.2–2.4	2.2	2.2–2.8	2.4	-1	0.36
Zoop. Biomass (cm^3)	13–392.0	51.5	28–330.0	180.5	-2.3	0.03

Table 3.2. Pearson product coefficient matrices for station 80.55 (nearshore) and station 80.80 (offshore), where values above the – are correlation coefficients and values below are p-values, which were used to test the significance of the correlations.

	Zoo. Biomass	**Year**	**Temp**	**Chl *a***	**NO_3**	**$\delta^{15}N$**	**Upwell.**
	Station 80.55						
Zoo. Biomass (cm^3)	-	-0.59	-0.33	0.03	0.23	0.07	0.50
Year	**0.017**	-	0.37	-0.49	-0.35	0.29	-0.51
Temp (°C)	0.108	0.052	-	-0.22	-0.80	0.36	-0.54
Chl *a* (µg L^{-1})	0.482	0.065	0.282	-	0.05	-0.16	0.24
NO_3 (µM L^{-1})	0.182	0.085	**0.000**	0.411	-	-0.60	0.48
$\delta^{15}N$ (‰)	0.460	0.141	**0.043**	0.290	**0.011**	-	-0.40
Upwelling	**0.040**	**0.011**	**0.008**	0.219	**0.016**	**0.041**	-
	Station 80.80						
Zoo. Biomass (cm^3)	-	-0.48	-0.71	0.48	0.09	-0.41	0.47
Year	**0.019**	-	0.47	-0.46	-0.46	0.23	-0.47
Temp (°C)	**0.002**	**0.011**	-	-0.71	-0.43	0.48	-0.63
Chl *a* (µg L^{-1})	0.015	**0.009**	**0.001**	-	0.56	-0.38	0.51
NO_3 (µM L^{-1})	0.176	0.030	0.044	**0.014**	-	-0.36	0.23
$\delta^{15}N$ (‰)	**0.033**	0.092	**0.017**	**0.020**	**0.049**	-	-0.20
Upwelling*	**0.012**	**0.012**	**0.003**	**0.012**	0.119	0.084	-

Discussion

Environmental Conditions

The recent warm water anomalies (WWA) in the CCLME, which were associated with a marine heatwave and strong El Niño, were unprecedented in regard to the length and intensity of these events. The marine heatwave persisted through the El Niño event, which led to different background conditions in the CCLME compared to previous El Niño events. This is evidenced by recent work which illustrates that recent warming off Central California was not restricted to coastal regions of the CCLME, unlike previous strong El Niño events (Jacox et al. 2017). Rather, the warming was more pervasive and extended farther offshore. Along line 80 of the CalCOFI sampling grid, where our samples were collected, temperature anomalies were +0.5°C in certain areas, and the warming was more evident at 50 m compared to the surface.

In our five-year sampling period at stations 80.55 and 80.80, we found significant differences in temperature, upwelling, and zooplankton biomass. Although the nitrate and chlorophyll *a* concentrations were not significantly different between the WWA and neutral periods, the ranges of these parameters were more restricted during the WWA. With typical upwelling conditions in the CCLME, chlorophyll *a* and nitrate values are higher during the spring compared to other seasons, which is important for the development of the spring bloom in the CCLME. However, during the warm anomalies, these values were depressed in the spring samples, which indicates that primary production was limited during this period. Furthermore, there was a decrease in zooplankton biomass at our sampling stations, suggesting that decreases in nutrient concentrations that limited primary production propagated to zooplankton consumers in the ecosystem.

δ^{15}N and Trophic Position

The CCLME is a highly dynamic marine ecosystem, which was apparent from the high variability in the δ^{15}N values of *Calanus pacificus* over our five-year sampling period. There was no significant seasonal or temporal trend in δ^{15}N values, however, our sampling period was likely too short to capture seasonal δ^{15}N patterns in the CCLME. The highest δ^{15}N values of our sampling period were from samples collected during the fall of 2012. Interestingly, 2012 was not an anomalous year due to warming, but there was a pervasive salp bloom in the CCLME (Smith et al. 2014). Since salps are filter-feeding organisms with high clearance rates, it is possible that they consumed high amounts of phytoplankton, which led to greater competition of resources among other zooplankton species and a diet switch for *C. pacificus*, which could explain the high δ^{15}N values in the fall of 2012.

Because the anomalous conditions in 2012 were not related to the marine heatwave or ENSO event and was outside the scope of our study, we also excluded these samples from our sampling period and re-analyzed our data. When these samples were excluded, δ^{15}N increased during the WWA compared to the neutral period, and this trend was most evident in the spring samples. We were particularly interested in spring samples because their collection coincides with the spring bloom in the CCLME and previous studies (Rau et al. 2003, Ohman et al. 2012, Decima et al. 2013) have indicated an increase in δ^{15}N associated with previous El Niño conditions. Our study supports these previous findings, and indicates that the δ^{15}N increase began with the marine heatwave and persistent through the El Niño event, which suggests that other warming events can alter the δ^{15}N values of consumers in the CCLME.

Although we found an increase in bulk δ^{15}N values, we did not find a difference in TP between the neutral periods and WWA at our sample locations. At the nearshore station, TPs

were consistent across our sampling period, including in the fall of 2012. However, at the more offshore station, *C. pacificus* had an elevated TP in 2012 compared to other years in our sampling period but otherwise TPs were consistent across years. The lack of TP differences between the WWA and neutral period strongly supports previous studies indicating that nitrate availability drives $\delta^{15}N$ values of consumers in the CCLME (Ohman et al. 2012, Decima et al. 2013). This is further supported when evaluating the nutrient concentrations in relation to nitrogen isotope data, as the $\delta^{15}N$ values of *C. pacificus* varied inversely with nitrate concentrations, particularly at the more nearshore station. These results suggest that the increase in ^{15}N in *C. pacificus* was likely driven by nitrate availability, where $\delta^{15}N$ values can be explained by an increase in phytoplankton utilization relative to supply via Rayleigh isotope fractionation, which is in agreement with previous studies (Waser et al. 1998, Ohman et al. 2012). Unlike previous research, our study used $\delta^{15}N$ values from source amino acids, which can be used as a proxy for the base of the food web to evaluate the mechanisms driving the $\delta^{15}N$ values of *C. pacificus*. In the subset of samples that we analyzed for CSIA-AA, the source amino acid $\delta^{15}N$ values closely mirrored the patterns in bulk $\delta^{15}N$ values, which provides strong evidence that phytoplankton $\delta^{15}N$ values at the base of the food web were driving the increase in *C. pacificus* $\delta^{15}N$ values during the WWA.

We did find elevated TPs of *C. pacificus* at the offshore sampling station during 2012, which may indicate a diet difference in 2012 compared to other years. Since salp abundances were unusually high in the summer and fall of 2012, these results may suggest a diet switch by *C. pacificus* during a period of nutrient limitation and increased competition among zooplankton. However, the $\delta^{15}N$ value of source amino acids were also higher in samples from the fall of 2012 compared to other years, which indicates that nitrogen cycling processes baseline $\delta^{15}N$ values of

phytoplankton were driving the difference in δ^{15}N and TP in 2012, rather than a change in *C. pacificus* diet. It is possible that our results indicate contributions of both baseline and diet shifts of *C. pacificus* in 2012, which we attribute to the pervasive salp bloom in our study area during that time, but warrants future investigation.

Conclusions

Overall, the results from this study indicate that the WWA began in late 2013-early 2014 at my sampling stations and persisted through to spring of 2016. In addition to increased water temperature, upwelling intensity and zooplankton biomass were significantly reduced. In addition, the range of chlorophyll a and nitrate concentrations were restricted during the WWA, which indicates nutrient limitation and reduced primary and secondary production. The δ^{15}N values of *C. pacificus* in my sampling period were variable, but were generally higher during the WWA and this trend was most evident in spring samples. However, values δ^{15}N values were highest during the fall of 2012, which was not related to warming. There was no change in *C. pacificus* trophic position associated with the WWA, but source amino acid δ^{15}N values increased, which suggests that trends in bulk isotope values can be attributed to increased phytoplankton utilization of nitrate relative to supply, rather than food web elongation.

Overall, my results demonstrate how environmental variability such as El Niño events, or large environmental anomalies like the persistent marine heatwave, can alter ecosystems through reduced upwelling, which limits nutrient availability to phytoplankton and ultimately reduces available energy to consumer biomass. Although we did not find differences in food web structure, isotope values do not provide information about prey availability or abundances. Furthermore, *C. pacificus* could have exhibited a prey switch to different phytoplankton or

microzooplankton species of similar trophic status, which would not result in a difference in the trophic position of *C. pacificus*, but could affect their biomass or population dynamics. It is therefore possible that there were differences in food web length during the WWA that we could not detect with our analyses. Future studies that couple stable isotopes of higher trophic level predators with in situ diet data or prey abundance data could provide useful insight into how these anomalies propagate up to top predators and ultimately influence their reproductive success during periods of unfavorable conditions.

Acknowledgements

We would like to thank all employees and volunteers on California Cooperative Oceanic Fisheries Investigations surveys, who collect zooplankton and hydrographic data used in this study. We would also like to thank Scripps Institution of Oceanography's Pelagic Invertebrate Collection and Linsey Sala for assistance with species identification, and the University of Hawaii's Isotope Geochemistry Laboratory for assistance in sample preparation and isotope analyses. This is School of Ocean Science and Technology contribution number ####. This research was funded by the University of California San Diego.

Chapter 3, in full, is being prepared for submission: 2018, Hetherington, ED and Kurle, CM. The dissertation author is the primary investigator and author of this paper and the co-author list for this paper has not been finalized.

