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Use of Stable Isotope and Trace Metal Signatures to Track the Emigration of Female Blue Crabs, Callinectes sapidus, from Tampa Bay

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Use of Stable Isotope and Trace Metal Signatures to Track the Emigration of Female Blue Crabs, *Callinectes sapidus*, from Tampa Bay

by

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A thesis submitted in partial fulfillment
of the requirements for the degree of
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Dedication

This thesis is dedicated to my parents Robert and Susan Williams. They push me to be my best so that I may, one day, change the world.

Acknowledgments

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Of course the guidance of my advisors has been invaluable to me throughout this process. Thank you, Kendra Daly, Ernst Peebles, and Ryan Gandy; the knowledge that you have imparted to me, I will carry with me throughout my career. Finally, I could not have accomplished everything that I have without the encouragement and wisdom of my parents, Robert and Susan, and the support of my friends and my siblings, Rheyn, Starr, and Hale.

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Abstract

The blue crab, *Callinectes sapidus*, supports a successful fishery in the Atlantic Ocean and throughout the Gulf of Mexico, with a total landing of 8,158,788 lb. and a total value of $10,562,128 for the state of Florida during 2012 (FWC 2012 Annual Landings Summary). An accurate and comprehensive understanding of the blue crab's life history and seasonal migration behavior is essential in defining effective management strategies for the fishery. Tag recapture studies and ultrasonic tracking methods for studying blue crab migrations are costly in terms of time and resources. In this study an alternative approach, microchemical natural tagging, was successfully used to determine a female's mating habitat. This approach assumes that the exoskeleton of the post-terminal molt female blue crab reflects the mating habitat's chemical signature and that the chemical signals are stable over time. To test these hypotheses, mature female blue crabs were collected from two Tampa Bay locations. Collected crabs were placed in tanks for 29 days, a subset was sacrificed at T = 0 and then twice per week, and the exoskeletons were analyzed via Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and Elemental Analyzer Infrared Mass Spectrometry (EA-IRMS) to observe the stability of the exoskseletal chemical signature over time. Over the 29 day time series, no significant change in the concentrations of Li, Ca, and Ba, or the isotopic ratios of $^{13}C/^{12}C$ ($\delta^{13}C$) and $^{15}N/^{14}N$ ($\delta^{15}N$) were observed (ANOVA p-value > 0.05). A Canonical Analysis of Principal Coordinates (CAP)-based discriminate analysis with leave-one-out cross-validation collectively compared Li concentrations, $\delta^{13}C$, and $\delta^{15}N$ among five Tampa Bay locations, producing a confusion matrix successfully classifying field collected crabs into: Alafia River 33%, Little Manatee River 71%,

Palm River 67%, Safety Harbor 30%, and Skyway Fishing Pier 83%, with an overall classification success of 66%. These results suggest that the largest biomass component of the migratory pulse collected near the mouth of Tampa Bay was dominated by crabs originating from an area not widely harvested by commercial fishermen, as relatively few of the migrating females were matched to riverine locations that were intensively fished. Instead, most appeared to originate from open waters of Tampa Bay. It is possible that low densities of blue crab inhabiting a large area that is not commercially fished, effectively shields a proportion of the individuals in the Tampa Bay estuary from economic exploitation, creating a density-dependent natural harvest refugium.

Introduction

The blue crab, *Callinectes sapidus* (family Portunidae), supports both commercial and recreational fisheries in the Atlantic Ocean (Millikin and Williams 1984) and throughout the Gulf of Mexico (Adkins 1972, Perry et al. 1984, Steele and Perry 1990). Maintaining a sustainable fishery is a mandate of the Magnuson-Stevenson Fisheries Management Act and at the core of marine fisheries management. To this end, the development of new technologies for determining linkages between fished populations is essential in ascertaining the connectivity and defining management units for accurate stock assessments. The objective of this thesis was to investigate the migratory behavior of post-copulatory female blue crabs. These data would provide fisheries managers with a better understanding of migration patterns, stock linkages and essential mating regions to help inform management.

Ovigerous female blue crabs from Tampa Bay migrate offshore to spawn (Oesterling 1976, Steele 1991). Linking emigrating females at the mouth of Tampa Bay back to the locations of mating would aid in the determination of essential mating habitat for this species in the region. Tagging and ultrasonic tracking techniques for studying blue crab migrations are costly in terms of time and resources. These methods have been used on numerous occasions in areas such as Chesapeake Bay (Wolcott and Hines 1990, Turner et al. 2003, Aguilar et al. 2005) and North Carolina estuaries (Carr et al. 2004). Alternative lower cost tracking and higher sample size methods using the natural tags of trace elements and isotopic ratios of $^{13}C/^{12}C$ ($\delta^{13}C$) and $^{15}N/^{14}N$ ($\delta^{15}N$) have the potential to meet this goal. If successful, the use of natural tags to determine the contribution of female blue crabs from various regions to the spawning stock of crabs in the state

would be a critically important component to be accounted for by marine fisheries managers (Jones et al. 1990).

Life Cycle and Migration

The life cycle of the blue crab begins when an ovigerous female releases larvae (zoeae) into mid- to high-salinity waters, either directly at the mouth of an estuary, or in higher salinity open-water spawning grounds (Kennedy and Cronin 2007). The zoeae molt through up to seven or eight stages in high-salinity waters and then metamorphose to the last larval, or megalops, stage before seeking out and settling in estuarine environments. Once blue crab megalopae reach nearshore waters they take advantage of flood-tide transport by using vertical migration behavior in relation to in-coming tides and actively travel down the salinity gradient (selective tidal-stream transport, Forward et al. 2003). Settled juvenile blue crabs seek out low salinity waters, which include areas rich in submerged aquatic vegetation. After another series of ecdyses, or molts, females attract mature males. Mating takes place during the final, or terminal, molt (Jivoff and Hines 1998).

Soon after molting, while the outer cuticle is still soft, the male mates with the female and the sperm is stored to later fertilize her eggs (Wenner 1989). After mating, a female absorbs the surrounding water and minerals to harden the exoskeleton. The cuticle is extensively calcified in this process, thus, establishing the chemical composition of the carapace (Roer and Dillaman 1984, Cameron 1985) and potentially recording the chemical signature of the mating environment. The microchemistry of the hardened cuticle is assumed to be unchanging over time between molts and after the terminal molt, but this has not been fully documented.

After the terminal molt, a female remains in low-salinity waters until she has acquired enough calories to replenish the expended energy from the recent ecdysis, as well as to prepare for migration to spawning grounds (Turner et al. 2003). During this time, newly mature females are commercially harvested using baited traps placed on the floor of riverine mating habitats with high adult population densities.

Once a fertilized female is prepared for the migration, she begins traveling toward higher salinity waters via selective tidal-stream transport, in the form of short bursts of swimming and passive surface drifting during ebb tides (Tankersley et al. 1998, Forward et al. 2003, Aguilar et al. 2005), followed by downward vertical migration and walking on the sediment during flood tides (Carr et al. 2004, Hench et al. 2004). Females taking advantage of fast moving surface currents are vulnerable to collection by recreational fishermen using dip-nets. Once a female reaches high-salinity spawning grounds, she will fertilize her eggs amassed in a 'sponge', which then develop and later hatch as zoeae (Jones et al. 1990, Tankersley et al. 1998, DiBacco and Levin 2000).

Ecdysis and Trace Metal Substitution

The exoskeleton of *Callinectes sapidus*, during the intermolt period, is composed of four distinct layers. These layers include from external to internal: epicuticle, exocuticle, endocuticle, and membranous layer (Roer and Dillaman 1984). The three outer layers, collectively referred to as the cuticle, each contain calcium carbonate ($CaCO_3$) in some proportion, along with protein, lipoprotein, and chitin (Kennedy and Cronin 2007). Through early premolt, the membranous layer breaks down and a new epicuticle and exocuticle are formed in its place during late premolt. These new layers are soft and missing calcite, the dominant mineral form of $CaCO_3$ in

the exoskeleton (Cameron 1985). Once the old cuticle is shed, a new endocuticle is formed beneath the new exocuticle in the early postmolt period, and water is absorbed through the gills and gut to assist in the expansion and calcification of the new cuticle (Neufeld and Cameron 1994). During calcification, the calcium from the surrounding water is used to add calcite to the new cuticle, causing it to harden (Cameron 1985).

Trace amounts of divalent metals from the water are substituted into the organic integument in place of Ca^{2+} (Lea and Spero 1992, Finch and Allison 2007). Concentrations of some metals found in blue crab tissues are regulated by metabolic processes, while others reflect ambient environmental conditions. Natural variation in metal concentrations in the water and consumed organic tissues can be caused by geography and seasonal runoff effects (Olowoyo et al. 2010, Ayas and Ozogul 2011), as well as anthropogenic inputs (Karouna-Renier et al. 2007, Ayas and Ozogul 2011). A variety of anthropogenic factors have been documented as contributing to localized variation in aquatic metal concentrations, including pesticides, fertilizers, and urban pollution (Karouna-Renier et al. 2007, Ayas and Ozogul 2011, Mutlu et al. 2011). Inductively Coupled Plasma Mass Spectrometry (ICP-MS) is a high-precision analytical instrument that can quantify metal concentrations. This technique has been previously used on homogenized blue crabs (Yang and Swami 2007) and, while it has never been completed on crab exoskeleton separate from other tissues, ICP-MS has the potential to detect minute metal concentration differences within the cuticle of crabs originating from different geographic locations.

Stable Isotope Composition

Isotopes of carbon and nitrogen have been extensively used to investigate trophic relationships and habitat use of marine organisms (Smith and Epstein 1971, DeNiro and Epstein 1978, Fantle et al. 1999, Dittel et al. 2006). Heavy isotopes have an additional neutron that decreases the zero-point energy of their bonds. A decrease in zero-point energy increases the amount of energy required to break those bonds, leading to isotopic fractionation in metabolic chemical reactions (Sharp 2007). The ratios of $^{13}C/^{12}C$ ($\delta^{13}C$) and $^{15}N/^{14}N$ ($\delta^{15}N$), expressed in units per mil (‰), are indicators used for investigating which primary producer forms the base of a particular food web (i.e., the "basal resource") and the trophic level of an animal within a particular trophic pathway (DeNiro and Epstein 1978, Peterson and Fry 1987, Tieszen et al. 1983). Food webs which rely largely on typical terrestrial C3 plants generally have a $\delta^{13}C$ value around -27‰ (Smith and Epstein 1971, Sharp 2007), while C4 plants exhibit a less depleted value of -13‰. Marine phytoplankton as a basal resource tends to fall somewhere in the middle and, while being highly variable, can often be observed around -22‰ to -20‰ (Haines and Montague 1979, Peterson and Fry 1987). $\delta^{15}N$ values reflect nutrient source, such as atmospheric deposition and runoff, biological (trophic) fractionation, and anthropogenic input (Incze et al. 1982, Fantle et al. 1999, Sigman et al. 2001).

Each step above primary producers in the food web results in more enriched values of $\delta^{13}C$ and $\delta^{15}N$. Each increase in trophic level results in a rise of 0‰ to 1.5‰ $\delta^{13}C$ and 2.3‰ to 3.4‰ $\delta^{15}N$ observed in the consumer (Peterson and Fry 1987, McCutchan et al. 2003, Dittel et al. 2006). This well documented enrichment factor has been applied to blue crabs, while investigating trophic relationships and habitat use (Fantle et al. 1999, Bucci 2003, Bucci et al. 2007). These studies almost exclusively target tissues with an isotopic turnover rate that

constantly adjust to reflect environmental characteristics, such as skeletal muscle (Bucci 2003), hepatopancreas, gill tissue (Llewellyn and Payre 2011), or homogenized whole crabs (Fantle et al. 1999, Dittel et al. 2000), rather than analyzing cuticle material separate from other tissues.

Approach

The microchemical approach applied in this study has been used with great success to investigate the geographic life histories of fishes (Thorrold et al. 1998, Campana 1999, Thorrold et al. 2002), but, to the author's knowledge, has never been conducted on crustaceans. A fish's otolith serves as an ideal microchemical storage mechanism and creates a life-long record of the organism's environmental and metabolic fluctuations. The blue crab, however, does not possess any material that is retained for its entire life, due to tissue turn-over and ecdysis.

The goal of this study was to establish whether the hardened cuticle of a post-terminal molt female blue crab could be used to determine the specific location of an individual's mating habitat. The initial hypothesis was that female blue crabs migrating out of the mouth of Tampa Bay towards the Gulf of Mexico would be a mix of individuals from the four major northern Tampa Bay riverine mating habitats previously identified by local commercial blue crab fishermen (i.e. Alafia River, Little Manatee River, Palm River, and Safety Harbor). A microchemical tracing technique was applied to a species that is difficult and costly to track, and whose spawning migrations are poorly known in many parts of its extensive range. The specific objectives of this research were to:

1. Identify the metals and stable isotopes that are chemically stable in the exoskeleton of the blue crab, *Callinectes sapidus*.

2. Use an exploratory chemical analysis to identify which metals and stable isotopes are most useful in chemically differentiating mating habitats in Tampa Bay.
3. Determine whether a microchemical approach can be used on exoskeletal material to determine the mating habitats of migrating post-copulatory female blue crabs.

Methodology

Field Collection

Mature female blue crabs were sampled from five locations around Tampa Bay, Florida, in 2011 and 2012 to experimentally determine the chemical signature and stability of a mature female blue crab's cuticle. In 2011, individuals from the Alafia River (27°51'25.70''N, 82°22'31.76''W, n = 12), Palm River (27°56'48.47''N, 82°23'33.55''W, n = 9), Little Manatee River (27°41'25.05''N, 82°27'0.27''W, n = 10), and Safety Harbor (27°1'25.15''N, 82°40'40.02''W, n = 37) (Figure 1) were purchased from commercial blue crab fishermen (coordinates are approximate). In addition, individuals swimming in surface waters near the mouth of Tampa Bay towards the Gulf were collected from the Skyway Fishing Pier (27°38'3.42''N, 82°40'0.93''W, n = 65) by Florida Fish and Wildlife Research Institute using dip-nets during a post-sunset outgoing tide. Within 24 hours of collection, all individuals were sacrificed and dissected using ceramic and plastic tools to avoid metal contamination; followed by preservation of the exoskseletal material from the carapace by freezing (-80° C).

In 2012, individuals were collected from the Little Manatee River (n = 54) and the Skyway Fishing Pier (n = 43) in the same manner as described above. A subset of individuals from the Little Manatee River (n = 20) and Skyway Fishing Pier (n = 11) were immediately sacrificed and processed, while the remaining mature females were placed in 2,460 liter (650 US gallon) tanks located at the Florida Fish and Wildlife Stock Enhancement Research Facility (SERF) to determine the longevity of chemical signatures.

Figure 1. Map of Tampa Bay with collection sites indicated (Google Earth).

Time Series Captive Animal Study

A time-series tank study using captive crabs was conducted to determine whether chemical constituents of blue crab exoskeletons varied over time. Mature female blue crabs were collected from the Little Manatee River (n = 34) and the Skyway Fishing Pier (n = 32) and placed into eight tanks for each source region (16 tanks total) on May 8, 2012. Salinity of the water started at 35.3 and decreased over four days down to 13.6 - 15.3, which was similar to that of Tampa Bay river mouth regions. Between May 14 and June 6, 2012, two to four individuals

from each of the two source groups were sampled and processed, as described previously, twice per week. Individuals were fed cigar minnows three times per week, and a 100% water exchange (salinity 10 - 12) was carried-out in each tank once a week. Deceased individuals were disposed of without being sampled. A water sample mixed from five randomly chosen tanks was collected three times per week to observe the elemental variability in the water. Water samples were immediately filtered (0.22 μm) to remove particulate matter, and acidified with 16M nitric acid (HNO_3) and refrigerated, to prevent precipitation or absorption onto the sides of the vial.

Sample Preparation

Frozen cuticle samples were thawed, cleaned with deionized water and sonicated, then placed in a drying oven (70^o C) for 36 hours. Samples were submerged in 2% nitric acid for 36 hours. The un-digested insoluble material was removed, rinsed and sonicated, dried in a drying oven (70^o C), and packaged in tin capsules. The carbon and nitrogen isotopic composition of the insoluble material was measured by continuous-flow isotope ratio mass spectrometry using a Carlo-Erba 2500 Series-II Elemental Analyzer (EA) (Carlo-Erba Instruments Ltd., Milan, Italy) coupled to a *ThermoFisher Delta+XL Isotope-Ratio Mass Spectrometer* (IRMS) (Thermo Fisher Scientific Inc., Bremen, Germany). Measurements were normalized to international scale (AT-Air for $\delta^{15}N$, VPDB for $\delta^{13}C$) using United States Geological Survey (USGS) 8573 and USGS 8574 L-glutamic acid Reference Materials. Analytical uncertainties of ±0.1‰ for $\delta^{13}C$ and ±0.16‰ for $\delta^{15}N$ were estimated for samples collected in 2011, and ±0.27 for $\delta^{13}C$ and ±0.21‰ for $\delta^{15}N$‰ for 2012, based on n = 27 replicated measurements of an isotopically homogenous lab standard.

The remaining nitric acid, containing the dissolved material, was spiked with internal standards indium, scandium, and bismuth (1,000 ppb), and diluted with additional nitric acid to a factor of 35 for the dissolved material solution, and a factor of 10 for the internal standards. These liquid samples were analyzed for elemental concentrations with an Agilent 7500cx Inductively Coupled Plasma Mass Spectrometer (ICP-MS) (Agilent Technologies Inc., Delaware, United States) equipped with an octopole reaction system (ORS) for helium mode, a MicroMist nebulizer, and a double-pass (Scott-type) quartz spray chamber, Peltier-cooled to 2^{o}C.

Two methods of analysis were used to process samples through the ICP-MS: quantitative method and semi-quantitative method. The semi-quantitative method is an exploratory technique that returns concentrations for a large number of elements. Acquired counts-per-second (cps) are converted to concentrations [ppb] using a conversion database. The factory pre-set semi-quantitative factors in the database were corrected using a single standard run with the dissolved and diluted samples in order to compensate for machine sensitivity. The quantitative method returns concentrations for fewer elements, but is considered to have higher precision because a calibration line of varied concentrations for each element of interest is run with the samples and used to convert acquired cps to concentration, rather than using a database. Samples from 2011 were analyzed using the semi-quantitative method, while 2012 samples were analyzed using the quantitative method.

Data and Statistics

Output concentrations from the ICP-MS were normalized to the mass of dry sample digested in nitric acid. All samples were run in duplicate through the EA-IRMS, and ratios that

were more than two standard errors apart, indicating a mechanical failure or heterogeneous sample, were removed from analysis.

All statistical analyses and graphs were conducted using MATLAB (version 7.12.0.635[R2011.a], Natick, Massachusetts: The MathWorks Inc.), the Fathom toolbox for MATLAB (Jones 2011), and Microsoft Excel (2007, Redmond, Washington). Permutation-based distribution-free ANOVA variants (Anderson 2011) were used to determine if there were any significant changes in the chemical composition of the cuticle during the 29 day captive animal tank study. In addition, these statistical tests were used to analyze data from the field study in order to compare a single concentration or isotopic ratio between multiple sampling locations of a single year, or a single location between years. Permutation-based distribution-free MANOVA variants (Anderson 2001) were used for pairwise comparisons between geographic locations using more than one analyte. Lastly, a Canonical Analysis of Principal Coordinates (CAP)-based discriminant analysis was used to identify elements that were most influential in differentiating among collection sites (Anderson and Willis 2003).

A CAP analysis begins with a principal coordinate analysis run on a distance or dissimilarity metric, in this case a Bray-Curtis Dissimilarity matrix, yielding a new matrix of orthogonal axes. A canonical analysis is run on an appropriate number of axes determined to maximize the classification success of a leave-one-out (LOO) cross-validation. The LOO procedure observes how distinct a geographic location's chemical signal is by generating a confusion matrix, which displays how successful the test was at reclassifying each blue crab into its original geographic location. $\alpha = 0.05$ was used for all statistical tests, and all permutation-based tests used 1,000 iterations.

Results

Field Study

Exploratory ICP-MS analysis of the 2011 samples returned concentrations for 79 elements. Forty-eight elements were removed from further analysis, because their concentrations were below detection limits or the element suffered from spectral interferences. The remaining 31 elemental concentrations were combined with $\delta^{13}C$ and $\delta^{15}N$ (Table 1, Figure 2), and a CAP analysis was conducted to identify elements that were most influential when chemically differentiating among sampling locations. A two-dimensional distance-based visualization (Figure 3) was generated in which each point represented a single female blue crab along two canonical axes, describing 80.5% (axis I) and 19.5% (axis II) of the between-group variation from all 33 input variables. Corresponding correlation vectors (Figure 4) display proportional contributions to separating sampling locations, and the LOO cross-validation generated confusion matrix (Table 2) displays classification success for placing each individual back into its original geographic group, with a total success of 54%.

Input variables Li, $\delta^{13}C$, $\delta^{15}N$, Na, Mg, Al, V, Zn, As, Sb, Cs, Ba, and U had the largest correlation vector magnitudes along canonical axis I (Figure 3), and were identified as having the greatest potential for generating unique chemical signatures for each collection site. These elements were selected for investigation into whether they were chemically stable in the cuticle of a mature female blue crab over time and would, therefore, retain the chemical properties of the mating habitat during a spawning migration.

Table 1. Referenced elements and corresponding atomic symbols

Element	Atomic Symbol
Lithium	Li
Carbon	C
Nitrogen	N
Sodium	Na
Magnesium	Mg
Aluminum	Al
Calcium	Ca
Vanadium	V
Iron	Fe
Nickel	Ni
Copper	Cu
Zinc	Zn
Arsenic	As
Strontium	Sr
Antimony	Sb
Cesium	Cs
Barium	Ba
Lead	Pb
Uranium	U

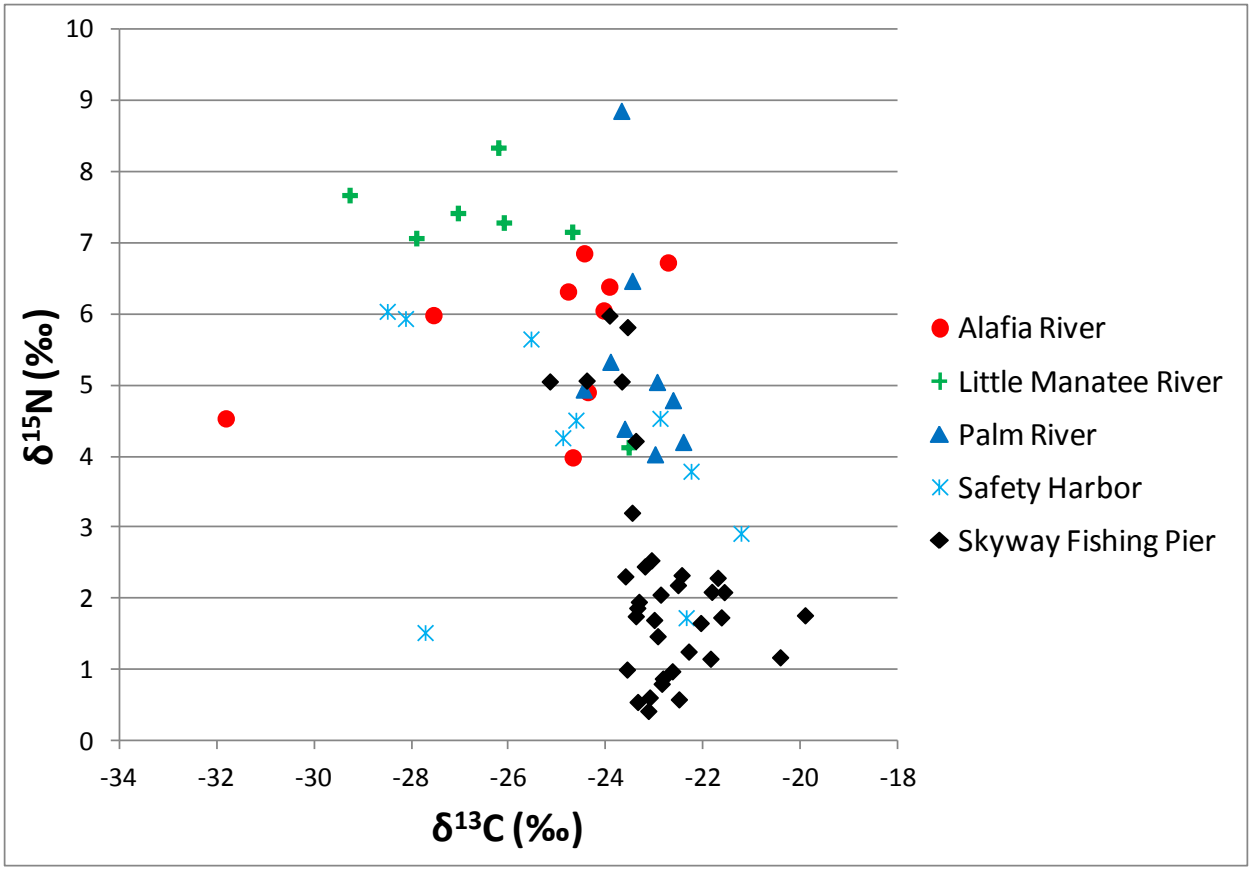

Figure 2. $\delta^{13}C$ and $\delta^{15}N$ in the cuticle of mature female blue crabs collected from five Tampa Bay locations in 2011. Each point is a single crab.

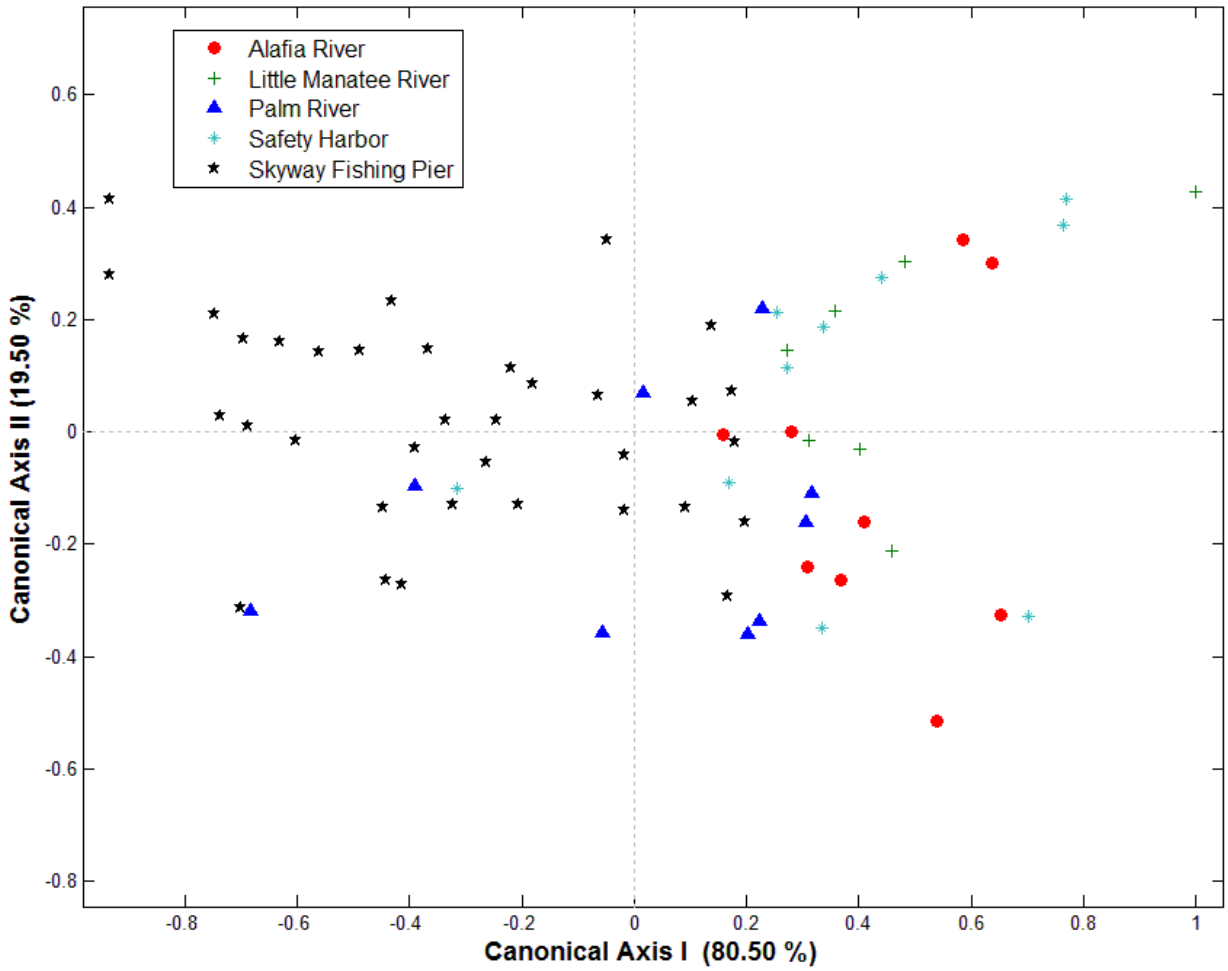

Figure 3. CAP-based Canonical Discriminant Analysis of 33 input variables from 2011 field study. Each point represents a single crab.

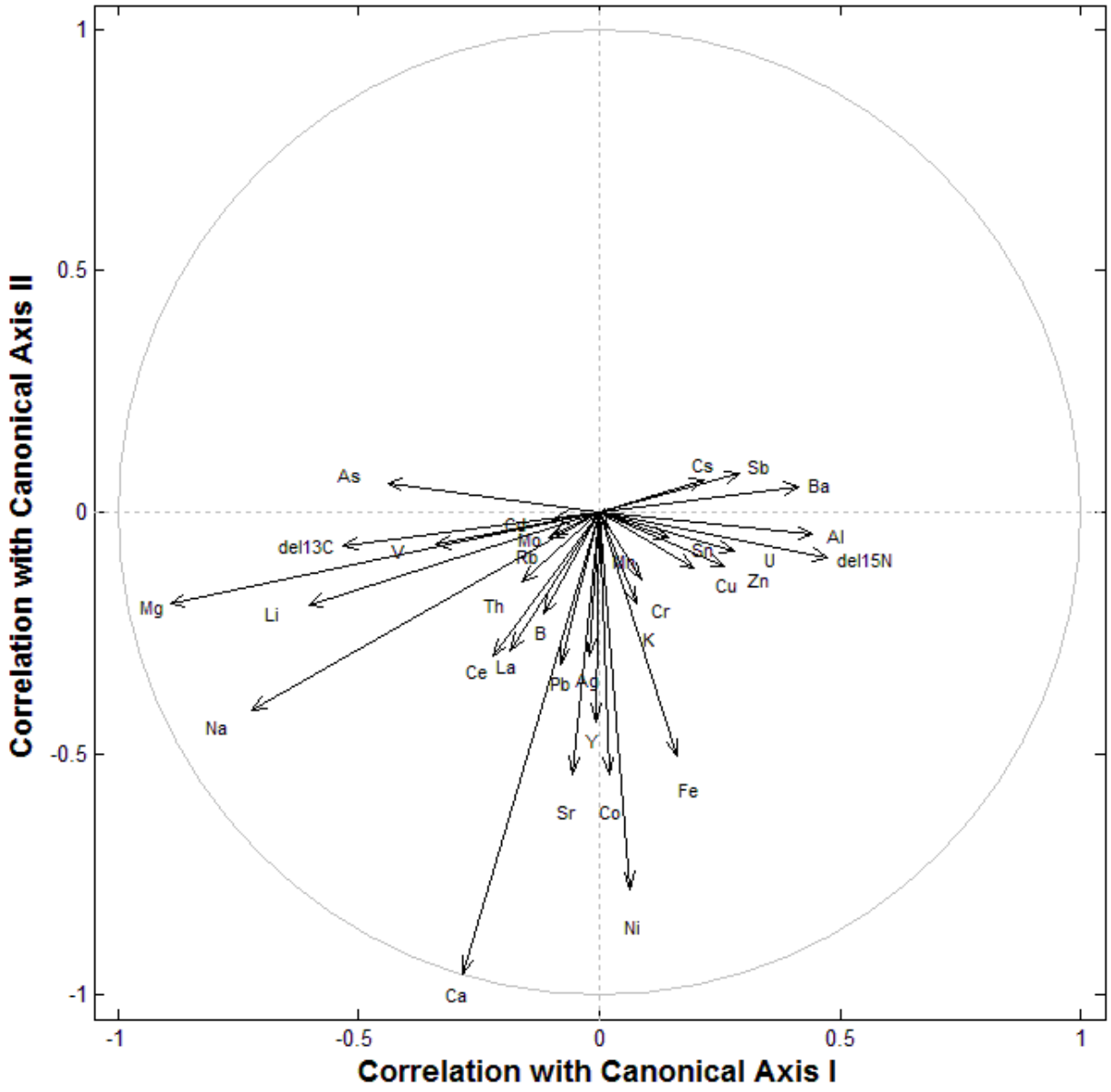

Figure 4. Correlation vectors corresponding to CAP analysis of 2011 field study (Figure 3). Vector directions and magnitudes depict underlying gradients of elemental concentrations and isotope ratios, and are proportional to each analyte's contribution toward separating individuals collected at differing sites.