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CHAPTER 4

Effects of chemical preservation on bulk and amino acid isotope values of zooplankton, fish, and squid tissues

Abstract

Carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotope values from archived tissues are useful in ecological studies to establish historic baselines and reconstruct the long-term trophic ecology of organisms. However, archived specimens are often stored in chemical preservatives and it is imperative to understand how preservation potentially alters tissue isotopic compositions before using historical samples in future studies. We evaluated the effects of chemical preservatives on $\delta^{13}C$ and $\delta^{15}N$ values of a) tuna (*Thunnus albacares*) and squid (*Dosidicus gigas*) muscle tissues that were fixed in formaldehyde and stored in ethanol for up to two years and b) two copepod species, *Calanus pacificus* and *Eucalanus californicus*, which were preserved in formaldehyde for 24-25 years. Additionally, we used compound-specific isotope analysis of amino acids (CSIA-AA) on a subset of our samples to test the effects of chemical preservation on amino acid $\delta^{15}N$ values. Tissues in formaldehyde-ethanol had higher $\delta^{15}N$ values (+1.4, *D. gigas*; +1.6 ‰, *T. albacares*), higher $\delta^{13}C$ values for *D. gigas* (+0.5‰), and lower $\delta^{13}C$ values for *T. albacares* (-0.8‰) compared to frozen samples. $\delta^{15}N$ values from whole copepods stored in formaldehyde for 25 years were not different than those from frozen samples, although $\delta^{13}C$ values from both species were lower (-1.0 ‰ for *E. californicus* and -2.2 ‰ for *C. pacificus*) than those from frozen samples. Mean amino acid $\delta^{15}N$ values from chemically preserved tissues were largely within 1‰ of frozen tissues, but phenylalanine $\delta^{15}N$ values from some chemically preserved samples were altered to a larger extent (range: 0.5-4.5 ‰). The changes in $\delta^{15}N$ values associated with chemical preservation were mostly minimal, suggesting that storage in formaldehyde or ethanol will not affect the interpretation of $\delta^{15}N$ values used in ecological studies. The effects of chemical preservation on $\delta^{13}C$ values were more variable, where the direction and magnitude of change differed. Preservation effects on amino acid $\delta^{15}N$ values were mostly minimal, which is

promising for future studies. However, there were substantial differences in phenylalanine $\delta^{15}N$ values between frozen and chemically preserved tissues. We speculate that changes in phenylalanine $\delta^{15}N$ values resulted from interference in chromatographic resolution of this compound rather than alteration of its isotopic composition due to chemical preservation.

Introduction

Evaluating the effects of chemical preservation on carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotope values from organismal tissues is essential for the best application of stable isotope data from archived samples. Stable isotope analysis is a powerful tool that is often used to answer questions about trophic ecology, energy flow, food web dynamics(DeNiro & Epstein 1981, Hansson et al. 1997, Hobson 1999), and more recently, to evaluate habitat use patterns of migratory animals(Olson et al. 2010, Seminoff et al. 2012, Vander Zanden et al. 2015, Tomaszewicz et al. 2017). Long-term tissue collections and museum specimens are extremely useful for reconstructing past food webs and addressing questions about ecological changes over time. However, samples are often preserved in chemicals such as ethanol or formaldehyde. These preservatives prevent bacterial growth and preserve the structural integrity of tissues, which allows for morphological examination of preserved organisms (Srinivasan et al. 2002, Huang & Yeung 2015). If one is interested in reconstructing the ecology of an organism using isotopic analyses from archived specimens, it is therefore imperative to understand if preservatives and long-term fixatives can alter $\delta^{13}C$ and $\delta^{15}N$ values.

In addition to the analysis of isotope values from bulk tissues or whole organisms (i.e., bulk isotope analysis), there is an increasing use of compound-specific isotope analysis of amino acids (CSIA-AA) in ecological studies, whereby the $\delta^{15}N$ values of amino acids in a consumer's

tissue are analyzed, as specific amino acid $\delta^{15}N$ values provide deeper insights than analyzing bulk tissue alone (Popp et al. 2007, Chikaraishi et al. 2009). Some amino acids (e.g., alanine, glutamic acid) are isotopically fractionated during transamination, causing a consumer's tissue to become enriched in ^{15}N relative to its prey. These are called 'trophic' amino acids, as they reflect the diet of the consumer (Popp et al. 2007). Conversely, 'source' amino acids (e.g., phenylalanine, lysine) show little isotopic fractionation, as their primary metabolic pathway does not cleave or form nitrogen bonds. Thus, source amino acids reflect the nitrogen isotope composition at the base of the food web (Popp et al. 2007, Chikaraishi et al. 2009) which is governed by different patterns in nitrogen cycling processes (e.g., nitrogen fixation, denitrification, nitrification) in the region. Although a myriad of physiological and biochemical reactions can affect $\delta^{15}N$ values of amino acids in organisms (O'Connell 2017 2017), these basic observations are sufficient to interpret many ecological relationships.

Amino acid $\delta^{15}N$ values can therefore help determine whether variation in bulk $\delta^{15}N$ values from consumers is due to dietary changes or they reflect biogeochemical changes that occurred at the base of the food web and influenced isotopic values of consumers. Not only has CSIA-AA been used to successfully evaluate the trophic positions of consumers and trace nitrogen flow through ecosystems, but in recent years has been used to reconstruct past food webs and detect environmental variability and changes in oceanographic conditions (Sherwood et al. 2011, Décima et al. 2013). Since CSIA-AA is expensive and time consuming compared to bulk isotope analysis, few studies have tested the effects of chemical preservation on the $\delta^{15}N$ of amino acids (Hannides et al. 2009, Ogawa et al. 2013). Such information is crucially important for the application of CSIA-AA in studies that rely on chemically preserved archived samples.

There are several mechanisms through which chemical fixatives and preservatives can alter stable isotope values. The tissues can either take up carbon or nitrogen from the fluid, or the preservative can promote the leaching of carbon or nitrogen from tissue. Since formaldehyde and ethanol do not contain nitrogen, they cannot add nitrogen to samples, although preservatives can break nitrogen bonds in tissues, which may affect stable isotope values. During long-term preservation in formaldehyde, carbon from formaldehyde can be added to the tissue (Kiernan 2000). Here, we would anticipate higher carbon content, carbon to nitrogen (C/N) ratio, and a change in $\delta^{13}C$ values, in chemically preserved tissue compared to the same sample stored frozen.

Formaldehyde is non-coagulant fixing agent that reacts with proteins to form intermolecular cross-links (Kiernan 2000, Huang & Yeung 2015), which preserves the cellular organization and structure of the tissue. Cross-linking of proteins and other molecules occurs when a methylene bridge (-CH_2) is formed by an aldehyde combining with proteins, usually by binding to nitrogen(Kiernan 2000, Srinivasan et al. 2002, Huang & Yeung 2015; Figure 4.1). Since formaldehyde has a low molecular weight (30 g/mol), it can quickly penetrate and bind to tissue, although the formation of methylene bridges occurs more slowly. Once the methylene bridges are formed, they are thought to remain stable, however, if they are not formed, unfixed proteins can be denatured and coagulated by ethanol or other dehydrating solvents (Kiernan 2000, Srinivasan et al. 2002, Huang & Yeung 2015). If carbon is added to the tissue when formaldehyde binds to protein, the direction and magnitude of change in $\delta^{13}C$ values would depend on the isotopic composition of the formaldehyde, which can vary depending on the chemical stock, relative to the $\delta^{13}C$ value of the tissue. Some studies have suggested an alternative mechanism for the effects of formaldehyde on stable isotope values, where

formaldehyde can hydrolyze proteins and promote the leaching of compounds that are enriched in ^{13}C compared to lipids, which leaves the preserved tissue relatively depleted in ^{12}C. Leaching of tissue lipids into a preservative solution could also bias $\delta^{13}C$ values.

Ethanol is a non-cross-linking reagent, which preserves nucleic acids better than aldehydes, and is therefore a common preservation technique for samples that may be used for genetic analyses. However, ethanol may also affect the carbon content and $\delta^{13}C$ values of tissues (Srinivasan et al. 2002). Ethanol can extract lipids and partition them into ethanol based on their solubility (Hayes 2001). lipids, which are depleted in ^{13}C) relative to proteins, carbohydrates and nucleic acids that comprise animal tissues (Bosley & Wainright 1999, Sarakinos et al. 2002). If ethanol removes lipids from samples, we would expect a decrease in carbon content, C/N ratios, and an increase in $\delta^{13}C$ values of preserved samples (McConnaughey & McRoy 1979).

Although there are clear mechanisms through which chemical preservation can alter stable isotope values, the effects of preservatives on isotopic compositions of tissues have been inconsistent across studies, which highlights the need for further research, particularly for CSIA-AA which has become an increasingly-used approach in ecological and biogeochemical studies. Generally, previous research demonstrates that formaldehyde or ethanol preservation have little effect on bulk $\delta^{15}N$ values, but can substantially alter bulk $\delta^{13}C$ values, where the direction and magnitude of change varies across studies (Kaehler & Pakhomov 2001, Arrington & Winemiller 2002, Rau et al. 2003, Ruiz-Cooley et al. 2011). Our objectives were to perform experiments that would contribute to a better mechanistic understanding of the effects of chemical preservation techniques on $\delta^{15}N$ and $\delta^{13}C$ values and investigate the effects of preservation on amino acid $\delta^{15}N$ values. Many vertebrate tissues are fixed first in formaldehyde then preserved long-term in 70-95% ethanol, but few studies have evaluated the effects of this specific technique on $\delta^{15}N$ and

$\delta^{13}C$ values. We evaluated the effects of formaldehyde fixation followed by storage in ethanol for up to two years on bulk $\delta^{15}N$ and $\delta^{13}C$ values and amino acid $\delta^{15}N$ values from tuna and squid.

To use museum specimens for stable isotope analysis, it is also important to evaluate longer-term effects of preservation, so we also analyzed the $\delta^{15}N$ and $\delta^{13}C$ values from copepods preserved frozen or stored in formaldehyde for 24-25 years. Testing the effects of long-term formaldehyde preservation on stable isotope values from zooplankton is particularly relevant as archived marine invertebrates are common in government, university, and museum collections and could be especially useful for reconstruction of past trophic baselines and oceanic conditions. We hypothesized that formaldehyde and formaldehyde-ethanol preservation would have little effect on $\delta^{15}N$ values of organismal tissues, including the $\delta^{15}N$ values of amino acids, but that both preservation techniques would alter the $\delta^{13}C$ values of squid, tuna, and copepod samples.