Table 2. Thirty-three input variable CAP test LOO cross-validation confusion matrix for five 2011 field study sampling locations. Bold text indicates percent crabs successfully classified into respective groups. Total classification success: 54%

	Alafia River	**Little Manatee River**	**Palm River**	**Safety Harbor**	**Skyway Fishing Pier**
Alafia River	**56**	22	11	11	0
Little Manatee River	29	**14**	0.	57	0
Palm River	22	0	**44**	11	22
Safety Harbor	20	30	10	**30**	10
Skyway Fishing Pier	0	0	20	9	**71**

Time-Series Captive Animal Study

ICP-MS analysis of the 2012 captive animal study samples returned concentrations for 17 elements; Al, V, and Sb were eliminated as concentrations were below detection limit. EA-IRMS analysis yielded $\delta^{13}C$ and $\delta^{15}N$. Figures 5 - 20 present all metal and stable isotope data for cuticle and tank water during the one-month study. Concentration of Fe in tank water was below detection limit and $\delta^{13}C$ and $\delta^{15}N$ were not collected for tank water due to funding limitations.

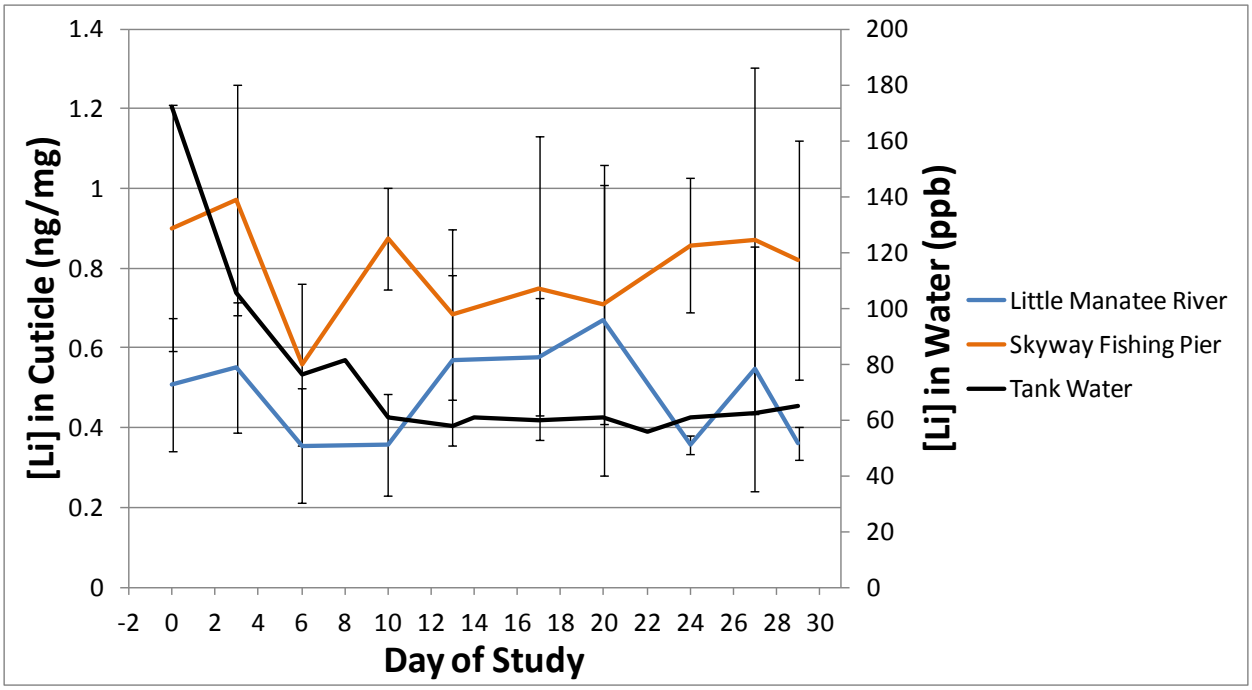

Figure 5. Lithium concentration ([Li]) in blue crab cuticle and tank water over 29 day captive animal study in 2012. Error bar is one standard deviation.

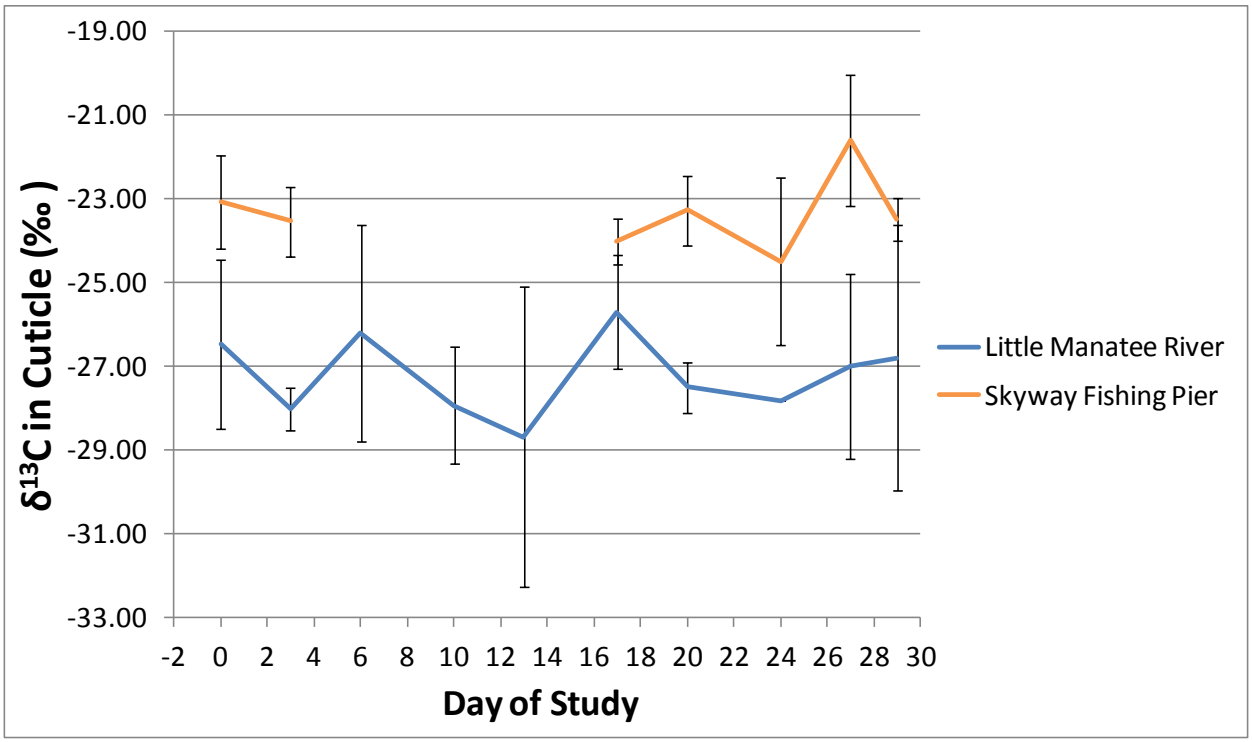

Figure 6. $\delta^{13}C$ in blue crab cuticle over 29 day captive animal study in 2012. Error bar is one standard deviation.

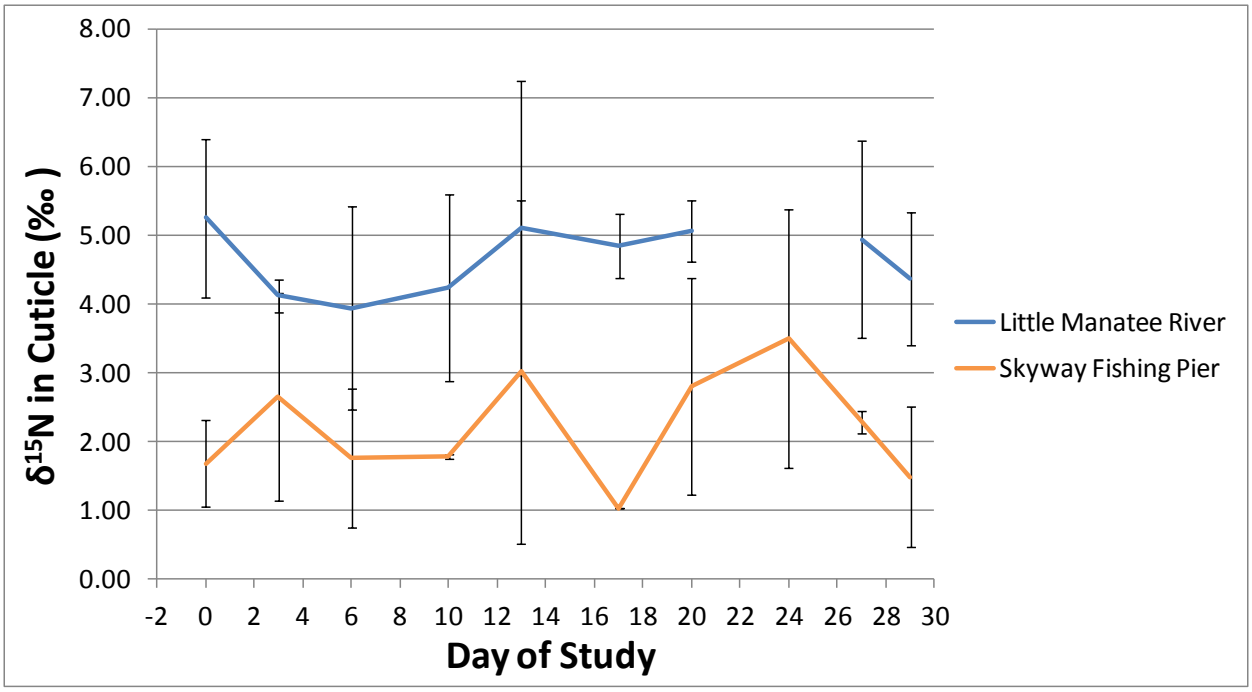

Figure 7. $\delta^{15}N$ in blue crab cuticle over 29 day captive animal study in 2012. Error bar is one standard deviation.

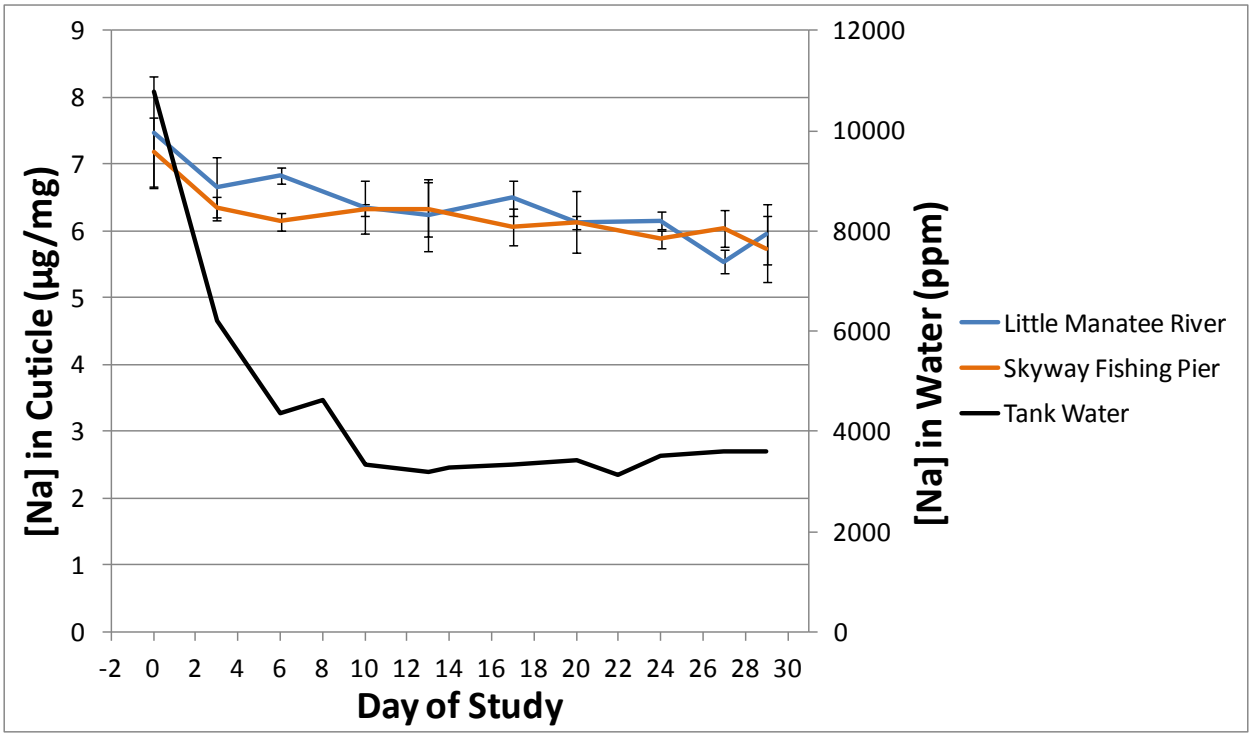

Figure 8. Sodium concentration ([Na]) in blue crab cuticle and tank water over 29 day captive animal study in 2012. Error bar is one standard deviation.

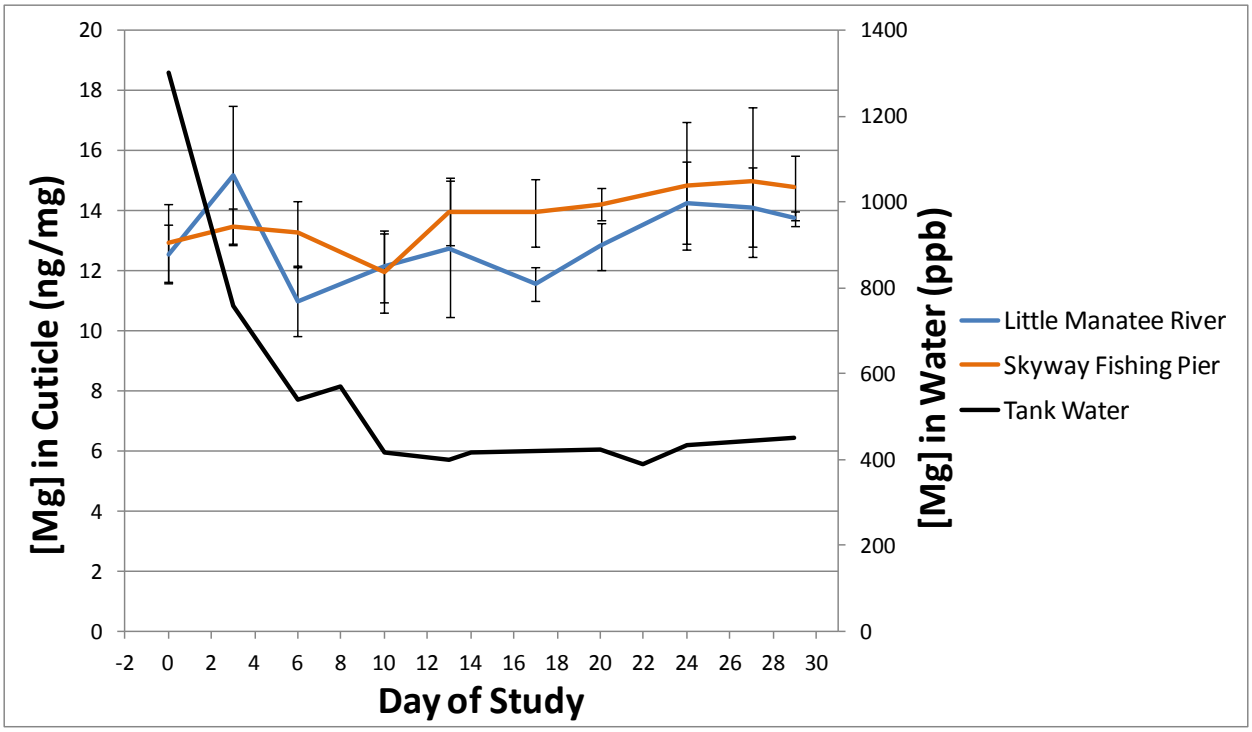

Figure 9. Magnesium concentration ([Mg]) in blue crab cuticle and tank water over 29 day captive animal study in 2012. Error bar is one standard deviation.