Materials and Methods

Sampling Method and Preservation Techniques

It is often necessary to fix samples following their collection and freezing is a widely-used method of storing samples with minimal effects on stable isotope values (Barrow et al. 2008, Jesus et al. 2015, Wolf et al. 2016). However, freezing is often unavailable during field studies and is typically not the method used for archiving museum specimens when the preservation of anatomical features is required. To investigate effects of formaldehyde fixation-ethanol preservation on organismal isotope values, we compared this protocol to preservation via freezing, using three specimens each of yellowfin tuna, *Thunnus albacares*, and Humboldt squid,

Dosidicus gigas, which are both commercially and ecologically significant consumers in pelagic, marine food webs. Tuna and squid specimens were collected on recreational fishing vessels and frozen intact until later processing. To test for differences between frozen and formaldehyde-ethanol preserved samples within each specimen, we collected a muscle tissue sample from each individual (n = 3 *T. albacares* and 3 *D. gigas*) and divided each sample into six, approximately 1 g subsamples. We collected samples from the dorsal side adjacent to the dorsal fin for each *T. albacares* and from the dorsal mantle muscle (with skin removed) for each *D. gigas*. We followed protocol for preservation of vertebrate tissues from Scripps Institution of Oceanography (H.J. Walker, *pers. comm.*). All non-frozen samples were initially fixed in 3.7% formaldehyde (deionized water to 37% formaldehyde ratio was 1:9) for approximately 48 hours, followed by two deionized water rinses over two days. Samples were then stored in 95% ethanol for specific time intervals: one week, one month, three months, six months, and two years. After samples were removed from the preservatives, they were lyophilized for 24 hours, homogenized, and weighed into tin capsules for bulk stable nitrogen and carbon isotope analysis.

Following the analysis of the formaldehyde-ethanol experiment, we separately tested the effects of formaldehyde and ethanol on tuna and squid muscle. Our sampling procedures were the same as our initial experiment, although a different bottle of formaldehyde was used, as this experiment was conducted two years after our initial experiment. We recognize that the δ^{13}C values of formaldehyde can vary between individual bottles and suppliers. Since our initial experiment indicated that the length of preservation was not a significant factor, we preserved samples from three tuna specimens and three squid specimens for one month by either fixing them in formaldehyde or preserving them in ethanol, for comparison with isotope values from frozen samples from the same specimens.

We also investigated effects of longer-term storage in formaldehyde on bulk isotope values from marine zooplankton using samples collected by the California Cooperative Oceanic Fisheries Investigations (CalCOFI) off central California in March and April of 1991 and 1992 on lines 80 and 83 of the CalCOFI sampling grid (see calcofi.org for map of sampling locations). Zooplankton were collected by bongo tows, consisting of two nets with a 0.71-m diameter (Ohman & Smith 1995) that were towed obliquely to 210 m. Following collection, one bongo net was fixed in a 3.7 % formaldehyde-seawater solution, buffered with sodium borate, and the other was frozen at -80°C until further analysis. In 2016, we slowly thawed frozen samples and sorted adult females of two copepod species, *Calanus pacificus* and *Eualanus californicus*. These species were selected for several reasons. Firstly, previous results from an 11-year experiment indicated there was a negligible difference in δ^{15}N values of these species between formalin-preserved and frozen specimens (Rau et al. 2003). Secondly, *C. pacificus* and *E. californicus* are abundant zooplankton species in our collection area and are easily identifiable, even when smaller structures are obscured by the freezing and thawing process. We analyzed five paired bongo samples, and due to the small body size of copepods, we pooled 20 *C. pacificus* and 10 *E. californicus* from each sample (following reference 18) to ensure that we had an adequate mass required for isotope analysis. We lyophilized the samples for 24 hrs, then homogenized and weighed them into tin capsules for stable isotope analysis.

To evaluate effects of formaldehyde fixation-ethanol preservation on amino acid δ^{15}N values, we selected a subset of samples to analyze for CSIA-AA. We selected two each of the *T. albacares* and *D. gigas* specimens, and analyzed matched samples that were either frozen or preserved for two years following the formaldehyde-ethanol protocol, and one each of the tuna and squid that were preserved for six months in formaldehyde-ethanol (n=10). For zooplankton,

we selected two frozen *C. pacificus* samples and their paired formaldehyde-preserved samples (n = 4) for CSIA-AA.

Sample and Data Analysis

We analyzed samples for their bulk δ^{15}N and δ^{13}C values, % C, % N, and the ratio of carbon to nitrogen (C/N ratio) at the Stable Isotope Laboratory at the University of California, Santa Cruz. We report stable isotope values in δ notation relative to PDB and atmospheric N_2. The subset of *T. albacares*, *D. gigas*, and *C. pacificus* samples selected for nitrogen CSIA-AA were analyzed at the University of Hawaii's Stable Isotope Biogeochemistry Laboratories. Samples were prepared for CSIA-AA by acid hydrolysis followed by derivatization of amino acids (see Popp et al. 2007, Hannides et al. 2013 for details). Samples were hydrolyzed (6N HCl, 150 °C for 70 minutes), esterified (4:1 isopropanol:acetyl chloride), and derivatized (3:1 methylene chloride:trifluoroacetyl anhydride), and were analyzed by GC-IRMS (Gas Chromatography-Isotope Ratio Mass Spectrometry) using Trace gas chromatograph and a Thermo Delta V Plus mass spectrometer through a GC-C III combustion furnace (980℃), reduction furnace (680℃), and a liquid nitrogen cold trap. Samples were injected (split/splitless, 5:1 split ratio) with a 180℃ injector temperature and a helium flow rate of 1.4 mL min^{-1}.

We analyzed samples for CSIA-AA in triplicate. We co-injected each sample with internal reference compounds (norleucine and aminoadipic acid) with known δ^{15}N values tope and corrected sample amino acid δ^{15}N values relative to these internal references. For quality control, we also analyzed an amino acid suite, with known δ^{15}N values of 12 amino acids, before and after each triplicate sample run. The δ^{15}N values of 18 amino acids were analyzed, however, some amino acids were not abundant enough to quantify their δ^{15}N values. Here, we report

results from 13 amino acids grouped into three categories: metabolic, source, and trophic amino acids. Analytical errors for amino acid $\delta^{15}N$ values based on triplicate sample analysis were mostly under 1.0 ‰ but ranged from 0.03 to 1.36 ‰.

We conducted data analyses using the statistical software R (Team 2016). We tested effects of formaldehyde-ethanol preservation on *T. albacares* and *D. gigas* muscle first by testing the potential for an interactive effect of time and species ($\delta^{15}N$ ~Time*Species and $\delta^{13}C$ ~Time*Species) to determine whether we could group samples from *T. albacares* and *D. gigas* together or analyze them independently. Then, we used one-way repeated measures ANOVA to evaluate the isotopic differences between frozen samples and those that were chemically preserved for different lengths of time. For the long-term zooplankton formaldehyde experiment, we used paired t-tests to compare the $\delta^{13}C$ and $\delta^{15}N$ values between frozen and formaldehyde-preserved *C. pacificus* and *E. californicus*. For amino acid $\delta^{15}N$ data, we used paired t-tests to compare isotope values from frozen and formaldehyde-ethanol preserved samples and used a sequential Bonferroni (Holm-Bonferroni) correction for multiple-comparisons.

Results

Tuna and squid preservation experiment (Bulk isotope values)

We found significant differences between frozen and chemically preserved tissues over time (Figure 2, Table 1). There was no significant interaction between time and species, so we analyzed the bulk $\delta^{15}N$ of *T. albacares* and *D. gigas* together and found a consistent, significant increase in $\delta^{15}N$ values ($F_{(1,32)} = 16.94$, $p < 0.001$) with formaldehyde-ethanol preservation compared to frozen tissues (Figure 4.2a). The mean bulk $\delta^{15}N$ values from the formaldehyde-ethanol preserved samples of *D. gigas* and *T. albacares* were higher than those of the frozen

samples by 1.6 ‰ and 1.4 ‰, respectively. Tukey's pairwise comparisons demonstrated that the δ^{15}N values of frozen samples was significantly lower than those of formaldehyde-ethanol preserved samples (all $p < 0.0001$), but there were no differences between the δ^{15}N values of frozen tissues and those stored only in ethanol over the five different time periods ($p \geq 0.51$).

The effects of formaldehyde-ethanol preservation on the δ^{13}C values from *T. albacares* and *D. gigas* muscle were more variable. There was a significant interaction effect between species (Adj $R^2 = 0.71$, $p < 0.0001$), so we analyzed the δ^{13}C values separately for each. We found opposite trends where the δ^{13}C values of formaldehyde-ethanol preserved *D. gigas* muscle were significantly higher than those of frozen tissue, by a mean of 0.5 ‰ ($F_{(1,14)} = 19.45$, $p < 0.001$; Figure 2b; Table 4.1), whereas the δ^{13}C values from formaldehyde-ethanol preserved *T. albacares* tissues were lower by 0.6‰ to 0.9‰ than those of frozen tissues ($F_{(1,14)} = 5.23$, $p = 0.04$; Tukey's pairwise comparisons: $p \leq 0.02$; Figure 4.2b). There were no differences in δ^{13}C values of frozen tissues compared with tissues stored in ethanol over the five different time periods ($p \geq 0.75$).

We found no differences in %C or %N between formaldehyde-ethanol preserved and frozen samples for *T. albacares* (% C: $F_{(1,15)} = 0.90$, $p = 0.36$; % N: $F_{(1,15)} = 0.96$, $p = 0.34$) or *D. gigas* (% C: $F_{(1,14)} = 0.56$, $p > 0.1$; % N: $F_{(1,14)} = 0.27$, $p > 0.1$). We used C/N ratios as a proxy for lipid content, where higher ratios indicate more lipid-rich tissues. C/N ratios for squids and tuna were low (< 3.5), and were fairly uniform, ranging from 3.1-3.4 for both species. There was no difference in C/N ratios between formaldehyde-ethanol and frozen samples for *T. albacares* ($F_{(1,15)} = 0.38$, $p > 0.1$). However, the C/N ratios of *D. gigas* were lower in formaldehyde-ethanol preserved samples compared to frozen samples ($F_{(1,14)} = 9.50$, $p < 0.01$), although the change was small (0.1; Table 4.1).

In a subsequent experiment where we separately tested the effects of formaldehyde and ethanol on squid and tuna muscle tissue, we found that $\delta^{15}N$ values significantly increased with preservation in both formaldehyde and ethanol by ~1 ‰ (Table 4.1; Appendix). The effects of formaldehyde or ethanol preservation on $\delta^{13}C$ values were mixed, where $\delta^{13}C$ values of formaldehyde-fixed tissues decreased in both species but $\delta^{13}C$ values from samples preserved in ethanol increased by 1.5 ‰ in *D. gigas* and did not change in *T. albacares* (Table 4.1; Appendix). The C/N ratios of *D. gigas* and *T. albacares* increased with formaldehyde fixation but there was no change in C/N ratios with ethanol preservation (Table 4.1).

Long-term formaldehyde preservation of zooplankton (*bulk isotope values)*

The $\delta^{15}N$ values of the paired copepod samples that were preserved in buffered formaldehyde for 24-25 years were not significantly different than those from frozen copepods: *C. pacificus* (Mean ± SD: 10.1± 0.5 *versus* 10.0 ± 0.6, respectively; paired t-test: $t = 103$, $df = 4$, $p = 0.36$), and *E. californicus* (9.5 ± 0.3 *versus* 9.7 ± 0.2, respectively; paired t-test: $t = -0.80$, $df = 4$, $p = 0.47$; Figure 3a, Table 4.1). However, the $\delta^{13}C$ values of formaldehyde-preserved copepods were lower for *C. pacificus* ($t= 8.22$, $df = 4$, $p = 0.001$; mean difference = 2.2 ‰) and *E. californicus* ($t = 3.63$, $df = 4$, $p = 0.02$; mean difference 1.0 ‰; Figure 4.3b, Table 4.1) than those of the frozen samples. The C/N ratios were overall higher and more variable for *E. californicus* (range: 4.2-8.9, mean ± SD: 5.6 ±1.4) compared to those from *C. pacificus* (range: 3.9-4.6, mean ± SD: 4.2 ±0.2). The C/N ratios were lower in formaldehyde-preserved samples of *E. californicus* ($p < 0.01$) and there was a decrease in % C, although it was not statistically significant ($p = 0.06$). There was no difference in % N, % C, or C/N for *C. pacificus* (all $p > 0.1$) between frozen and formaldehyde preserved samples (Table 4.1).

Preservation effects on amino acid δ^{15}N values

Using paired t-tests, we found no significant differences between the amino acid δ^{15}N values from frozen and chemically preserved tissues (all $p > 0.05$). The differences in δ^{15}N values between frozen and chemically preserved samples were generally < 1 ‰, which lies within the error of CSIA-AA (Figure 4.4, Table 4.2). However, the δ^{15}N values of several amino acids were altered by more than 1.0 ‰, including valine and phenylalanine. Most notably, the δ^{15}N values of phenylalanine, the canonical source amino acid, was, on average, 3.6 ‰ higher in preserved tuna muscle and 1.8 ‰ higher in preserved *D. gigas* muscle compared to those from their frozen counterparts (Table 4.2). Our results were similar for *C. pacificus*, where 25 years of formaldehyde preservation minimally altered δ^{15}N values of most amino acids and paired t-tests showed no differences between formaldehyde preserved and frozen samples (p values > 0.1). However, there was a decrease of 3 ‰ in the δ^{15}N value of phenylalanine in one paired sample (Figure 4.4, Table 4.2).

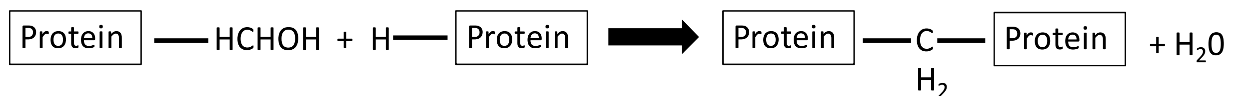

Figure 4.1. Modified from Kiernan (2000); Chemical reaction of formaldehyde (HCHOOH) cross-linking proteins through the formation of a methylene bridge (=CH_2).

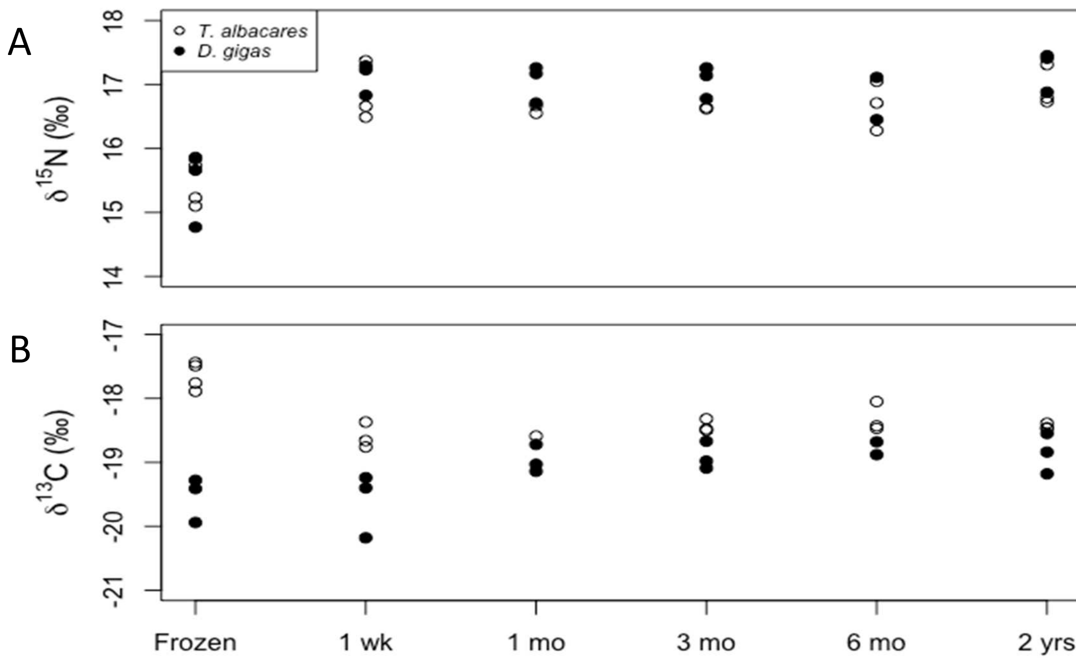

Figure 4.2. A) δ^{15}N values and B) δ^{13}C values from frozen tuna (*Thunnus albacares*) and squid (*Dosidicus gigas*) muscle and muscle fixed in formaldehyde and stored in ethanol for 1 week, 1 month, 3 months, 6 months, and 2 years.

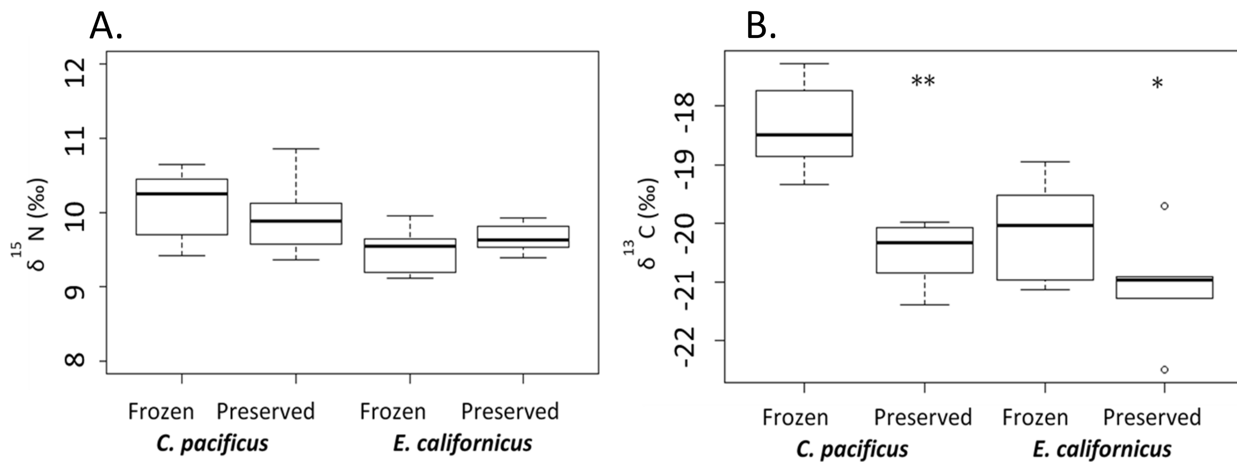

Figure 4.3. The bulk A) δ^{15}N and B) δ^{13}C values from the copepods *Calanus pacificus* and *Eucalanus californicus*, where paired samples were frozen or formaldehyde-preserved for 24-25 years. Mean ± 95% SD. An asterisk (*) indicates significant differences with $p < 0.05$ and ** indicates $p < 0.01$.

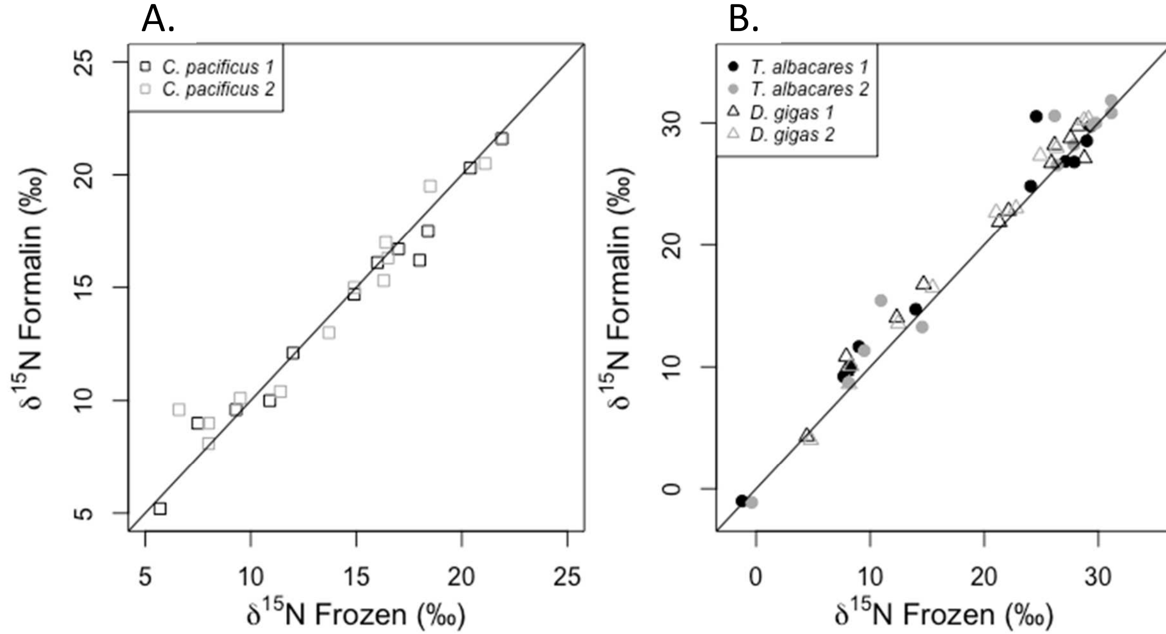

Figure 4.4. The $\delta^{15}N$ values of amino acids of frozen versus formaldehyde preserved A) *Calanus pacificus*, which were either frozen or stored in formaldehyde for 25 years and B) tuna (*Thunnus albacares*) and squid (*Dosidicus gigas*) muscle, where the $\delta^{15}N$ values of chemically preserved tissues were fixed in formaldehyde and stored for two years in ethanol. The black 1:1 line demonstrates where $\delta^{15}N$ values were expected if they were not altered by chemical preservation.