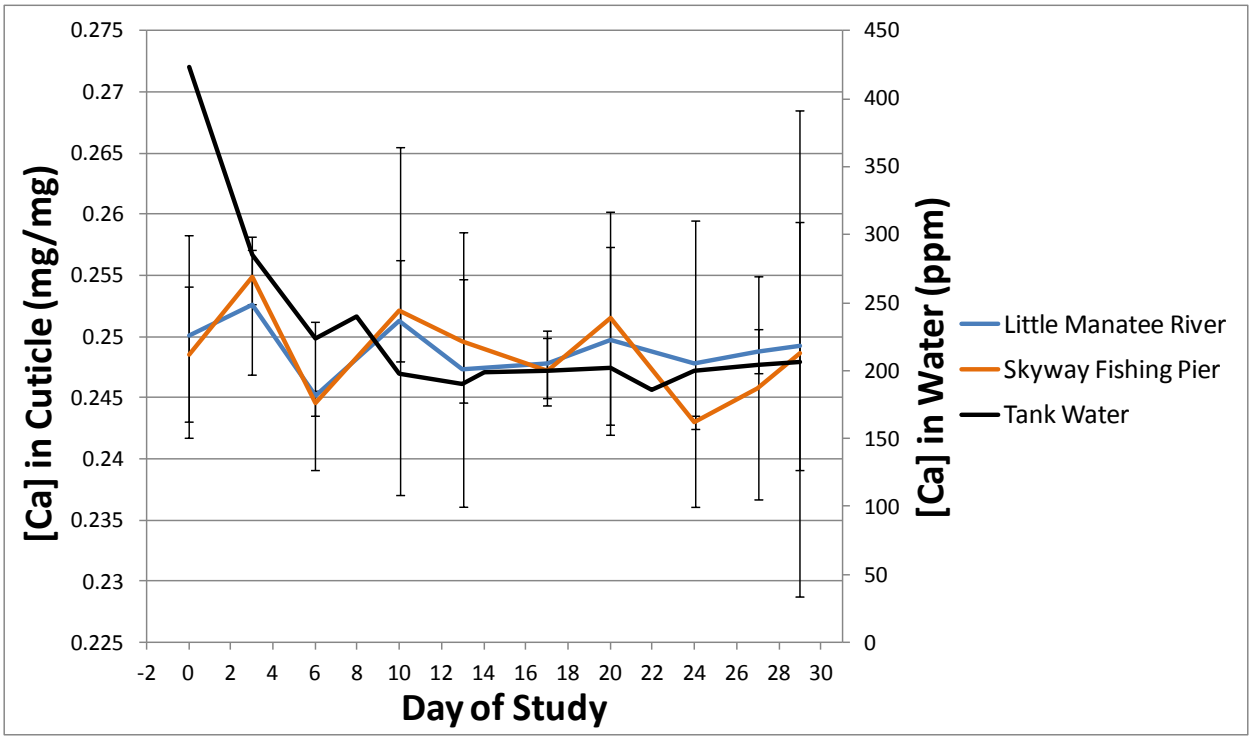

Figure 10. Calcium concentration ([Ca]) in blue crab cuticle and tank water over 29 day captive animal study in 2012. Error bar is one standard deviation.

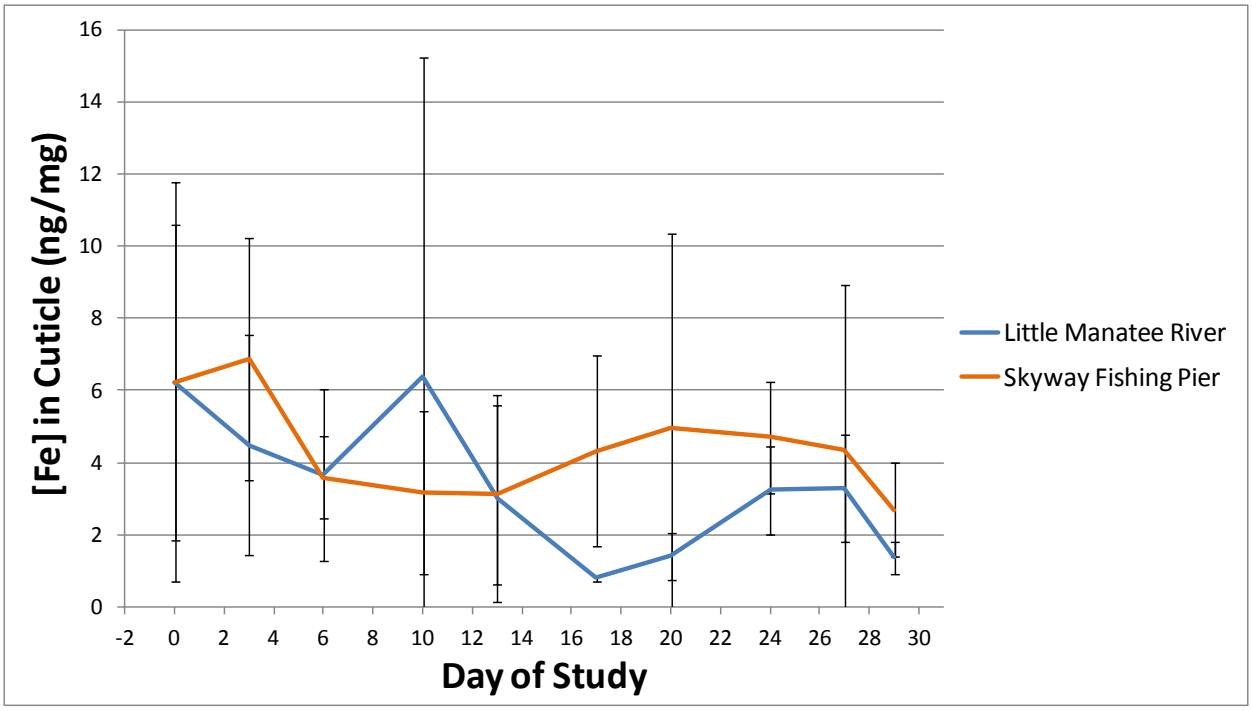

Figure 11. Iron concentration ([Fe]) in blue crab cuticle over 29 day captive animal study in 2012. Error bar is one standard deviation.

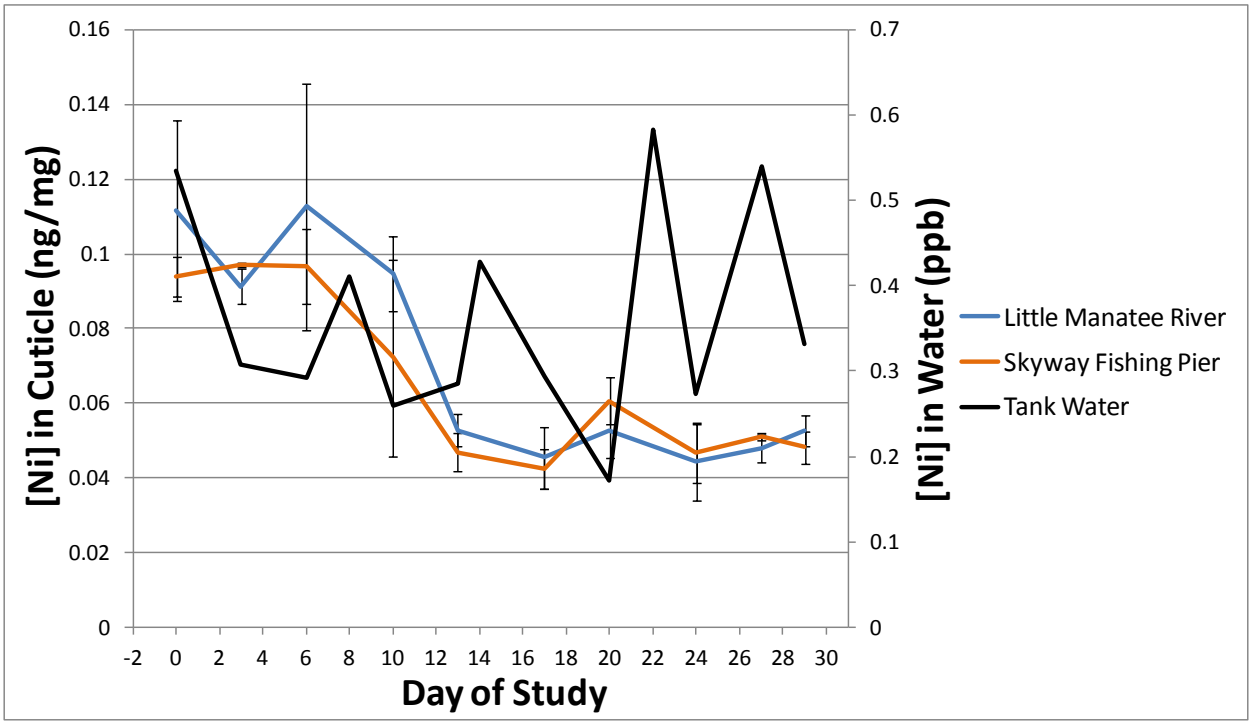

Figure 12. Nickel concentration ([Ni]) in blue crab cuticle and tank water over 29 day captive animal study in 2012. Error bar is one standard deviation.

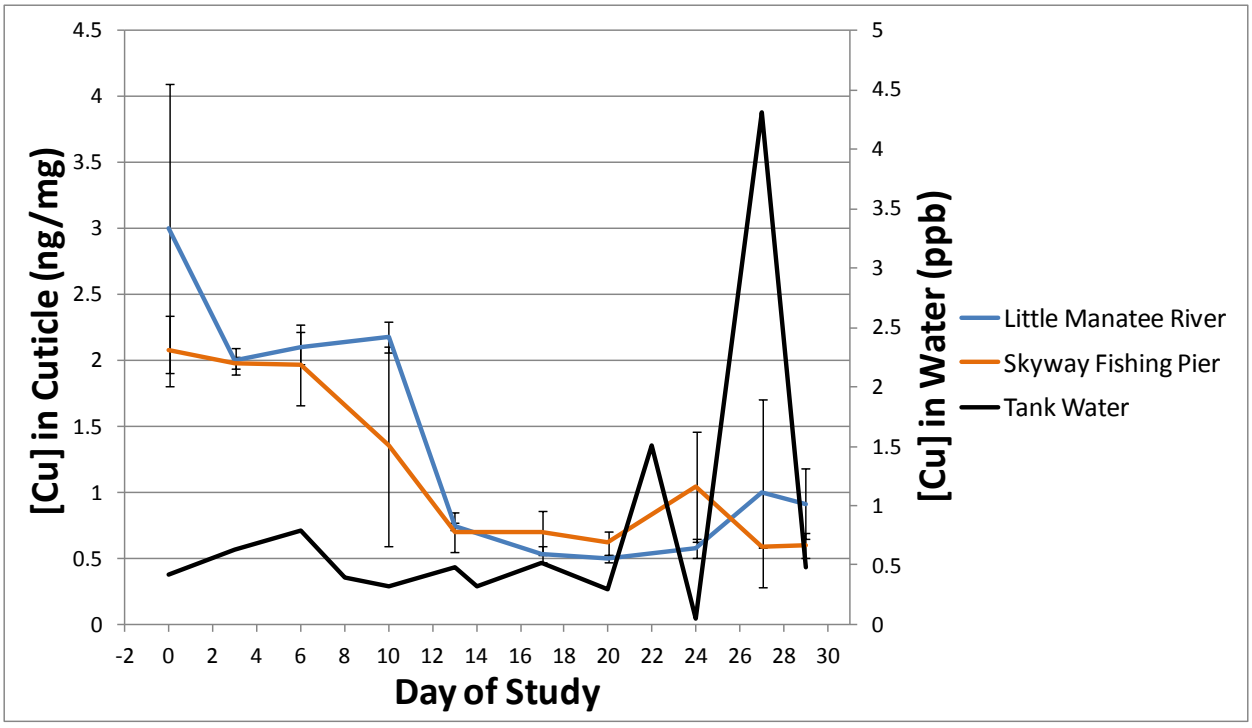

Figure 13. Copper concentration ([Cu]) in blue crab cuticle and tank water over 29 day captive animal study in 2012. Error bar is one standard deviation.

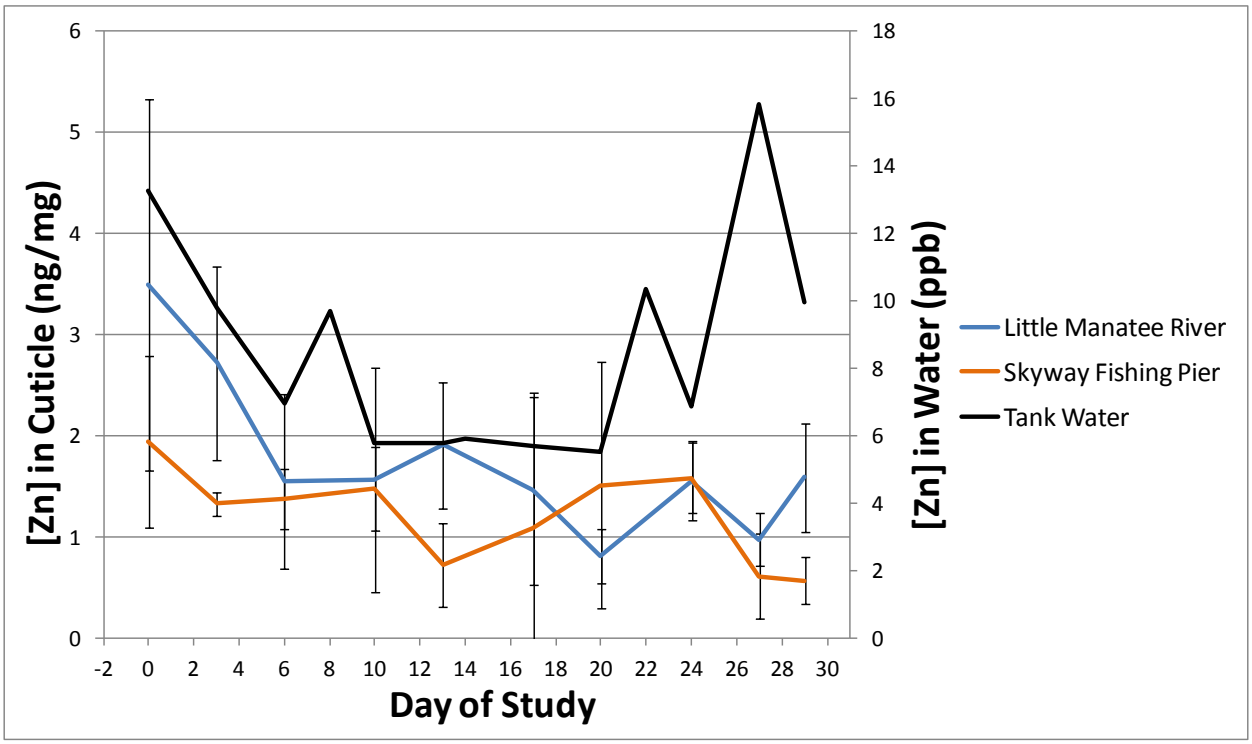

Figure 14. Zinc concentration ([Zn]) in blue crab cuticle and tank water over 29 day captive animal study in 2012. Error bar is one standard deviation.

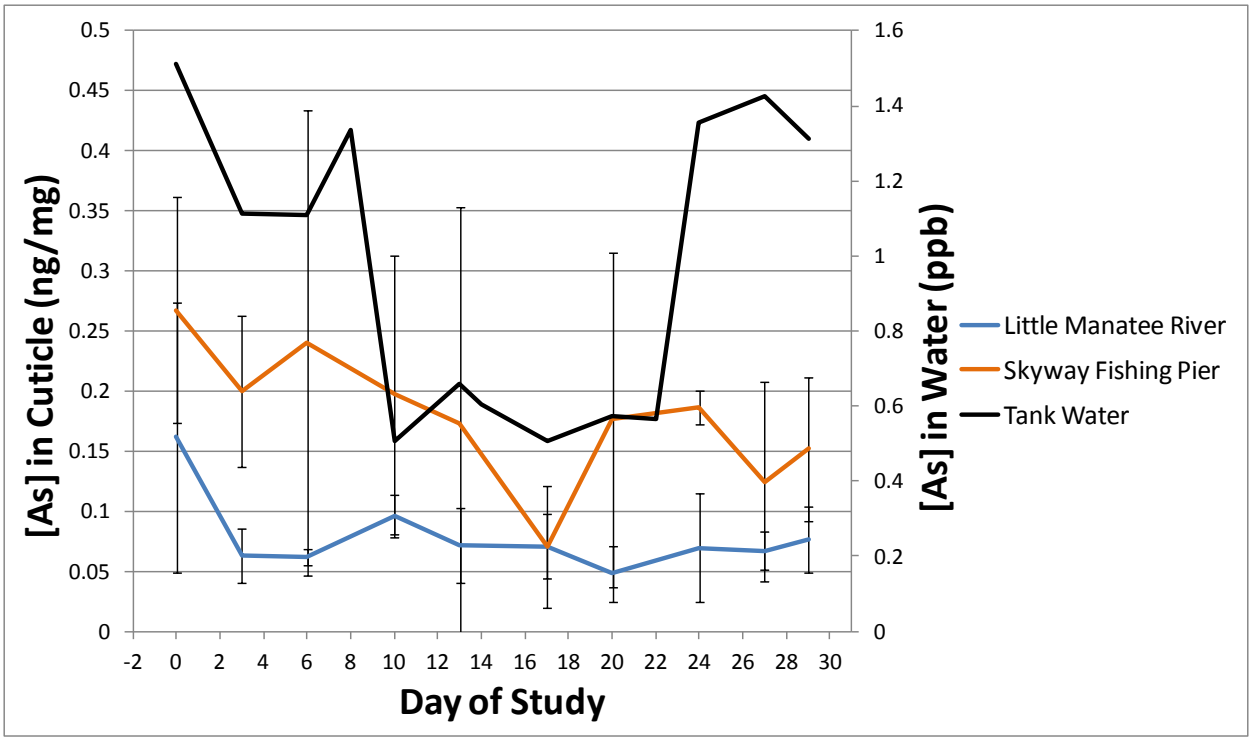

Figure 15. Arsenic concentration ([As]) in blue crab cuticle and tank water over 29 day captive animal study in 2012. Error bar is one standard deviation.