Table 4.1. Mean $\delta^{15}N$, $\delta^{13}C$, % N, and % C values from *Thunnus albacares* (n = 3) and *Dosidicus gigas* (n = 3) muscle tissue with standard deviations (± SD) for frozen samples and the means of the preserved samples for all treatments (1 week, 1 month, 3 months, 6 months, and 2 years), and the mean $\delta^{15}N$, $\delta^{13}C$, % N, and % C values from *Calanus pacificus* (n = 10; five frozen, five preserved) and *Eucalanus californicus* (n = 10; five frozen, five preserved) that were either stored in formaldehyde or frozen. Δ represent the difference in values of $\delta^{15}N$, $\delta^{13}C$, % N, and % C between frozen and preserved samples, where arrows indicate the direction of change, and an asterisk indicates statistical significant ($p < 0.05$).

Species	$\delta^{13}C$	% C	$\delta^{15}N$	% N	C/N	Δ $\delta^{13}C$	Δ %C	Δ $\delta^{15}N$	Δ %N	Δ C/N
D.g.										
Frozen	-19.5 ± 0.3	48.7 ± 9.5	15.4 ± 0.6	15.1 ± 2.8	3.2 ± 0.0					
F-E	-19.0 ± 0.3	57.5 ± 8.8	17.0 ± 0.1	18.3 ± 2.6	3.1 ± 0.1	↑0.5*	↑8.8	↑1.6*	↑3.2	↓0.1*
T.a.										
Frozen	-17.7 ± 0.2	58.2 ± 12.4	15.4 ± 0.4	18.4 ± 3.7	3.2 ± 0.1					
F-E	-18.5 ± 0.1	53.1 ± 5.2	16.8 ± 0.3	16.5 ± 1.7	3.2 ± 0.0	↓0.8*	↓5.2	↑1.4*	↓1.8	0
D.g.										
Frozen	-19.0 ± 0.2	44.5 ± 1.4	11.6 ± 0.3	13.8 ± 0.7	3.1 ± 0.1					
Formaldehyde	-20.2 ± 0.6	59.7 ± 6.1	12.7 ± 0.8	15.3 ± 2.1	3.9 ± 0.1	↓1.2*	↑15.2	↑1.1	↑1.5	↑0.8*
Ethanol	-17.5 ± 0.3	65.1 ± 26.2	12.9 ± 0.7	21.0 ± 8.5	3.2 ± 0.0	↑1.5*	↑20.6	↑1.3	↑7.2	↑0.1
T.a.										
Frozen	-16.5 ± 0.1	51.1 ± 6.5	12.1 ± 0.1	16.4 ± 2.2	3.0 ± 0.0					
Formaldehyde	-18.0 ± 0.2	58.1 ± 5.7	13.4 ± 0.2	17.5 ± 1.7	3.3 ± 0.0	↓1.5*	↑7.0	↑1.3*	↑1.1	↑0.3*
Ethanol	-16.5 ± 0.1	61.9 ± 11.1	13.0 ± 0.1	20.4 ± 3.7	3.1 ± 0.0	↑0.1	↑10.9	↑1.1*	↑4.0	↑0.1
C.p.										
Formaldehyde	-18.3 ± 0.8	34.3 ± 2.4	10.1 ± 0.5	8.2 ± 0.6	4.2 ± 0.1					
Ethanol	-20.5 ± 0.6	33.3 ± 3.4	10.0 ± 0.6	7.9 ± 0.9	4.2 ± 0.3	↓2.2*	↓1.0	↓0.1	↓0.3	0
E.c.										
Formaldehyde	-20.1 ± 0.9	25.9 ± 7.1	9.5 ± 0.3	4.1 ± 1.2	6.4 ± 1.5					
Formaldehyde	-21.1 ± 1.0	20.2 ± 4.4	9.7 ± 0.2	4.3 ± 1.1	4.8 ± 0.5	↓1.0*	↓5.7	↑0.2	↑0.2	↓1.6*

Table 4.2. Amino acid $\delta^{15}N$ values of paired *Thunnus albacares* (n = 5) and *Dosidicus gigas* (n = 5) muscle samples either frozen or initially fixed in formaldehyde then stored in ethanol (F/E) for six months (6 mo) or two years (2 yr), and paired *Calanus pacificus* samples that were either frozen or preserved in formaldehyde (F) for 25 years. The $\Delta\ \delta^{15}N$ values represent the differences in $\delta^{15}N$ between frozen samples and those chemically preserved for two years. We categorized amino acids into metabolic (Met), source, or trophic and we used standard three letter abbreviations for each amino acid. We report values for the 13 amino acids consistently detected on chromatographs, where nd = not detected and NA = not applicable.

		Met	Source					Trophic						
Sample/Treatment		**Thr**	**Gly**	**Lys**	**Meth**	**Phe**	**Ser**	**Ala**	**Asp**	**Glu**	**Leu**	**Iso**	**Pro**	**Val**
T.a. 1	Frozen	-22.4	-1.2	7.7	14	9	8.3	29.3	29	27.9	27.1	nd	24.1	24.6
T.a. 1	F/E- 2 yr	-22	-1	9.2	14.7	11.7	9.9	29.7	28.5	26.8	26.9	26.8	24.8	30.5
$\Delta\ \delta^{15}N$		**0.4**	**0.2**	**1.5**	**0.7**	**2.7**	**1.6**	**0.4**	**-0.4**	**-1.1**	**-0.3**	**NA**	**0.8**	**6**
T.a. 2	Frozen	-22.9	-0.4	8.1	14.6	10.9	9.5	31.1	31.1	29.8	27.8	29.5	26.4	26.2
T.a. 2	F/E- 6 mo	-21.8	-1.8	8.4	12.3	12.3	9.7	28.9	29.1	27.9	26.4	27.9	24.9	26.1
T.a. 2	F/E- 2 yr	-20	-1.1	8.7	13.3	15.4	11.3	30.8	31.8	30	28.2	29.9	26.5	30.6
$\Delta\ \delta^{15}N$		**2.9**	**-0.7**	**0.6**	**-1.3**	**4.5**	**1.8**	**-0.3**	**0.7**	**0.2**	**0.4**	**0.3**	**0.2**	**4.4**
D.g. 2	Frozen	-12.9	4.8	8.2	15.5	8.3	12.5	28.7	21	24.9	26.4	nd	22.8	29.1
D.g. 2	F/E- 2 yr	-15.5	4	8.6	16.5	10.1	13.6	30.2	22.7	27.3	28	nd	23	30.3
$\Delta\ \delta^{15}N$		**-2.6**	**-0.7**	**0.5**	**1**	**1.8**	**1.1**	**1.5**	**1.6**	**2.4**	**1.6**	**NA**	**0.2**	**1.2**
D.g. 1	Frozen	-15.8	4.4	7.9	14.7	7.9	12.3	28.2	21.3	25.9	26.2	27.6	22.1	28.8
D.g. 1	F/E- 6 mo	-17.3	2.7	10	17.4	11.9	12.9	27.1	20	24.2	25.6	25.6	20.7	26.9
D.g. 1	F/E- 2 yr	-16.9	4.3	11	16.8	9.7	14	29.7	21.9	26.7	28.2	28.7	22.8	27.1
$\Delta\ \delta^{15}N$		**-1.1**	**-0.1**	**3**	**2.1**	**1.8**	**1.7**	**1.6**	**0.6**	**0.8**	**2**	**1.2**	**0.7**	**-1.7**
C.p. 1	Frozen	7.5	12	9.3	nd	5.7	10.9	21.9	18.4	20.4	14.9	16	17	18
C.p. 1	F- 25 yr	9	12.1	9.6	nd	5.2	10	21.6	17.5	20.3	14.7	16.1	16.7	16.2
$\Delta\ \delta^{15}N$		**-1.5**	**0.1**	**0.3**	**NA**	**0.5**	**0.9**	**0.3**	**0.9**	**0.1**	**0.2**	**-0.1**	**0.3**	**1.8**
C.p. 2	Frozen	8	11.4	8	nd	6.6	9.5	21.1	16.4	18.5	13.7	14.9	16.3	16.5
C.p. 2	F- 25 yr	9	10.4	8.1	nd	9.6	10.1	20.5	17	19.5	13	15	15.3	16.3
$\Delta\ \delta^{15}N$		**-1**	**1**	**-0.1**	**NA**	**-3**	**-0.6**	**0.6**	**-0.6**	**-1**	**0.7**	**-0.1**	**1**	**0.3**

Discussion

Tuna and squid preservation experiment (Bulk isotope values)

Our two-year experiment showed that formaldehyde fixation followed by storage in ethanol significantly altered stable isotope values, as we found an increase in bulk $\delta^{15}N$ values of both species with chemical preservation. Although bulk $\delta^{15}N$ values were altered by chemical preservation, the changes were consistent and relatively small compared to ~3-5 ‰ changes that are typically used to detect trophic position changes in trophic ecology studies. Our results bolster those from previous isotope preservation studies, which have mostly reported small increases (~1-1.5 ‰) in $\delta^{15}N$ values associated with formaldehyde or ethanol preservation (Bosley & Wainright 1999, Sweeting et al. 2004, Bicknell et al. 2011, Ruiz-Cooley et al. 2011). There is no easily-identifiable mechanism to explain the observed changes in $\delta^{15}N$. Since formaldehyde does not contain nitrogen, there is no mechanism by which N could be incorporated into the tissue from formaldehyde. However, formaldehyde preservation could alter $\delta^{15}N$ if nitrogen bonds are broken during preservation. We found no evidence of this, as there were no changes in the N content between frozen and formaldehyde-ethanol preserved samples for either species (see below). It is possible that water-soluble, N-containing compounds (e.g., free amino acids or amines) may have been extracted from the tissues, causing the observed increase in $\delta^{15}N$ values, although a future study is needed to specifically test this hypothesis.