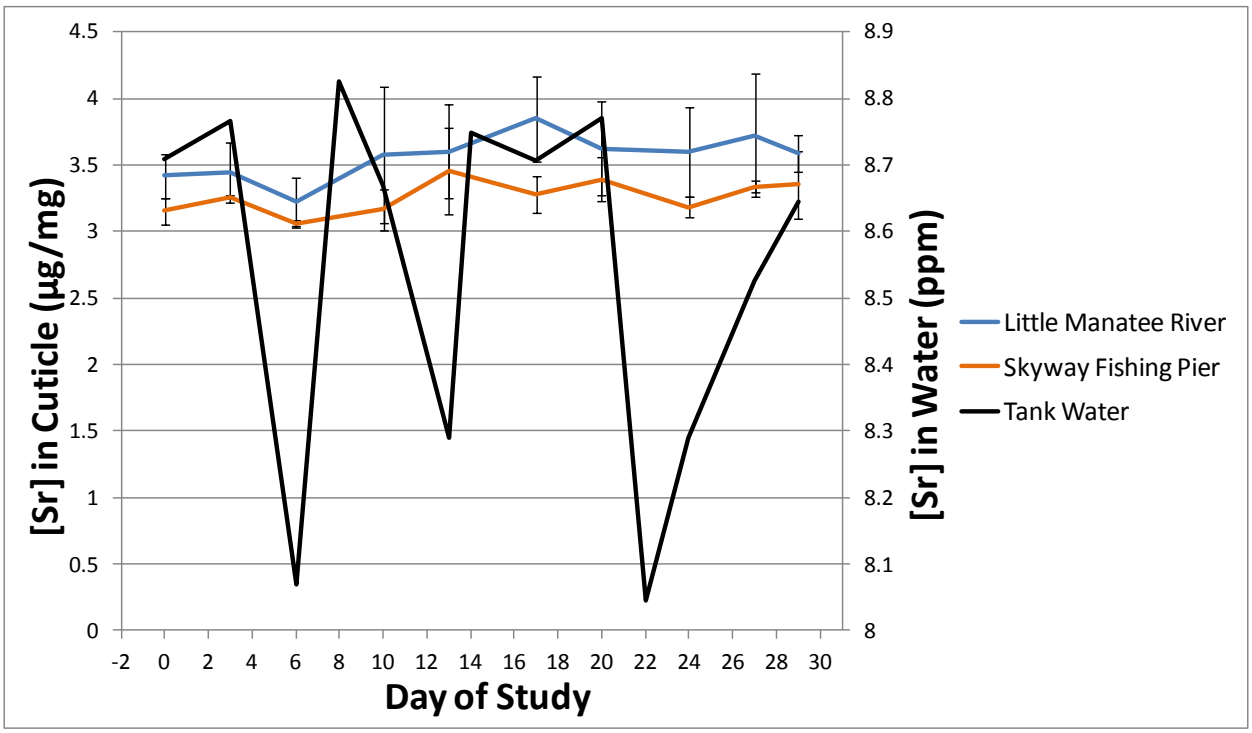

Figure 16. Strontium concentration ([Sr]) in blue crab cuticle and tank water over 29 day captive animal study in 2012. Error bar is one standard deviation.

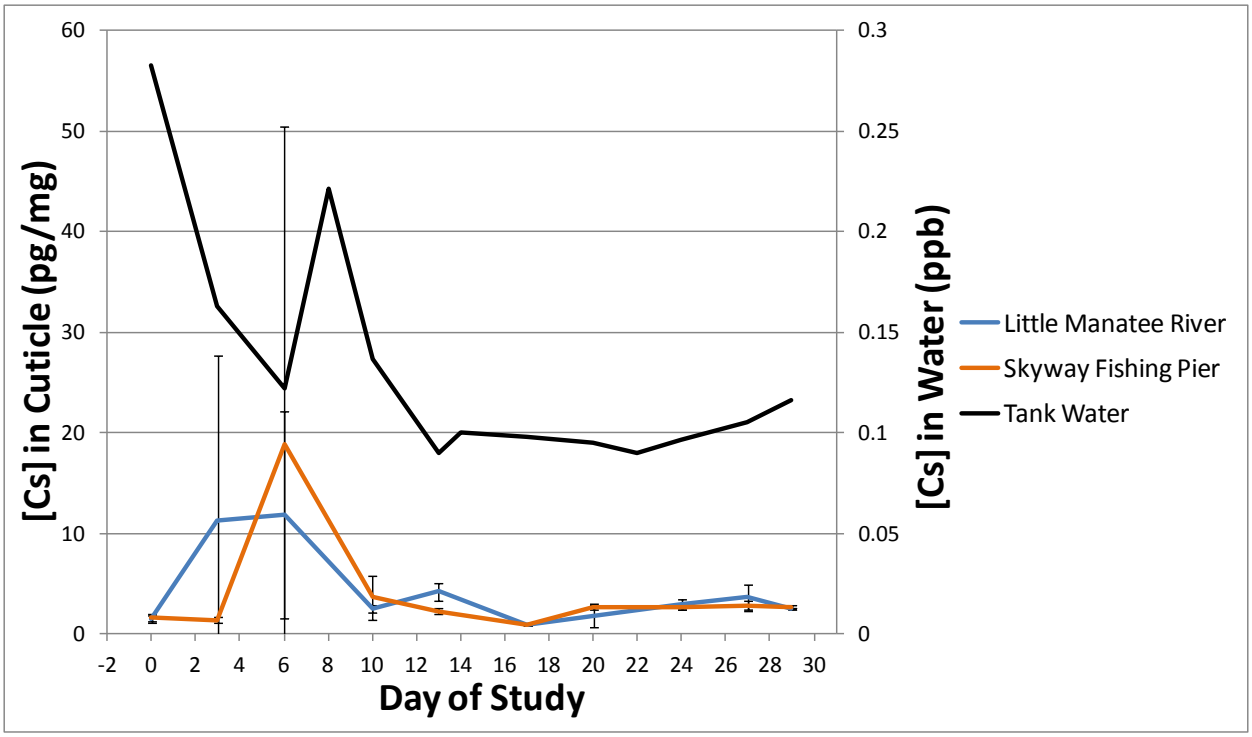

Figure 17. Cesium concentration ([Cs]) in blue crab cuticle and tank water over 29 day captive animal study in 2012. Error bar is one standard deviation.

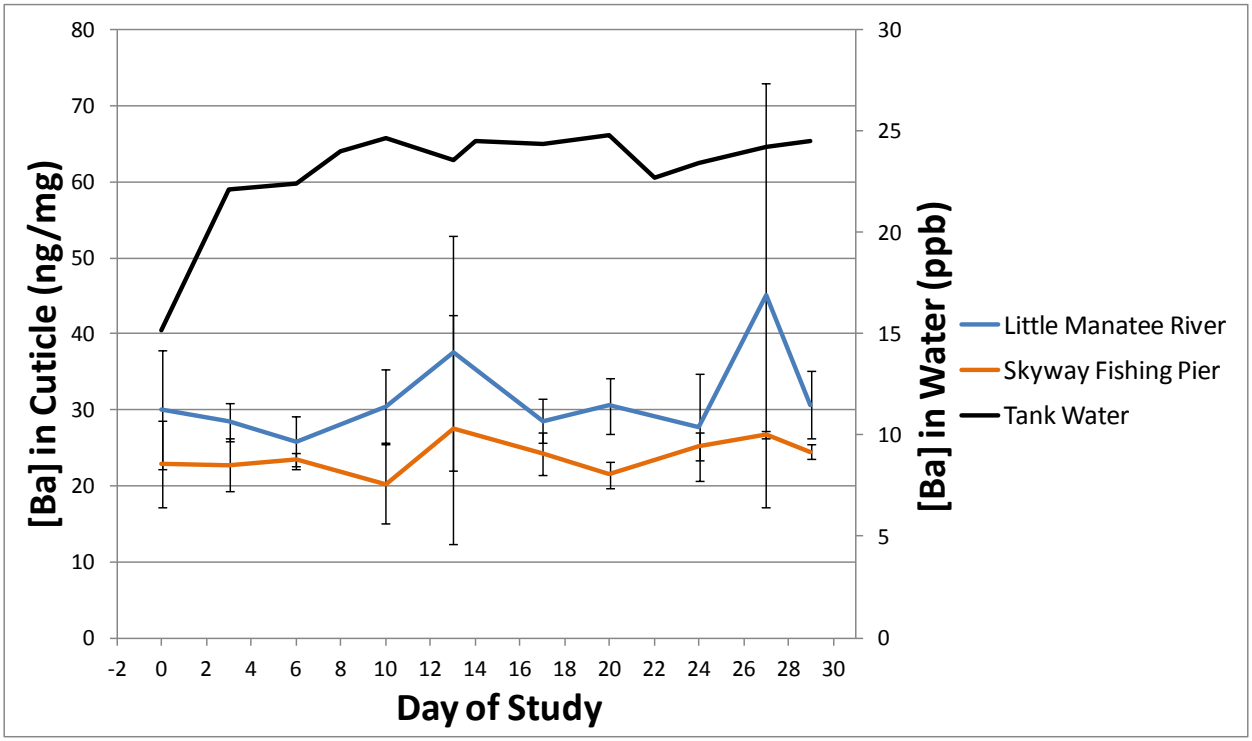

Figure 18. Barium concentration ([Ba]) in blue crab cuticle and tank water over 29 day captive animal study in 2012. Error bar is one standard deviation.

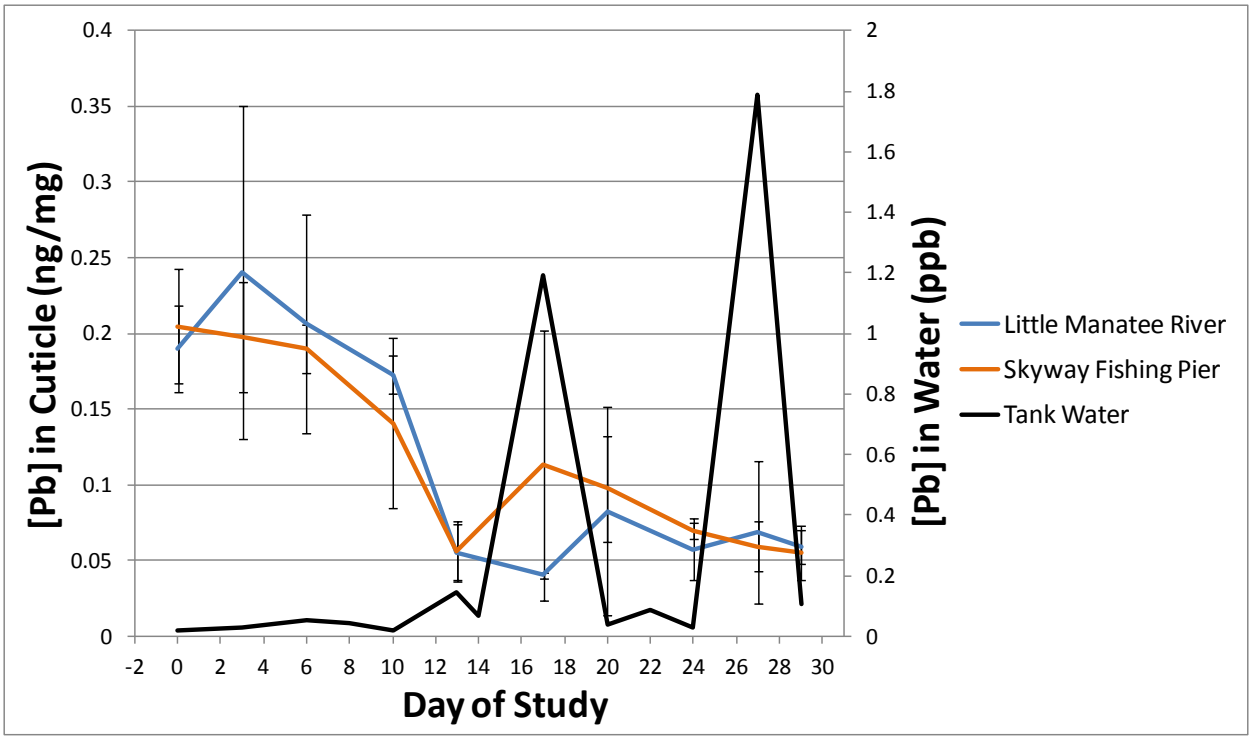

Figure 19. Lead concentration ([Pb]) in blue crab cuticle and tank water over 29 day captive animal study in 2012. Error bar is one standard deviation.

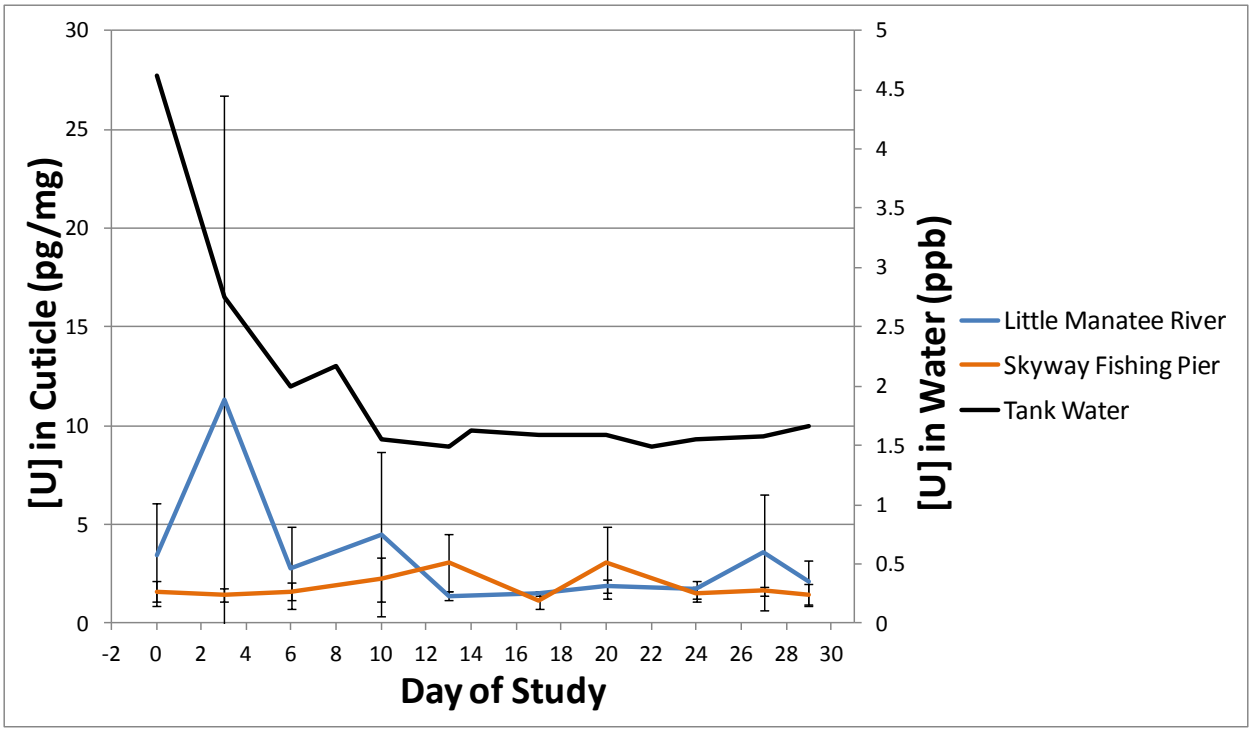

Figure 20. Uranium concentration ([U]) in blue crab cuticle and tank water over 29 day captive animal study in 2012. Error bar is one standard deviation.

In order to determine whether each element was chemically stable in blue crab cuticle, separate ANOVAs were carried-out for Li, $\delta^{13}C$, $\delta^{15}N$, Na, Mg, Zn, As, Cs, Ba, and U, comparing the means of each of the 10 collection days over the entire study period (Table 3); the two sampling locations were analyzed separately. The variables that did not show any significant difference among days of the study for both collection sites were Li, $\delta^{13}C$, $\delta^{15}N$, and Ba. Ca also showed no significant change, but was not identified as potentially useful in generating chemical signals for determining mating habitats due to the small magnitude of its correlation vector along canonical axis I in the 2011 CAP analysis (Figure 3). Grand means of $\delta^{13}C$ and $\delta^{15}N$, and concentrations of Li, Ba, and Ca, spanning five sampling locations from 2011 and two locations from 2012 are presented in Figures 21 - 25.

Table 3. Distribution-free ANOVAs for individual elements over the 29 day captive animal study in 2012 to determine which values were stable. Bold type indicates no significant difference among days, p-value > 0.05

	Little Manatee River	**Skyway Fishing Pier**
Element	**p-value**	**p-value**
Li	**0.196**	**0.593**
Na	0.001	0.001
Mg	0.003	0.041
Ca	**0.985**	**0.734**
Fe	0.008	**0.380**
Ni	0.001	0.001
Cu	0.001	0.001
Zn	0.007	0.007
As	0.007	**0.115**
Sr	**0.144**	0.021
Cs	0.001	0.001
Ba	**0.632**	**0.672**
Pb	0.001	0.001
U	**0.388**	0.032
$\delta^{13}C$	**0.874**	**0.128**
$\delta^{15}N$	**0.600**	**0.579**

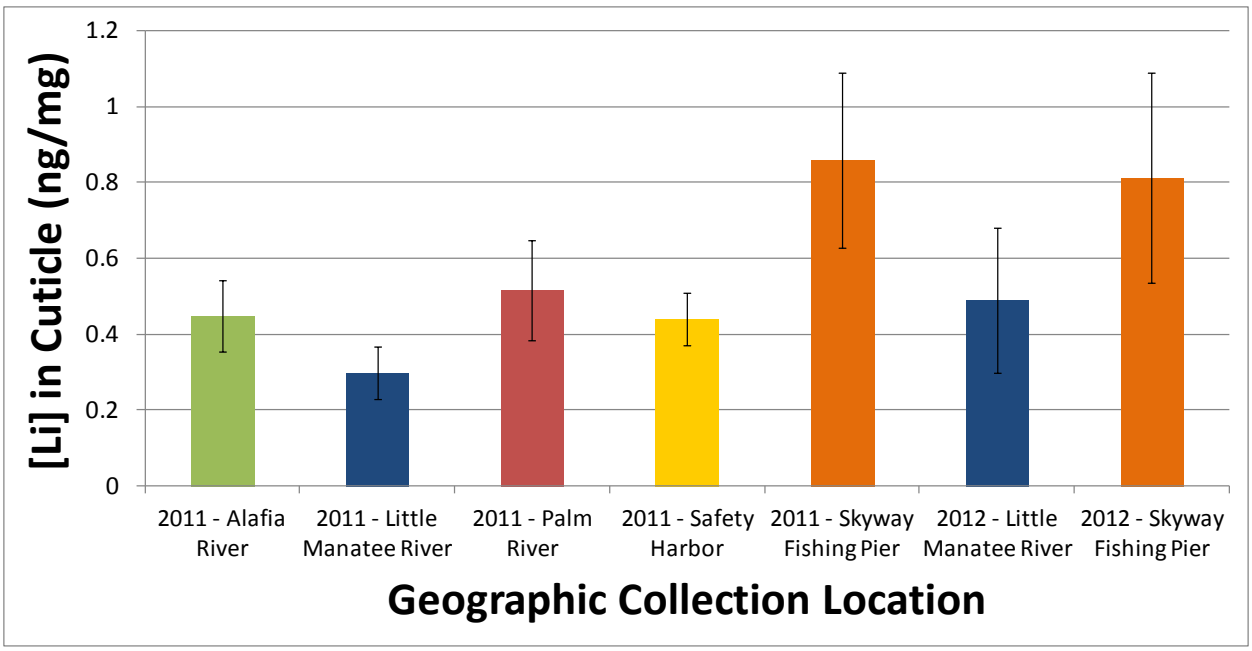

Figure 21. Lithium concentration ([Li]) grand means for five collection sites in 2011 and two sites in 2012, with error bars of one standard deviation.

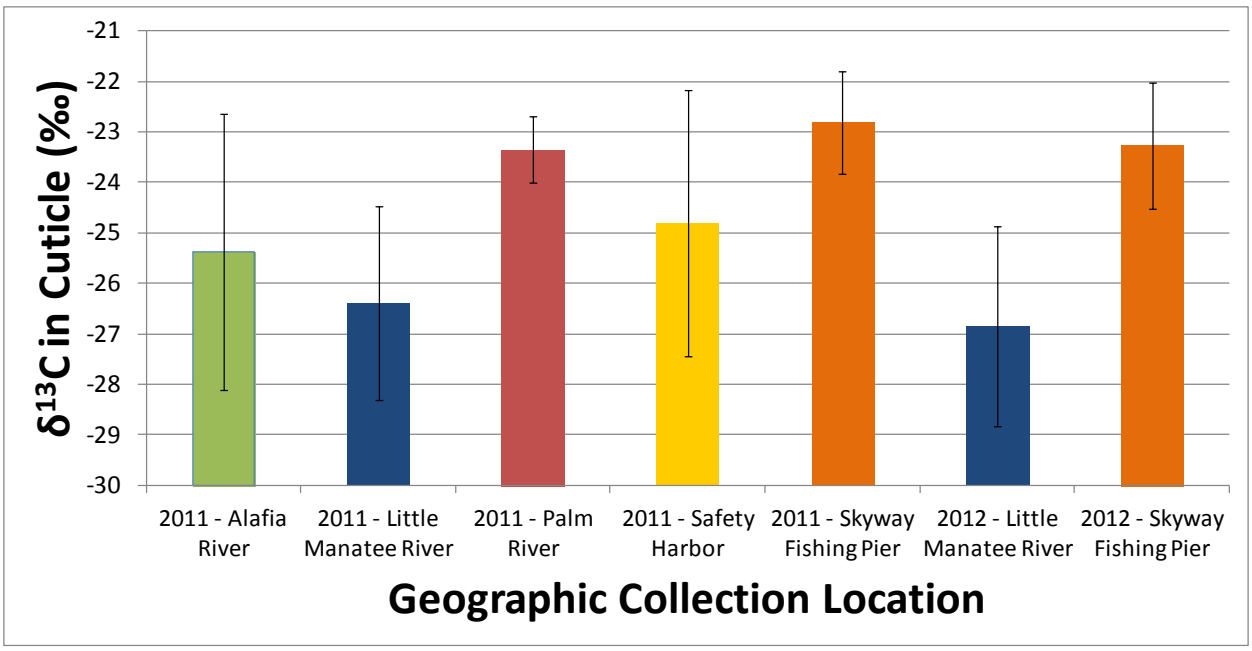

Figure 22. $\delta^{13}C$ grand means for five collection sites in 2011 and two sites in 2012, with error bars of one standard deviation.

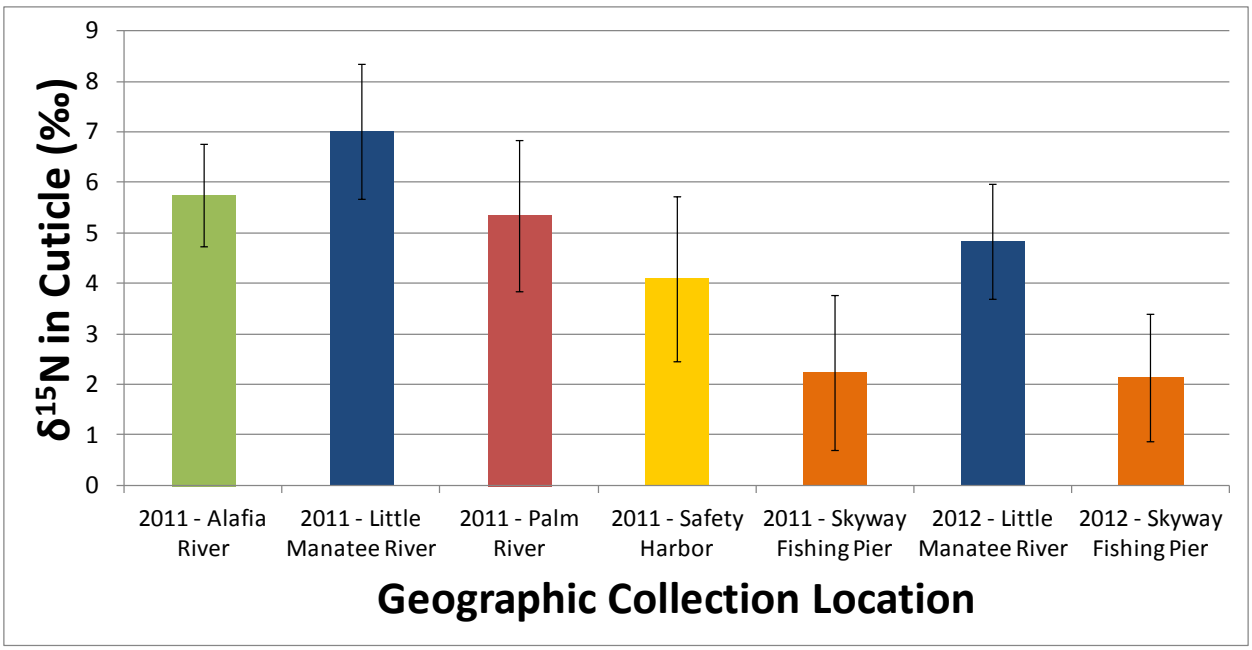

Figure 23. $\delta^{15}N$ grand means for five collection sites in 2011 and two sites in 2012, with error bars of one standard deviation.

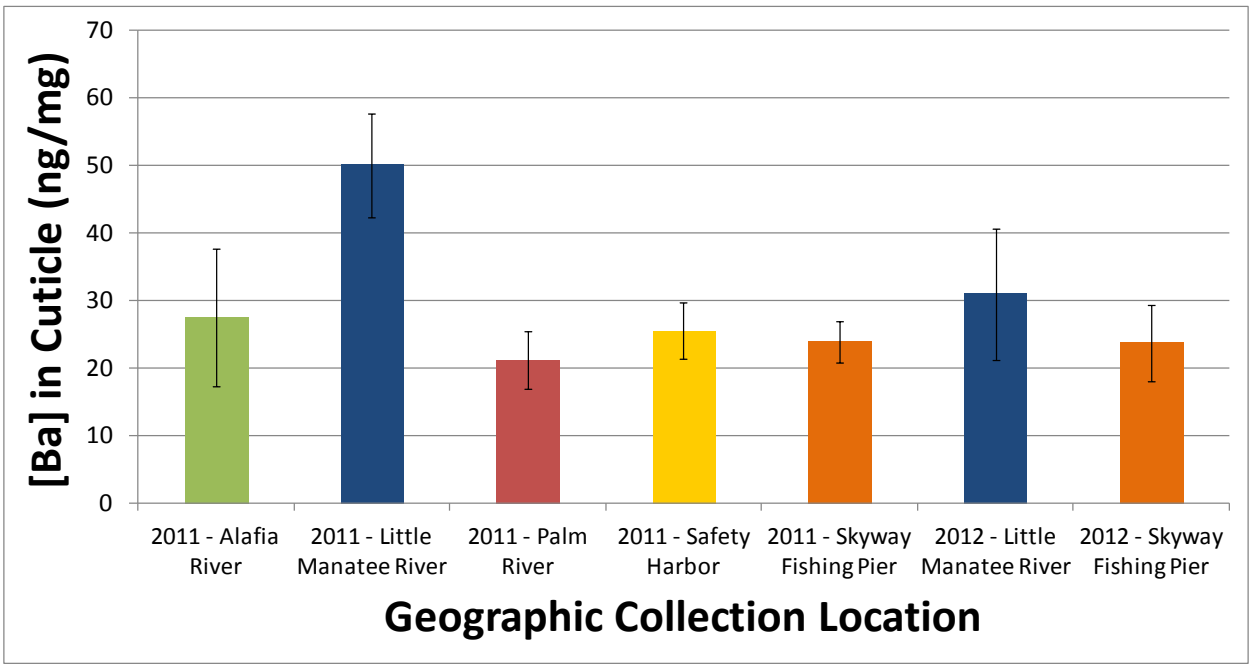

Figure 24. Barium concentration ([Ba]) grand means for five collection sites in 2011 and two sites in 2012, with error bars of one standard deviation.

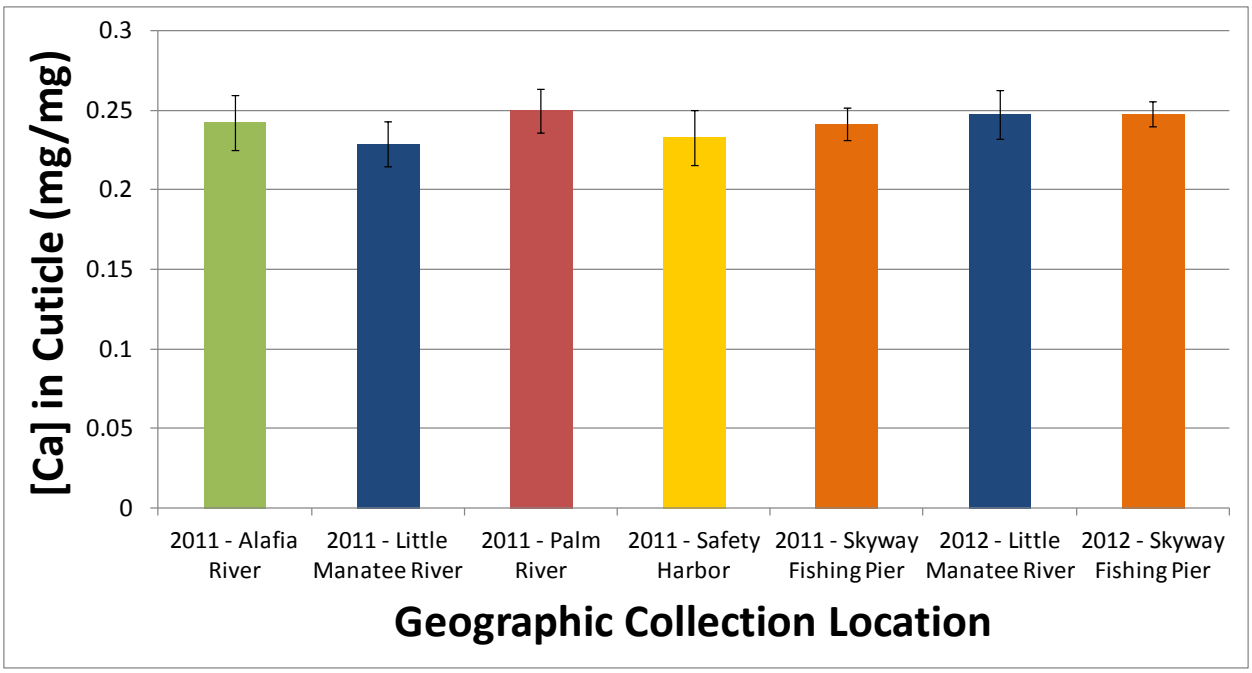

Figure 25. Calcium concentration ([Ca]) grand means for five collection sites in 2011 and two sites in 2012, with error bars of one standard deviation.

Refined Field Study

Li, $\delta^{13}C$, $\delta^{15}N$, and Ba showed no significant change in mature female blue crab cuticle over 29 days in tanks, indicating that signatures for these elements captured in the exoskeleton at the time of terminal molt and mating should be retained during the course of a spawning migration. To observe differences between sampling locations for each element previously determined to be chemically stable in blue crab cuticle, pairwise comparisons of five 2011 sampling locations using ANOVAs were completed one element at a time (Tables 4 - 7). The Little Manatee River was statistically different from all other locations (all p-values < 0.05) in terms of Li and Ba concentrations. In addition, the Skyway Fishing Pier was statistically different from all other locations (all p-values < 0.05) in terms of Li and $\delta^{15}N$.