Formaldehyde-ethanol treatment significantly altered $\delta^{13}C$ values of both *T. albacares* and *D. gigas*. The magnitude of change was similar in both species (< 1 ‰), but the direction of change differed. Tissues of *T. albacares* stored in formaldehyde had higher $\delta^{13}C$ values compared to frozen samples, whereas $\delta^{13}C$ values of *D. gigas* were lower in formaldehyde preserved tissues. It is possible that carbon was added to the tissue from the formaldehyde via the

cross-linking of proteins, which affected the $\delta^{13}C$ values. The isotopic composition of a mixture (i.e., preserved tissue) is the intermediate between the composition of its two endmembers (i.e., formaldehyde and tissue $\delta^{13}C$ values). As tissues take up formaldehyde, the $\delta^{13}C$ value of the tissue will shift towards that of the formaldehyde (Hobson et al. 1997, Sweeting et al. 2004, Bicknell et al. 2011). The direction and magnitude of change in $\delta^{13}C$ of the tissue is therefore dependent upon the amount of carbon added to the sample and the relative difference in $\delta^{13}C$ values of the tissue and formaldehyde.

We hypothesize that carbon was added from the formaldehyde to the tissue during the fixation process, which affected the $\delta^{13}C$ values of *D. gigas* and *T. albacares*. Since we found an increase in *D. gigas* $\delta^{13}C$ values and a decrease in *T. albacares* $\delta^{13}C$ values with preservation, this may suggest that the $\delta^{13}C$ value of the formaldehyde used for our experiment was between the *D. gigas* and *T. albacares* values (~-18.5 ‰), and the tissues were converging on the $\delta^{13}C$ value of formaldehyde. However, if carbon was added to the tissues, we would expect an increase in % C and C/N ratios in the preserved tissues. We detected no change in %C or %N for either species and no change in C/N ratios for *T. albacares*. The C/N ratio of *D. gigas* was marginally lower in the preserved samples (3.1) compared with the frozen samples (3.2). Although statistically significant, this decrease was very small. It is possible that our measurements were not precise enough to capture small changes in carbon content expected from the addition of formaldehyde. Alternatively, ethanol solubilizes lipids, so while formaldehyde added carbon to our samples, it is possible that long-term storage in ethanol masked this effect by removing carbon, which resulted in no change in % N or % C. The mechanistic explanations are difficult to disentangle when tissues are preserved in multiple chemical preservatives that alter tissues in different ways.

To separate the potential independent effects of formaldehyde and ethanol on tuna and squid muscle tissue, we conducted a short follow-up study. We found that ethanol and formaldehyde preservation had similar effects on δ^{15}N values, where δ^{15}N values of chemically preserved tissues were ~1 ‰ higher than frozen samples. For tissues preserved in formaldehyde only, we found a decrease in δ^{13}C and an increase in %C and C/N ratios for both *T. albacares* and *D. gigas*, which is consistent with formaldehyde fixation adding ^{12}C-enriched carbon to the tissues. We surmise that the tuna and squid tissues had δ^{15}N values greater than that of the formaldehyde used in these experiments and therefore we saw consistent trends in this experiment. For samples preserved in ethanol, δ^{13}C values of *D. gigas* were higher than frozen samples, although this trend was not apparent for *T. albacares*. *T. albacares* and *D. gigas* muscle has relatively low lipid content (C/N < 5), it is possible that ^{12}C-enriched lipids were removed from the preserved samples, which resulted in an increased in δ^{13}C values of *D. gigas*.

Overall, our two-year experiment indicated that the alteration of isotope values due to chemical preservatives occurred quickly upon preservation, then the isotope values stabilized, suggesting long-term storage in ethanol does not continue to alter tissue stable isotopic compositions. As tissues were fixed in formaldehyde for one week and stored in ethanol long-term, these results do not indicate potential effects of storage in formaldehyde on the δ^{13}C and δ^{15}N values from tissues, although the zooplankton portion of our study provides insight into long-term formaldehyde preservation. The magnitude of change in δ^{13}C values we observed from preserved tissues was slightly lower than the changes observed in the δ^{15}N values, however, small changes in δ^{13}C values are more likely to affect the interpretation of δ^{13}C values, as differences of ~ 1 ‰ can represent a trophic level change for the organism of interest.

Long-term formaldehyde preservation of zooplankton (*bulk isotope values)*

We found no differences in the $\delta^{15}N$ between frozen and formaldehyde-preserved copepod samples, indicating that $\delta^{15}N$ of copepods is minimally altered by long-term storage in formaldehyde. Our results support findings from a previous study with these same two species of copepods after 11 years preservation (Rau et al. 2003). A shorter-term study of calanoid copepods also found no change in $\delta^{15}N$ values of calanoid copepods after one year of storage in formaldehyde (Bicknell et al. 2011). Overall, these results are promising for future studies using formaldehyde-preserved, archived samples for nitrogen stable isotope analysis.

In contrast to $\delta^{15}N$ values, the $\delta^{13}C$ values of copepods were altered by storage in formaldehyde. The $\delta^{13}C$ values for both species decreased with formaldehyde preservation, but the difference between frozen and chemically-preserved samples was greater for *C. pacificus* than for *E. californicus*. Exactly this pattern, with nearly identical isotopic values as in the present study, was observed previously after 11 years preservation (Rau et al. 2003). There are several mechanisms that can explain changes in $\delta^{13}C$ values with formaldehyde preservation. First, as discussed above, formaldehyde could add carbon to the samples. This could explain a decrease in $\delta^{13}C$ values in the preserved tissue, which we found for both copepod species. The differences in the magnitude of change between *C. pacificus* and *E. californicus* can likely be explained by isotope mass balance, where the $\delta^{13}C$ values of preserved tissues converge on the $\delta^{13}C$ value of the formaldehyde used. We hypothesize that the $\delta^{13}C$ values of the formaldehyde used in this experiment and a previous study (Rau et al. 2003) were closer to that of *E. californicus* than *C. pacificus* and therefore formaldehyde fixation altered $\delta^{13}C$ values of *C. pacificus* more than those of *E. californicus*. If carbon was added to the tissues from formaldehyde, we would expect an increase in carbon content (% weight C) and C/N ratios,

which was not observed. There was no change in % C or C/N for *C. pacificus*, but the C/N ratio decreased with formaldehyde preservation in *E. californicus* samples and the % C decreased, although it was not statistically significant (Table 4.2).

An alternative mechanism for formaldehyde altering $\delta^{13}C$ values is through a loss of carbon from the tissue to the preservative. Some studies have suggested that compounds may leach out of the tissue upon formaldehyde fixation (Hobson et al. 1997, Bosley & Wainright 1999). Formaldehyde preferentially hydrolyzes proteins, which are enriched in ^{13}C compared to lipids. Thus, the preferential loss of ^{13}C could cause a relative increase in ^{12}C and decrease in $\delta^{13}C$ values with formaldehyde preservation (Bicknell et al. 2011). The observed changes in C/N ratio in formaldehyde-preserved samples may suggest that carbon was lost to the preservative, although one would expect that %N would decrease if proteins were hydrolyzed, which we did not find.

The mechanistic driver of the observed changes in $\delta^{13}C$ values is therefore somewhat unclear. Our sample size was small and overall, the $\delta^{13}C$ values, %C content, and C/N ratios were more variable in copepods, both in preserved and unpreserved samples, compared with squid and tuna muscle samples. This is unsurprising and can likely be attributed to the lipid content in copepods. While lipid content is uniformly low in squid and tuna muscle, the amount of lipid storage in copepods can vary between species, males and females of the same species, and temporally within or among species. Copepod lipid content can range from 2-73% of copepod dry weight (Blaxter et al. 1998, Ventura 2006) and high lipid content in tissues can result in lower $\delta^{13}C$ values than samples with low lipid content. In frozen samples, the C/N ratios of *E. californicus* were higher than that of *C. pacificus*, indicating that *E. californicus* may be more lipid-rich than *C. pacificus*, in agreement with previous results (Ohman 1988).

We did not remove lipids from our samples due to potential effects of lipid extraction on bulk δ^{15}N values reported in some studies (Mateo et al. 2008). To account for the potential variability in lipid content, which affects δ^{13}C values, we limited our analyses to adult females from springtime paired plankton tows. However, our samples were opportunistic, and we used copepods collected from different locations which may account for some of the variability in the δ^{13}C values among samples.

Our results bolster previous studies that reported a decrease in δ^{13}C values with formaldehyde preservation (Sarakinos et al. 2002, Barrow et al. 2008, Bicknell et al. 2011), specifically one study that reported a decrease of ~ 1 ‰ in δ^{13}C values of formaldehyde-preserved copepods (Bicknell et al. 2011). However, the δ^{13}C values from their study were less variable than ours, potentially because they extracted lipids from their samples. For a greater understanding of the mechanistic drivers of change in copepod δ^{13}C values, future studies should compare results between lipid-extracted and non-lipid-extracted samples. Techniques for lipid extraction using chloroform:methanol from zooplankton require close attention because non-lipid compounds can also be extracted (Ohman 1997).

Compound-specific isotope analysis of amino acids

The δ^{15}N values of most amino acids were minimally altered by chemical preservation, both in the formaldehyde-ethanol preserved tuna and squid samples and in the copepod samples preserved in formaldehyde. These results are promising for future CSIA-AA studies and provide further evidence that formaldehyde preservation does not affect δ^{15}N values of amino acids. However, we found a surprising trend in the δ^{15}N values of valine, where values were higher in formaldehyde preserved samples for *T. albacares*, but not for *D. gigas*. Additionally, the δ^{15}N

values of phenylalanine from formaldehyde-ethanol preserved samples were also substantially different than frozen samples in some samples from all three species. These changes were larger than those observed for most other amino acid δ^{15}N values. The results for phenylalanine are especially relevant, as it is frequently used as the canonical source amino acid to estimate trophic positions of species and to evaluate changes in baseline δ^{15}N values. Thus, understanding how and the degree to which δ^{15}N values of phenylalanine is altered by chemical preservation is pertinent to many CSIA-AA studies.