Table 4. Distribution-free ANOVAs comparing lithium concentrations for pairs of 2011 collection locations. Bold type indicates a significant difference between two sampling sites, p-value < 0.05

Collection sources		**p-value**
Alafia River	Little Manatee River	**0.001**
Alafia River	Palm River	0.234
Alafia River	Safety Harbor	0.952
Alafia River	Skyway Fishing Pier	**0.001**
Little Manatee River	Palm River	**0.001**
Little Manatee River	Safety Harbor	**0.002**
Little Manatee River	Skyway Fishing Pier	**0.001**
Palm River	Safety Harbor	0.139
Palm River	Skyway Fishing Pier	**0.001**
Safety Harbor	Skyway Fishing Pier	**0.001**

Table 5. Distribution-free ANOVAs comparing $\delta^{13}C$ for pairs of 2011 collection locations. Bold type indicates a significant difference between two sampling sites, p-value < 0.05

Collection sources		**p-value**
Alafia River	Little Manatee River	0.367
Alafia River	Palm River	**0.012**
Alafia River	Safety Harbor	0.627
Alafia River	Skyway Fishing Pier	**0.001**
Little Manatee River	Palm River	**0.001**
Little Manatee River	Safety Harbor	0.188
Little Manatee River	Skyway Fishing Pier	**0.001**
Palm River	Safety Harbor	0.144
Palm River	Skyway Fishing Pier	0.170
Safety Harbor	Skyway Fishing Pier	**0.002**

Table 6. Distribution-free ANOVAs comparing $\delta^{15}N$ for pairs of 2011 collection locations. Bold type indicates a significant difference between two sampling sites, p-value < 0.05

Collection sources		**p-value**
Alafia River	Little Manatee River	0.083
Alafia River	Palm River	0.382
Alafia River	Safety Harbor	**0.017**
Alafia River	Skyway Fishing Pier	**0.001**
Little Manatee River	Palm River	**0.039**
Little Manatee River	Safety Harbor	**0.007**
Little Manatee River	Skyway Fishing Pier	**0.001**
Palm River	Safety Harbor	0.099
Palm River	Skyway Fishing Pier	**0.001**
Safety Harbor	Skyway Fishing Pier	**0.002**

Table 7. Distribution-free ANOVAs comparing barium concentrations for pairs of 2011 collection locations. Bold type indicates a significant difference between two sampling sites, p-value < 0.05

Collection sources		**p-value**
Alafia River	Little Manatee River	**0.002**
Alafia River	Palm River	0.105
Alafia River	Safety Harbor	0.609
Alafia River	Skyway Fishing Pier	0.173
Little Manatee River	Palm River	**0.001**
Little Manatee River	Safety Harbor	**0.001**
Little Manatee River	Skyway Fishing Pier	**0.001**
Palm River	Safety Harbor	**0.029**
Palm River	Skyway Fishing Pier	**0.016**
Safety Harbor	Skyway Fishing Pier	0.242

In order to test whether a significant difference can be observed among five Tampa Bay sampling locations using the four variables determined to be chemically unchanging (Li, $\delta^{13}C$, $\delta^{15}N$, Ba), a MANOVA was run on 2011 field study data. This resulted in a p-value = 0.137 (Table 8), indicating that there was no significant difference among sampling locations when

considering all four variables simultaneously. To investigate an optimum combination of elements for chemically differentiating among sites, additional MANOVAs were conducted using five 2011 sampling locations, while removing elemental input variables one at a time (Table 8). The combinations {Li, $\delta^{15}N$, and Ba} and {Li, $\delta^{13}C$, and $\delta^{15}N$} yielded p-values = 0.001, indicating that a difference does exist among the chemical signatures of collection sites in these two cases.

Table 8. Distribution-free MANOVAs for combinations of input elements comparing five collection sites in 2011. Bold text indicates a significant difference exists among sampling locations, p-value < 0.05

Elements	**p-value**
Li, $\delta^{13}C$, $\delta^{15}N$, Ba	0.137
Ba, $\delta^{13}C$, $\delta^{15}N$	0.462
Li, $\delta^{13}C$, Ba	0.916
Li, $\delta^{15}N$, Ba	**0.001**
Li, $\delta^{13}C$, $\delta^{15}N$	**0.001**

The above analyses indicated significant differences exist for variables among the five collections sites. Pairwise analyses were conducted to determine which rivers can be distinguished. MANOVAs were conducted to compare pairs of geographic locations with input variables Li, $\delta^{15}N$, and Ba (Table 9). The Skyway Fishing Pier and the Little Manatee River were each significantly different from all other locations. In addition, MANOVAs were conducted to compare pairs of geographic locations with input variables Li, $\delta^{13}C$, and $\delta^{15}N$ (Table 10). The Skyway Fishing Pier and the Palm River were each significantly different from all other

locations. Neither of these two combinations of elemental inputs was able to identify significant differences among all five sampling locations.

Table 9. Distribution-free MANOVAs comparing pairs of 2011 collection locations, using input variables Li, $\delta^{15}N$, and Ba. Bold text indicates a significant difference between two sampling sites, p-value < 0.05

Collection sources		**p-value**
Alafia River	Little Manatee River	**0.003**
Alafia River	Palm River	0.106
Alafia River	Safety Harbor	0.245
Alafia River	Skyway Fishing Pier	**0.001**
Little Manatee River	Palm River	**0.001**
Little Manatee River	Safety Harbor	**0.001**
Little Manatee River	Skyway Fishing Pier	**0.001**
Palm River	Safety Harbor	**0.017**
Palm River	Skyway Fishing Pier	**0.001**
Safety Harbor	Skyway Fishing Pier	**0.014**

Table 10. Distribution-free MANOVAs comparing pairs of 2011 collection locations, using input variables Li, $\delta^{13}C$, and $\delta^{15}N$. Bold text indicates a significant difference between two sampling sites, p-value < 0.05

Collection sources		**p-value**
Alafia River	Little Manatee River	0.106
Alafia River	Palm River	**0.029**
Alafia River	Safety Harbor	0.152
Alafia River	Skyway Fishing Pier	**0.001**
Little Manatee River	Palm River	**0.003**
Little Manatee River	Safety Harbor	**0.013**
Little Manatee River	Skyway Fishing Pier	**0.001**
Palm River	Safety Harbor	**0.040**
Palm River	Skyway Fishing Pier	**0.001**
Safety Harbor	Skyway Fishing Pier	**0.001**

Once pairwise comparisons established significant differences between certain combinations of 2011 sampling locations, a CAP test was used to measure the strength of the overall chemical signal for each sampling site. A CAP-based canonical discriminant analysis with LOO cross-validation was carried-out on each {Li, $\delta^{15}N$, and Ba} and {Li, $\delta^{13}C$, and $\delta^{15}N$}, yielding respective principal coordinate visualizations (Figures 26, 28), correlation vectors (Figures 27, 29), and confusion matrices (Tables 11, 12).

LOO cross-validation of the 2011 Li, $\delta^{15}N$, and Ba data for five sampling locations successfully classified 64% of crabs to their correct geographic origin, while analysis of Li, $\delta^{13}C$, and $\delta^{15}N$ resulted in 66% classification success. Therefore, the latter was selected as the optimal elemental combination for distinguishing geographic locations. LOO classification success was variable using Li, $\delta^{13}C$, and $\delta^{15}N$, with successful classification for each sampling location being 33%, 71%, 67%, 30% and 83% for the Alafia River, Little Manatee River, Palm River, Safety Harbor, and Skyway Fishing Pier, respectively.

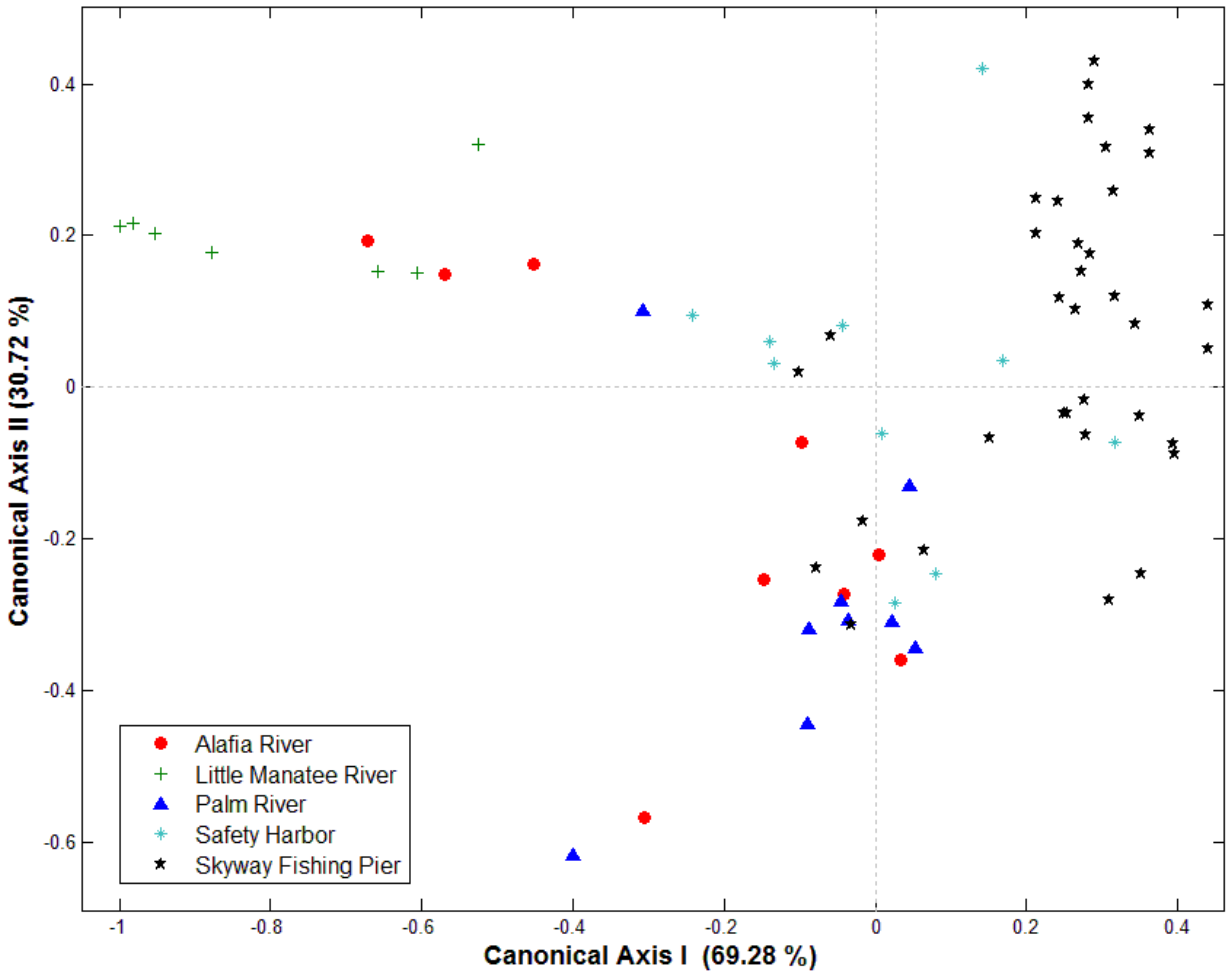

Figure 26. CAP output of Li, $\delta^{15}N$, and Ba from 2011 field study. Each point represents a single crab.

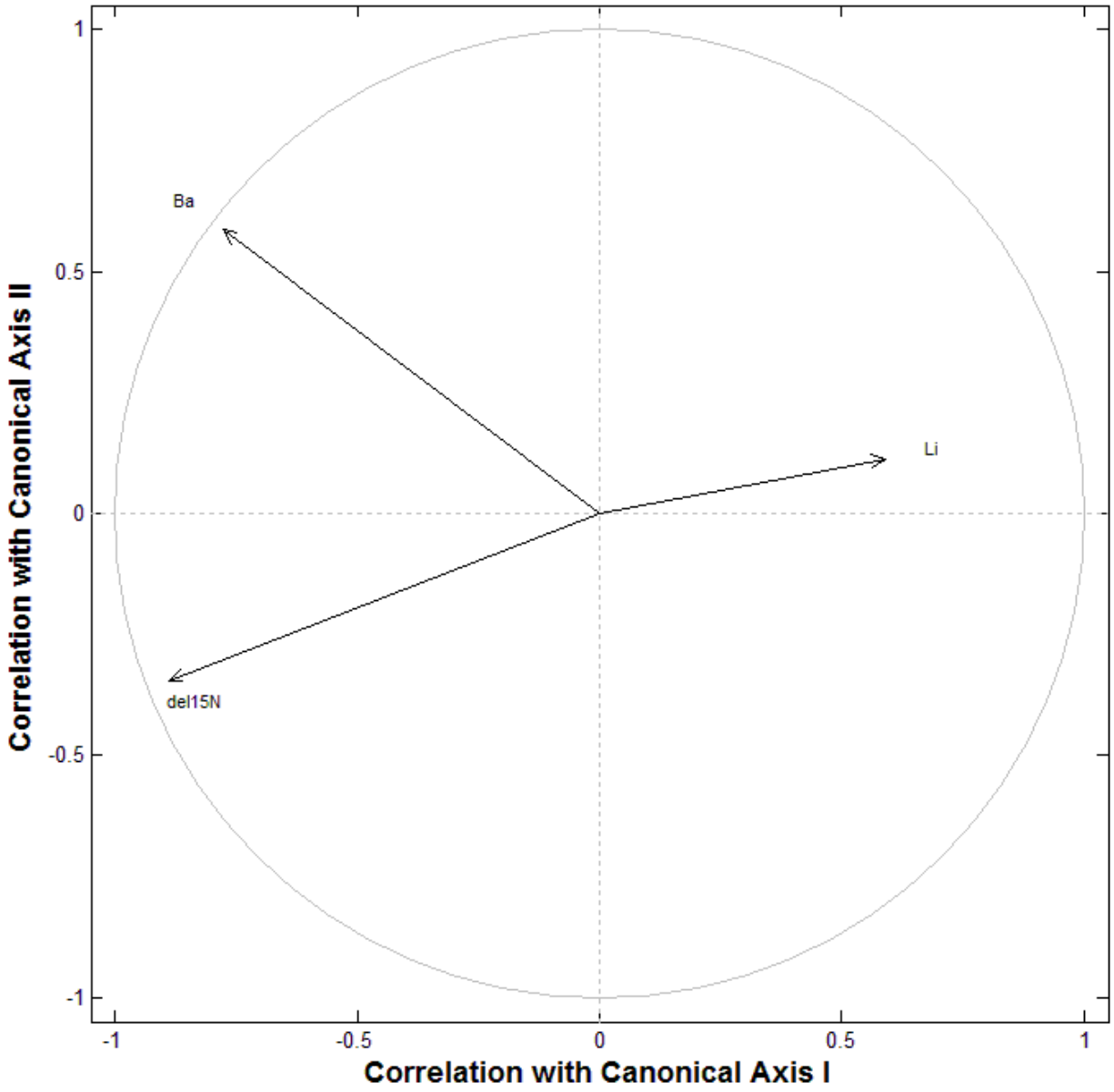

Figure 27. Correlation vectors corresponding to CAP analysis of 2011 field study Li, $\delta^{15}N$, and Ba data (Figure 26). Vector directions and magnitudes depict underlying gradients of elemental concentrations and isotope ratio, and are proportional to each analyte's contribution toward separating individuals collected at differing sites.

Table 11. Li, $\delta^{15}N$, and Ba CAP test LOO cross-validation confusion matrix for five 2011 sampling locations. Bold text indicates percent crabs successfully reclassified. Total classification success: 64%

	Alafia River	Little Manatee River	Palm River	Safety Harbor	Skyway Fishing Pier
Alafia River	**0**	33	56	11	0
Little Manatee River	0	**100**	0	0	0
Palm River	22	0	**67**	11	0
Safety Harbor	10	0	20	**40**	30
Skyway Fishing Pier	0	0	11	9	**80**

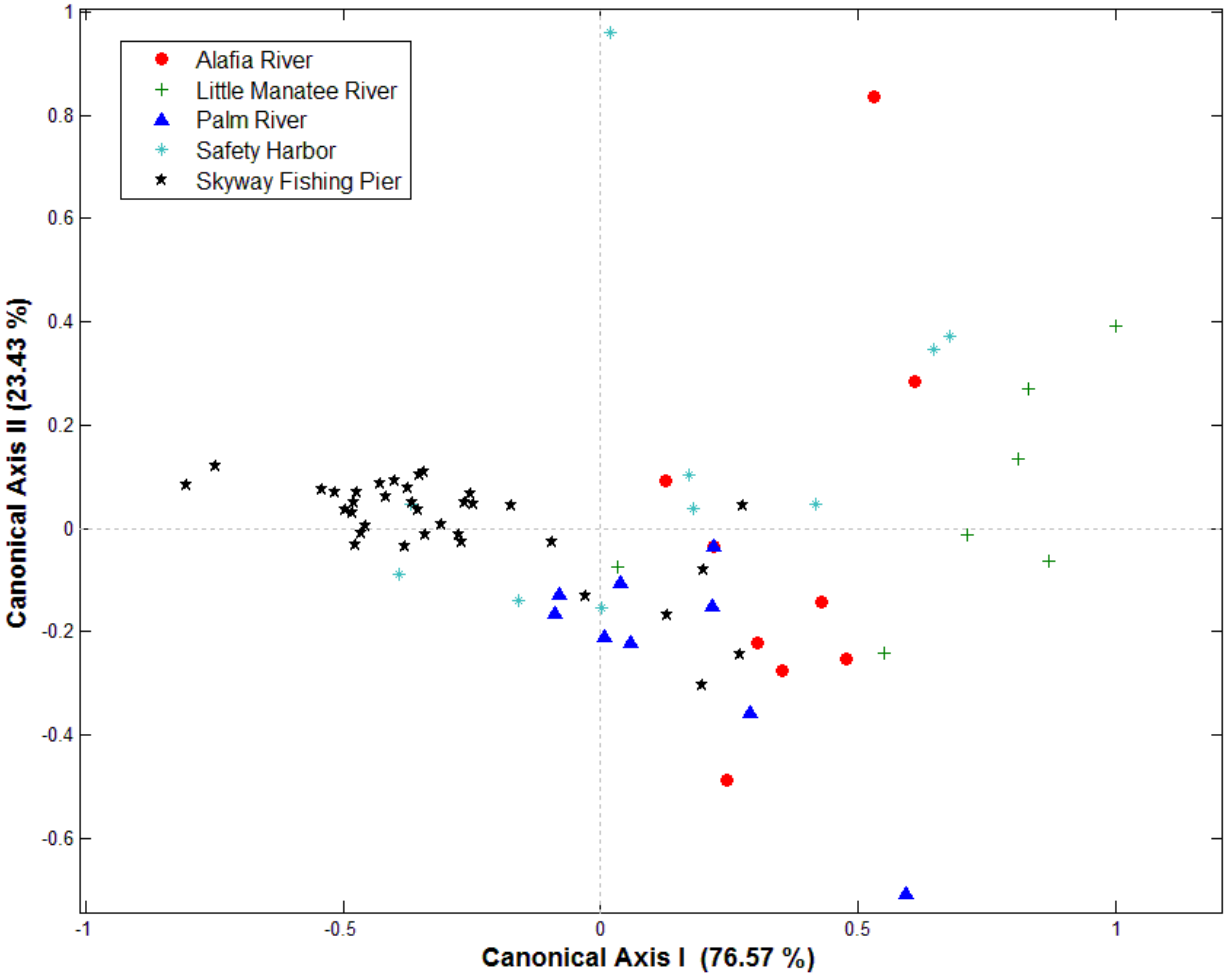

Figure 28. CAP output of Li, $\delta^{13}C$, and $\delta^{15}N$ from 2011 field study data. Each point represents a single crab.

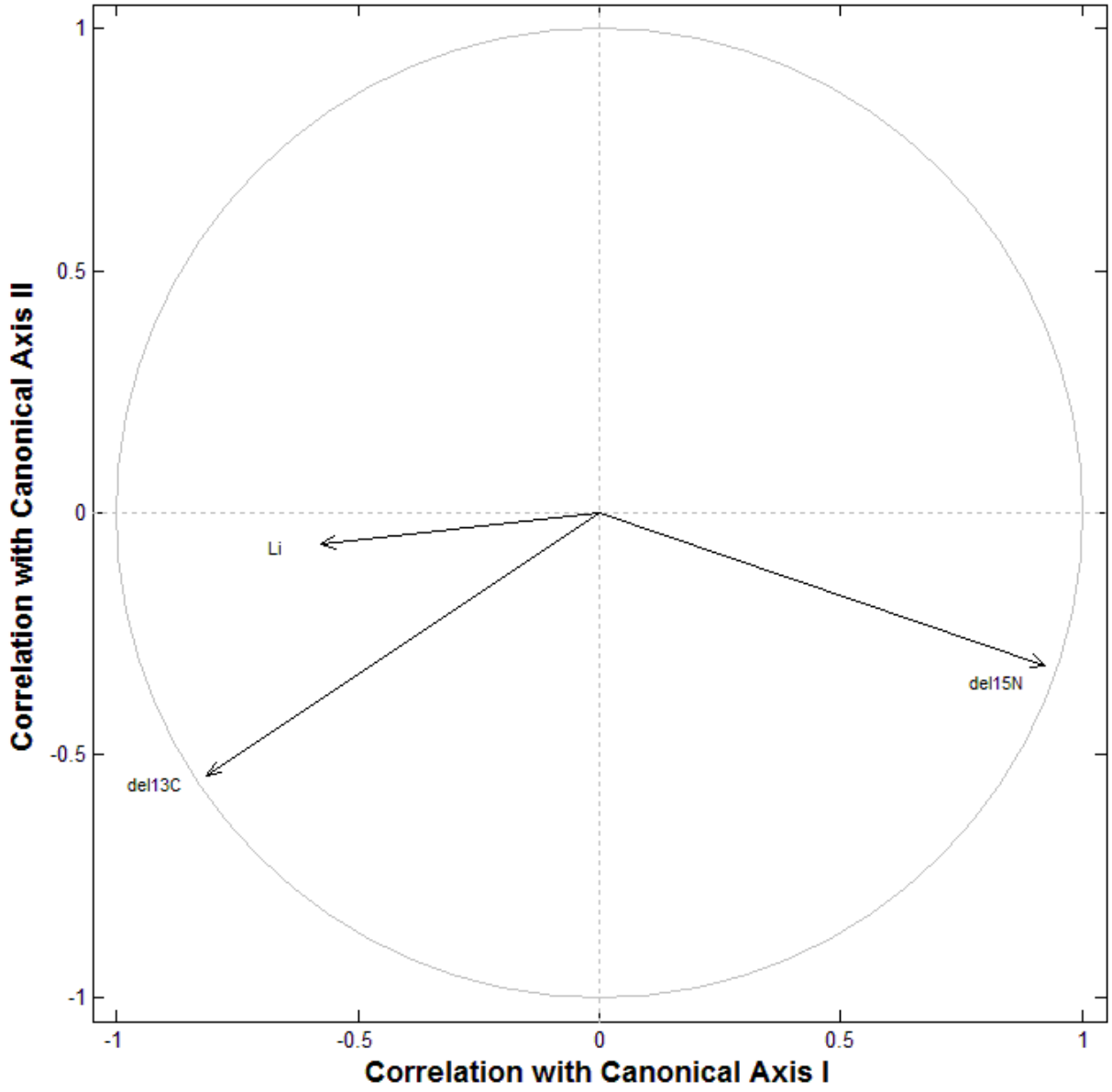

Figure 29. Correlation vectors corresponding to CAP analysis of 2011 field study Li, $\delta^{13}C$, and $\delta^{15}N$ data (Figure 28). Vector directions and magnitudes depict underlying gradients of elemental concentrations and isotope ratio, and are proportional to each analyte's contribution toward separating individuals collected at differing sites.

Table 12. Li, $\delta^{13}C$, and $\delta^{15}N$ CAP test LOO cross-validation confusion matrix for five 2011 sampling locations. Bold text indicates percent crabs successfully reclassified. Total classification success: 66%

	Alafia River	**Little Manatee River**	**Palm River**	**Safety Harbor**	**Skyway Fishing Pier**
Alafia River	**33**	22	33	11	0
Little Manatee River	14	**71**	14	0	0
Palm River	22	0	**67**	0	11
Safety Harbor	10	20	10	**30**	30
Skyway Fishing Pier	3	0	14	0	**83**

To determine whether the chemical signatures observed above were stable for each sampling location between years, ANOVAs were completed for each Li, $\delta^{13}C$, $\delta^{15}N$, and Ba, comparing 2011 and 2012 data for the Little Manatee River and Skyway Fishing Pier (Table 13). The Little Manatee River showed a significant difference between 2011 and 2012 for concentrations of Li, Ba, and $\delta^{15}N$. Chemical signatures in the cuticle of blue crabs collected at the Skyway Fishing Pier appeared to be stable between years.

Table 13. Distribution-free ANOVAs comparing 2011 and 2012 for separate analytes. Bold text indicates a significant difference between years for a metal or isotope ratio for an individual sampling site, p-value < 0.05

	Little Manatee River	**Skyway Fishing Pier**
Element	**p-value**	**p-value**
Li	**0.003**	0.290
$\delta^{13}C$	0.618	0.119
$\delta^{15}N$	**0.002**	0.591
Ba	**0.001**	0.479

Discussion

The initial hypothesis of this study was that female blue crabs migrating past the Skyway Fishing Pier towards the Gulf of Mexico would be a mix of individuals from the four major northern Tampa Bay riverine mating habitats previously identified by local commercial blue crab fishermen (i.e. Alafia River, Little Manatee River, Palm River and Safety Harbor). Instead, initial chemical analysis indicated that samples collected from the Skyway Pier were largely individuals that were not from known mating habitats and were, therefore, not regularly harvested by commercial fishermen. This necessitated testing the assumption that the blue crab cuticle is chemically stable after the terminal molt. Once certain chemical constituents were established as being chemically unchanging, analysis comparing individuals from separate collection sites within Tampa Bay confirmed that many female blue crabs emigrating from Tampa Bay may originate from a source that is separate from those regularly harvested by commercial blue crab fishermen.

The results show that some elements are chemically stable in the cuticle of a post-terminal molt female blue crab in terms of either elemental concentration or stable isotopic ratio. $\delta^{13}C$, $\delta^{15}N$, Li, Ca, and Ba showed no statistically significant change over the course of the 29 day captive crab experiment. This establishes that the analytical techniques using EA-IRMS and ICP-MS can be reliably conducted on blue crab exoskeleton isolated from the rest of the organism. Something to note is that the statistical tests used here to determine whether or not an analyte varied over the course of the captive animal study were extremely conservative, as is

generally desirable for fisheries management. Due to the conservative nature of this screening process, however, it is possible, if not likely, that some chemical constituents were eliminated from further analysis due to natural variation in the data being interpreted as chemical instability. Future studies could use a less conservative statistical screening method, as well as conduct a thorough quantitative metal analysis of blue crab cuticle over time to test for additional stable chemical constituents that were not detected during this experiment. With a comprehensive set of stable elemental concentrations and isotopic ratios, the chemical tagging technique used by this study could be applied elsewhere along the east coast of North and South America to study post-terminal molt blue crab migrations.

A graphical representation of isotopes $\delta^{15}N$ vs. $\delta^{13}C$ (Figure 2) shows clear isolation of samples originating from the Little Manatee River, serving as proof-of-concept that an elemental analysis of exoskeleton can be used to indicate the mating habitat of mature female blue crabs if the chemical signatures are distinct. The Little Manatee River showed statistical difference from the Palm River, Safety Harbor, and the Skyway Pier for each of these isotopes (Tables 9, 10). Enriched values of $\delta^{15}N$ for the Little Manatee River indicate runoff influence and anthropogenic input (Sigman and Casciotti 2001, Phillips and Gregg 2001), while corresponding $\delta^{13}C$ values are largely depleted indicating carbon input from terrestrial C3 plant organic matter (Smith and Epstein 1971, Peterson and Fry 1987). Values of $\delta^{13}C$ for samples from the other four sources mostly grouped around -25‰ to -21‰, with large amounts of overlap, indicating a higher proportion of carbon input from marine phytoplankton organic material (Haines and Montague 1979). Individuals collected from the Skyway Fishing Pier exhibited depleted values of $\delta^{15}N$ in comparison to all other sample sources, indicating less influence from run-off and anthropogenic input. Together, this suggests that individuals collected from the Skyway Fishing Pier did not

originate from one of the identified rivers as previously hypothesized, but may have originated from an area with marine phytoplankton carbon influence as a basal resource and low terrestrial run-off.

Lithium concentrations in individuals sampled from the Skyway Fishing Pier were elevated and exhibited significant differences compared to all other sample locations. While Li behavior and distribution in estuaries is largely unknown, this result strongly suggests that individuals sampled while undergoing spawning migration past the Skyway Fishing Pier originated from a non-riverine mating habitat. When analyzed collectively, Li, $\delta^{13}C$, and $\delta^{15}N$ indicate that individuals from the Skyway Fishing Pier are chemically different from those collected from the other four locations. The Skyway Pier had the highest rate of validation reclassification (83%), followed by the Little Manatee River (71%) and Palm River (67%). The chemical signatures from the Alafia River and Safety Harbor sites overlap with all other locations and, therefore, result in low classification rates of 33% and 30%, respectively.

A combination of isotopic and metallic microchemical data reveals that many post-terminal molt female blue crabs emigrating from Tampa Bay during this spawning pulse originated from a region that is not generally harvested by commercial fishermen. Instead, blue crabs undergoing spawning migrations appear to have originated from a mating habitat that has low anthropogenic input from freshwater runoff, and increased marine phytoplankton influence from Gulf of Mexico open waters, such as in the open waters of Tampa Bay, as opposed to contributing rivers or the shallow periphery.

Commercial blue crab harvesting is performed using a trapping technique, wherein a baited trap (or "pot") is set and left to soak for an extended period of time (Perry 1975, Perry et al. 1984). This technique is density-dependent, and fishermen naturally concentrate on areas of

high density in order to maximize their catch-per-unit-effort. If the density of harvestable blue crabs located on the floor of the Tampa Bay estuary was much lower than the density in the contributing rivers, then the diffuse individuals would be an unattractive investment of resources for commercial fishermen. Therefore, it is possible that low densities of blue crabs distributed across the estuary's large bottom area could dominate the biomass of emigrating females that ultimately contribute to the spawning stock in the Gulf of Mexico. The low density of the estuary group effectively shields those individuals from economic exploitation, creating a density-dependent natural harvest refugium.

Blue crabs maturing in the greater Tampa Bay estuary, including its tributaries, are capable of moving between nursery and mating habitats, from one river to another or from a low salinity river habitat to higher salinity open coastal waters. This suggests that Tampa Bay blue crabs may have a variety of habitat preferences and differing ecological histories. By harvesting individuals found in high density, low salinity habitats, economic pressures may be selecting against individuals with a (possibly genetic) predisposition to mate within estuarine tributaries.

Overall, the chemical tagging technique was able to successfully distinguish among sampling locations. Rainfall patterns and the timing of sample collection, however, may be a challenge when implementing this technique. In the Tampa Bay area, precipitation increases during the summer, resulting in increased freshwater input into the rivers and estuary. Additional water flow increases mixing between rivers and may homogenize the microchemical signatures, making geographic locations difficult to distinguish using this approach. Female blue crabs are known to use tides, with increased outgoing volume resulting from precipitation, to migrate into open water. Therefore the best timing for sample collection may be difficult to determine. In the

case of this study, ideal collection occurred in late spring after blue crabs had begun to mate, but just before strong precipitation might have homogenized mating habitat chemical signatures.

Over the two years of this study, the chemical signature for the Little Manatee River showed variation, while the Skyway Fishing Pier appeared to be chemically unchanged. This indicates that if the technique applied here is used over multiple mating seasons, the signature for each known mating habitat should be re-established each year, if not more frequently. Generating a database of known mating habitat chemical signatures over multiple years may be useful to track inter-annual variation, but a database generated from a single year for use in subsequent years without updates might not be defendable. While this would require sample collection from known mating habitats on a regular basis, the overall time and financial investment necessary for this microchemical migratory tracing technique may still be an improvement over more traditional tag-and-release or tracking methodologies.

One last challenge that might be encountered when implementing the microchemical tagging technique described here is the variability of chemical constituents being retained during molting. There is documentation of varied turn-over rates for $\delta^{13}C$ and $\delta^{15}N$ in blue crabs depending on the type of tissue (Llewellyn 2008). When stable isotopes are drawn from storage within the organism to form a new cuticle, the tissue from where the isotopes originated would determine how soon after relocation the isotopic signature of a newly formed exoskeleton would reflect the environment. When immature females move to a mating habitat, there can be a period where the cuticle reflects the previous molting habitat, while the organs are beginning to reflect the newly encountered environment and basal resource. If storage tissues have not fully turned-over to reflect the new habitat before contributing carbon, nitrogen, and trace metals to a new cuticle, then sampled post-terminal molt exoskeleton may not accurately indicate the mating

habitat. Future investigation into the storage tissues that contribute material to a new cuticle, as well as detailed surveys of the chemical signatures throughout the local range of the crab, could be used to determine whether sampled individuals are new arrivals to the mating habitat.

Conclusion

There were three major successful outcomes from this completed study. First, microchemical analysis technique can be reliably conducted on isolated exoskeleton of crustaceans, yielding a chemical snap-shot of a mature female blue crab's mating habitat. Secondly, a sub-set of elements were confirmed to be stable in the cuticle of the blue crab over time in a chemically variable environment, while others appeared to be more variable. Finally, the results indicate that the Tampa Bay blue crab stock may have a high biomass of individuals not being harvested by commercial blue crab fishermen. These individuals are likely located in open waters of the estuary and may dominate Tampa Bay's contribution to the Gulf of Mexico spawning stock, while being naturally shielded from economic exploitation.

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