There is no mechanism by which ^{15}N enriched phenylalanine could be added to the sample from formaldehyde. A potential explanation for how chemical preservation could alter the δ^{15}N values of phenylalanine is through the breaking of C-N bonds. During this process, phenylalanine would be lost from the analytical pool, and thus we would expect a decrease in the peak area of phenylalanine and an enrichment of ^{15}N in the preserved samples. To evaluate this possibility, we calculated the ratios of peak areas of phenylalanine to other amino acids that were minimally altered by preservation (e.g., glutamic acid and proline). The peak areas ratios were relatively constant, which implies there was no preferential loss of phenylalanine in the formaldehyde preserved samples and this is not a likely explanation for the difference in δ^{15}N between chemically preserved and frozen tissues.

The discrepancy in the δ^{15}N values of phenylalanine between frozen and chemically preserved samples may reflect our ability to chromatographically separate phenylalanine form other amino acids and N-containing compounds in order to measure δ^{15}N values rather than phenylalanine being altered by chemical preservation. Measurement of amino acid δ^{15}N values using GC-IRMS techniques requires baseline chromatographic separation of peaks of different compounds (Meier-Augenstein 2002, Sessions 2006). This is essential, as the peak areas of

masses 28 and 29 are used to calculate ion-current ratios, which are then compared to the ion-current ratio of reference materials of known isotopic composition to calculate the $^{15}N/^{14}N$ ratio of individual compounds (Meier-Augenstein 2002, Sessions 2006). Thus, to determine isotope ratios it is imperative to separate and accurately measure the entire peak without interference from fully co-eluting or partially co-eluting compounds. In many of our samples, there were unknown peaks that appeared on the mass 28 and 29 chromatograms near phenylalanine and these peaks limited our ability to measure the $\delta^{15}N$ values of phenylalanine. Interestingly, the unknown N-containing peak, which eluted between glutamic acid and phenylalanine using our derivatization method and chromatographic column, was 15 times more abundant relative to phenylalanine in the frozen sample and was lowest in the tissue that was in ethanol for two years. Therefore, it is possible that the 95% ethanol solution solubilized the interfering compound and improved our ability to achieve baseline chromatographic separation of phenylalanine for nitrogen isotope analysis. Unfortunately, the identification of the interfering compound was beyond the scope of this work.

The performance of compound-specific isotope analyses using GC-irMS is commonly limited by chromatographic resolution of individual compounds (Hayes et al. 1990, Kenig et al. 2000, Ohkouchi et al. 2017). Our results underscore the importance of the preparative chromatographic steps necessary to isolate a pure amino acid fraction from hydrolyzed tissues (e.g., see recommendations by Ohkouchi et al. 2017). Even though we included a solvent extraction of our hydrolysate, these results suggest that we might have further purified our amino acid fraction by further extraction using 95% ethanol. We hypothesize that changes in the $\delta^{15}N$ values of phenylalanine can be attributed to chromatographic isolation and our ability to measure the $\delta^{15}N$ values rather than formaldehyde alteration of the $\delta^{15}N$ values of phenylalanine. Future

studies might focus on testing gas chromatographic columns with different stationary phases, which could result in greater chromatographic separation of the interfering peaks and phenylalanine, or employ alternative derivatization techniques to isolate amino acids in the samples prior to analysis (see Ohkouchi et al. 2017).

Conclusions

Overall, our results indicate that formaldehyde and ethanol can produce small changes in the δ^{15}N values of tissues, which is consistent with findings from other studies (Hobson et al. 1997, Bosley & Wainright 1999, Kaehler & Pakhomov 2001). Formaldehyde does not contain nitrogen and thus this preservation method is not expected to greatly affect δ^{15}N values from organismal tissues. However, formaldehyde by itself or in combination with 95% ethanol may promote leaching or solubilization of compounds in tissues that can alter δ^{15}N values (Hobson et al. 1997, Bosley & Wainright 1999). Although we did find changes in δ^{15}N values in the formaldehyde-ethanol experiment, they were relatively uniform. Our findings generally concur with the literature, where increases in δ^{15}N values associated with formaldehyde or ethanol preservation are less than the 2 to 5 ‰ change used to detect trophic level differences in food web studies. Thus, the alteration of δ^{15}N values with formaldehyde fixation-ethanol preservation may or may not be ecologically significant, as we found increases of ~1.5 ‰, which can affect the interpretations of isotope data in ecological studies when trophic enrichment is lower than ~3 ‰. Long-term storage of calanoid copepods in formaldehyde did not alter δ^{15}N values, which is promising for future studies.

Since the δ^{13}C values in our study were altered in inconsistent ways, using δ^{13}C from preserved tissues should be avoided unless there is a mechanistic understanding of how the

preservative specifically alters $\delta^{13}C$ values of the tissue of interest. Using a fixative-preservative combination (e.g., formaldehyde-ethanol) complicates data interpretation, as it is difficult to disentangle the independent and cumulative effects of multiple preservatives on stable isotope values. Our results suggest that formaldehyde may have altered tissues in different ways, both through adding carbon to the samples and through the leaching of compounds from the tissue. Although correction factors can be useful, they should not be developed without a mechanistic understanding of how the preservation method alters $\delta^{13}C$ values. We recommend testing the $\delta^{13}C$ value of formaldehyde, using the same bottle of formaldehyde throughout an experiment, and removing lipids with appropriate caution to obtain more uniform $\delta^{13}C$ values and C/N ratios. Additionally, future studies evaluating preservation effects of amino acid $\delta^{13}C$ values would be useful to determine if these values are altered with preservation.

This study provides both promise and reason for caution for future studies that aim to use amino acid $\delta^{15}N$ values from preserved specimens. The long-term zooplankton study showed no change in $\delta^{15}N$ values after 25 years of storage in formaldehyde, in agreement with a previous experiment lasting 11 years (Rau et al. 2003) Additionally most measurements of amino acid $\delta^{15}N$ values from preserved tissues were within the typical 1‰ error associated with CSIA-AA. However, future studies that illuminate the differences we found in some valine and phenylalanine $\delta^{15}N$ values would be useful. We hypothesize that the differences we found are reflective of analytical uncertainty rather than preservation altering $\delta^{15}N$, but future studies testing this hypothesis would helpful for the best use of stable isotope data in ecological studies.

Acknowledgements

We would like to thank Robert Olson and Leanne Duffy at the Inter-American Tropical Tuna Commission for sample collection and preparation. We would also like to thank Natalie Wallsgrove and the University of Hawaii's Isotope Geochemistry Laboratory for assistance in sample preparation and isotope analyses. This research was funded by the University of California San Diego. This is School of Ocean Science and Technology contribution number ####.

Chapter 4, in full, is being submitted to Rapid Communications in Mass Spectrometry in July of 2018, Hetherington, ED, Kurle, CM, Ohman, MD, and Popp, BN. The dissertation author is the primary investigator and author of this paper.

Appendix

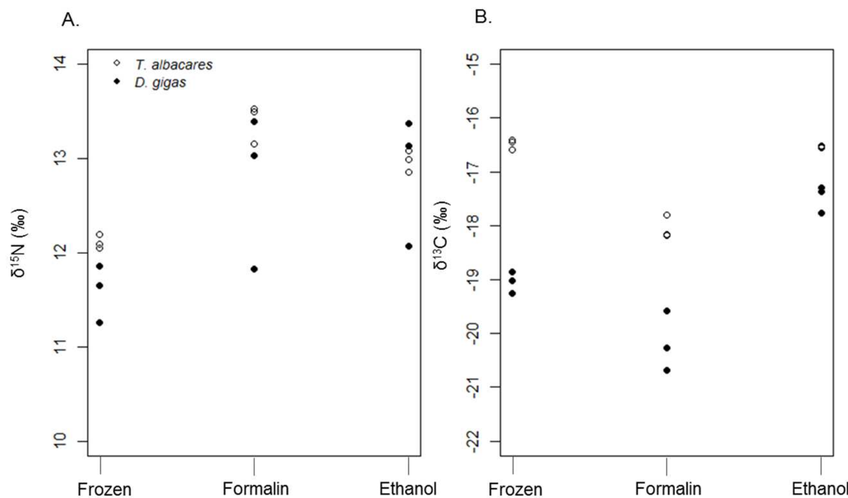

Figure 4A.1. A) δ^{15}N values and B) δ^{13}C values from frozen versus preserves muscle tissue from *Thunnus albacares* and *Dosidicus gigas,* where samples were either stored in formaldehyde or ethanol for one month.

Table 4A.1. Test statistics for the follow-up experiment shown in Figure 4A.1, where we tested the change in isotope values, %C, %N, and C/N ratios among preservation treatments (frozen, formaldehyde, and ethanol) using ANOVAs.

Species	Variable	Df	F	p
Thunnus albacares	$\delta^{15}N$	2,6	61.73	< 0.0001
	$\delta^{13}C$	2,6	130.6	< 0.0001
	% N	2,6	1.82	0.24
	% C	2,6	1.38	0.32
	C/N	2,6	1.61×10^{32}	< 0.0001
Dosidicus gigas	$\delta^{15}N$	2,6	3.58	0.09
	$\delta^{13}C$	2,6	40.26	< 0.001
	% N	2,6	1.73	0.26
	% C	2,6	1.41	0.31
	C/N	2,6	108.2	< 0.0001

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CONCLUSION

The objectives of this dissertation were to evaluate the contribution of trophic and foraging ecology on the populations of leatherback turtles in two ocean basins, and to investigate how environmental variability can alter food web dynamics in the California Current Large Marine Ecosystem (CCLME), which is a critical habitat for leatherback turtles and other top predators of conservation or commercial interest (Benson et al. 2007, Benson et al. 2011, Bailey et al. 2012, Block et al. 2011, Hazen et al. 2013). As many populations of large pelagic fishes, marine mammals, and sea turtles continue to decline from an array of anthropogenic threats, understanding the energy pathways that support their biomass, and how these pathways may change over time is important for the conservation and management of marine resources. However, obtaining dietary data on threatened species that inhabit pelagic ecosystems and migrate through multiple jurisdictions present many challenges. In my dissertation research, I used stable isotope analyses on consumer tissues to answer questions about foraging ecology and marine food webs.

In Chapter 1, I used an eighteen-year collection of archived blood samples from a nesting group of North Atlantic leatherback turtles for stable isotope analysis. My objective was to evaluate patterns in foraging ecology from 1992-2010, a period in which this leatherback population was steadily increasing and rebounding from a population decline (Dutton et al. 2005). I was particularly interested in how the foraging quality or oceanographic conditions within the North Atlantic could impact this population and potentially contribute to its population success. There was no change in trophic ecology (measured through trophic position (TP) estimates) over time and TP estimates for North Atlantic leatherbacks in this study were nearly identical to those from a declining Pacific population. However, nitrogen isotope values

significantly decreased over time, which may be linked to changes in nitrogen cycling biogeochemistry over my sampling period.

My study overlapped with a positive phase of the North Atlantic Oscillation (NAO), which is used to describe broad-scale patterns in sea surface temperature throughout the North Atlantic Ocean. The NAO index was related to leatherback isotope values and nesting parameters, indicating that leatherbacks nested more frequently and laid a higher number of clutches during positive NAO phases. I hypothesize that during the positive phase of the NAO, warm sea-surface temperature trends in leatherback foraging areas created favorable conditions for gelatinous zooplankton and an overall steady prey supply for leatherbacks in this region, which contributed to their population recovery.

In this chapter, I also used nitrogen isotope values of individuals that were sampled over multiple nesting seasons to evaluate leatherback fidelity to distinct foraging areas. I found that the nesting population as a whole uses multiple foraging areas and migrates to the nesting beach from one of several regions, while individual turtles exhibit fidelity to certain areas. My findings provide useful information about leatherback habitat use and support previous satellite telemetry data from North Atlantic leatherbacks (Hays et al. 2006, Fossette et al. 2010). These data are useful for agencies managing leatherback populations and working to limit leatherback bycatch in regions where they overlap with fisheries.

Future research should focus on evaluating potential differences in leatherback prey abundances among their foraging hotspots. To specifically test my hypothesis that environmental conditions in the North Atlantic were beneficial for leatherbacks in the North Atlantic and contributed to their population recovery, studies should examine the trends in leatherback prey

(gelatinous zooplankton) abundances, or a proxy for primary and secondary production, in distinct leatherback foraging areas over time.

Unlike the North Atlantic leatherback population, Pacific populations are declining and in danger of extinction (Spotila et al. 2000, Tapilatu et al. 2013). My second chapter focused on the foraging ecology of western Pacific leatherbacks, which nest in Indonesia and migrate to the California Current Large Marine Ecosystem (CCLME), presumably to forage on seasonally abundant gelatinous prey. I was interested in potential differences in foraging ecology between Atlantic and Pacific populations and the impacts of diet, trophic ecology, and habitat use on the population dichotomy between leatherback conspecifics. I used stable isotope analyses on leatherbacks and their potential gelatinous prey collected in the CCLME from 2003-2016. In addition to answering key questions about leatherback foraging ecology, I was also interested in evaluating the trophic structure of gelatinous zooplankton in the CCLME, as they are an understudied food web component in many ecosystems and can provide important information on energy flow pathways that support leatherback biomass.

I found no change in nitrogen isotope values (or TPs) for leatherbacks over our sampling period, indicating relative food web or trophic stability. However, carbon isotope values, which can be used to assess habitat use patterns, were higher in 2005 compared to other years. My results suggest that in 2005, leatherbacks were foraging in more nearshore areas, which support data from aerial surveys showing a restricted geographic range for leatherbacks during that year. I hypothesize this change in habitat use can be attributed to anomalous environmental conditions, where upwelling was reduced and delayed in 2005, creating unfavorable conditions for leatherback prey, which I illustrated through examining an upwelling index in my study area.

The isotope mixing model, which used to evaluate the contributions of different prey items to leatherback diet, indicated that leatherbacks in the CCLME selectively feed on the scyphozoan *Chrysaora spp.*, which also supports the limited data on direct observations and animal-borne cameras (Peterson et al. 2006, Benson et al. 2007). However, my results also suggest that leatherbacks are consuming salps or other low-trophic level, filter-feeding organisms. I hypothesize that in nearshore areas, leatherbacks selectively feed on *Chrysaora spp*., but in offshore habitats where large scyphozoans are less abundant, they consume filter-feeding salps and pyrosomes, particularly when they are densely aggregated.

In this chapter, I was also interested in the trophic structure of the gelatinous energy pathways that support leatherback biomass in the CCLME. I used multiple methods for estimating the TPs of leatherbacks and gelatinous zooplankton, and highlighted the limitations and advantages of each approach. Overall, we found agreement between approaches, where TP estimates for leatherbacks were higher than their gelatinous prey, there was trophic and niche overlap between some scyphozoans at intermediate trophic levels, and salps occupy the lowest TP.

This chapter provided information useful for managers, particularly for the isotope values and TP estimates which, can be incorporated into food web or dynamic ocean management models. These tools are used to better understand the CCLME food web, predict food web changes under climate change scenarios, and predict leatherback habitat use patterns to minimize their interactions with fisheries.

In Chapter 2, my data suggest that leatherback habitat shifted during a period of reduced or delayed upwelling, although their trophic ecology was stable over time. However, we were unable to collect leatherback samples in 2014 or 2015 to evaluate leatherback trophic ecology

associated with recent oceanographic anomalies. Previous studies have suggested that Pacific leatherbacks are more vulnerable to population declines because of environmental variability associated with El Niño-Southern Oscillation in the Pacific, which may limit their ability to acquire the energy needed for reproduction (Saba et al. 2007, Saba et al. 2008). Furthermore, many previous studies have documented the wide-ranging biological responses to El Niño events in the CCLME, including the reduction of primary production, and increased mortality of top predators like marine mammals, sea lions, and birds (Barber & Chavez 1983, Barber et al. 1996, Chavez et al. 2002, Lindegren et al. 2018).

The recent warm water anomalies (marine heatwave and strong El Niño event) in the Northeastern Pacific Ocean provided an opportunity to assess the effects of warming on the CCLME and its potential for altering food web structure and energy flow to consumers. In Chapter 3, I evaluated potential differences in the trophic ecology of an abundant copepod, *Calanus pacificus*, during the warm water anomalies, as it is an important zooplankton species for transferring energy between phytoplankton and higher trophic level consumers. Specifically, I used trophic position estimates from stable isotope analyses of *C. pacificus* as a proxy for food web length to assess potential differences in food web structure during the warm water anomalies.

My results indicate that warming began in late 2013-early 2014 at my sampling stations and persisted through to spring of 2016. In addition to increased water temperature, upwelling and zooplankton biomass were significantly reduced. There was also a decrease in the maximum values of chlorophyll *a* and nitrate concentrations during the warm anomalies, which indicates nutrient limitation and subsequently reduced primary and secondary production. The $\delta^{15}N$ values of *C. pacificus* in my time series were variable, but overall increased with the onset of warming

and persisted through the ENSO event. Although I found an increase in $\delta^{15}N$ values, which was most apparent in the spring samples, there was no change in trophic position. Rather, the source amino acid $\delta^{15}N$ values also increased during the warm anomalies. These results suggest the trend in bulk $\delta^{15}N$ values were attributed to nitrogen cycling dynamics, likely an increased nitrate utilization compared to supply, rather than food web elongation or changes in food web structure.

Overall, my results demonstrate how environmental variability such as El Niño events, or large environmental anomalies like the persistent marine heatwave, can alter ecosystems through reduced upwelling, which limits nutrient availability to phytoplankton and ultimately reduces available energy to consumer biomass. Although we did not find differences in food web structure, isotope values do not provide information about prey availability or abundances. Furthermore, *C. pacificus* could have exhibited a prey switch to different phytoplankton or microzooplankton species of similar trophic status, which would not result in a change in the trophic position of *C. pacificus*, but could affect their biomass or population dynamics. It is therefore possible that there were differences in food web length that we could not detect with our analyses. Future studies that couple stable isotopes of higher trophic level predators with in situ diet data (e.g., scat or stomach contents analysis) or prey abundance data could provide useful insight into how these anomalies propagate up to top predators and ultimately influence their reproductive success during periods of unfavorable conditions.

The final chapter of my dissertation is a methodological contribution to the field of isotope ecology. As ecosystems are degraded human activity, many scientists aim to establish historic baselines so we can track ecosystem changes over time. Stable isotope values from previously collected and archived specimens may be valuable for reconstructing past food web

structure and foraging ecology of species. However, archived and museum specimens are often stored in chemical preservatives (e.g., formaldehyde, ethanol) and therefore, it is essential to evaluate the effects of these preservation methods on the stable isotope values of organisms before using them to reconstruct historical food web dynamics. Chapter 4 provides useful information for researchers aiming to use stable isotope analysis on archived specimens that have been stored in chemical preservatives.

I conducted experiments to test the effects of formaldehyde and ethanol on the nitrogen and carbon isotope values of tunas, squids, and copepods. Tuna and squid specimens were initially fixed in formaldehyde for one week and then stored in ethanol for up to two years. For copepod samples, I compared paired versus frozen zooplankton samples that were stored in formaldehyde for 24-25 years. Overall, the changes in nitrogen isotope values associated with chemical preservation were mostly minimal, suggesting that storage in formaldehyde or ethanol likely will not affect the interpretation of nitrogen isotope values used in ecological studies. However, preservation in formaldehyde and ethanol had more variable and substantial effects on carbon isotope values, where the direction and magnitude of change differed among species and treatments. Chemical preservation effects on amino acid isotope values were relatively small (< 1 ‰), which is promising for future studies. However, I found differences in phenylalanine isotope values (3-5 ‰), between frozen and chemically preserved tissues, which is particularly notable as phenylalanine is the canonical source amino acid used to establish isotopic baselines and estimate trophic position. I speculate that changes in phenylalanine resulted from interference in chromatographic resolution of a compound rather than alteration of its isotopic composition due to chemical preservation but future research is needed to test this hypothesis.

My dissertation research demonstrates the importance of archived and museum specimens for monitoring and assessing long-term trends in the trophic and foraging ecology of species and evaluating ecosystem responses to environmental perturbations. The preservation experiments contribute practical knowledge for future studies on how to best use archived specimens in ecological studies to reconstruct baselines and evaluate the trophic ecology of species over time.

There are many difficulties in predicting species and ecosystem responses to environmental variability and intensifying climate change scenarios, as there are multiple interactive mechanisms that regulate ecosystem structure and the population dynamics of predators in marine ecosystems. In my dissertation, I was able to assess how foraging ecology influences two populations of leatherback turtles and the mechanisms through which warm water anomalies affect an important upwelling ecosystem. This work has applications for management of a threatened species my results can be used and incorporated into food web, ecosystem, or habitat models that improve our understanding of leatherback turtles and the CCLME. Furthermore, this dissertation contributes to our understanding of community ecology, and provides insight into the effects of environmental variability on food web ecology and energy flow pathways to consumers.

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