Rethinking Food Web Tracing: A Conceptual Framework Utilizing a Multi Biomarker Approach to Better Understand Trophic Relationships

by

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A Dissertation Submitted in Partial Fulfillment of the Requirements for the Doctoral Degree in Molecular Biosciences

Middle Tennessee State University

May 2025

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Acknowledgments

I would like to thank Dr. Jason Jessen and the Molecular Biosciences program at MTSU for giving me the guidance necessary to navigate my way through graduate school.

I would like to thank all my committee members for coming together for this dissertation research. Each member brought a unique perspective, and I am extremely grateful for their guidance and feedback throughout this process. Thank you to Dr. Frank Bailey at MTSU for agreeing to step in as my committee chair, encouraging me to think critically about experimental design, and always being there for any questions or advice. Thank you to Dr. Jeff Leblond for helping develop the methodology for lipid and fatty acid work done throughout my experiments and also for being an amazing repairman for the GC-MS. Thank you to Dr. Dan Hua, who introduced me to the freshwater mussel world and the applications of my work to conservation biology, which I have loved every moment of. I am forever grateful for my rare opportunity to work with her and the Tennessee Wildlife Resources Agency during my PhD. Thank you to Dr. Marc Mills, who gave a unique look at how applied ecotoxicology happens in the "real world" outside of academia.

I would like to thank my primary mentor, Dr. Ryan Otter, for helping to develop me into the scientist I am today. He has been essential in my journey, and I couldn't ask for a better, more understanding mentor.

I would like to thank all the undergraduate students who have helped with spider and insect sampling as well as sample processing and analysis. I would especially like to thank Addison Plummer and Andrew Hetrick, both of whom were essential in getting this work done. I know they will become great scientists, and I look forward to seeing what they accomplish.

I would also like to thank my family and friends, all of whom have been so supportive during this period of my life. I would especially like to thank my mom, who introduced me to the world of biology at a young age and encouraged my curiosity of the natural world.

I would like to thank my amazing husband, Gary Landaverde, for supporting me through my crazy decision to return to school during a global pandemic. Thank you for making this dream possible for me and then being there for me through all the highs and lows.

ABSTRACT

In this dissertation, a conceptual framework for tracing trophic interactions and providing more clearly defined food web reconstructions is introduced, the Tracer Trichotomy. This framework involves the use of three separate trophic tracing methods to inform trophic interactions: stable isotopes (carbon and nitrogen), polyunsaturated fatty acid biomarkers, and bioaccumulative contaminants (replaced with stress/ health biomarker in conservation scenarios). The use of the Tracer Trichotomy provides a more complete and accurate understanding of trophic pathways and contaminant transport than the use of any individual method. In this dissertation, four separate projects are described, each assessing environmental or individual life cycle variables that could impact the values of these food web tracers.

The environmental effects associated with time of year and annual time changes are assessed in a sentinel spider species in chapters I and II. In chapter I, it was found that spider mass and length steadily increased from April to September to a maximum average value of 0.078 ± 0.03g, then decreased in October. Seasonal trends were observed for carbon and nitrogen stable isotopes, with significantly decreased signatures occurring late in the active season. Overall, methyl mercury concentrations (range: 12.1-134.4 ng/g) and the methyl:total mercury ratio (range: 49-98% methyl mercury) increased throughout the active season, with higher variability observed at the end of the active season. These results indicated that seasonality impacted several important endpoints and that spiders collected during the end of the active season may not be representative of spiders during the entire active season. In chapter II, it was found that carbon and nitrogen stable isotopes, ω3:ω6 ratios, and mercury concentrations differed significantly in tetragnathid spiders between consecutive years at the same site location.

These results indicated that enough variation occurs annually that the data of one year cannot be applied to following years at the same sampling site using tetragnathid spiders.

In chapters III and IV, the individual life cycle variable of metamorphosis and altered metamorphosis processes are assessed in laboratory-reared and field-caught mayflies and an endangered species of freshwater mussel. In chapter III, the results showed that the $\delta^{15}N$ and %N increased significantly with metamorphosis in both laboratory-reared *N. triangulifer* and field-collected *Heptageniidae* mayflies. There were no significant differences in polyunsaturated fatty acid profiles between larval and adult stages of field-collected mayflies; however, there was a significant increase in % arachidonic acid in laboratory-reared individuals. The results of this study indicate that the metamorphosis has a significant impact on food web tracers in laboratory and field mayflies, which should be considered when using mayflies or potentially other emergent aquatic insects in calculations connected to ecological risk assessments. In Chapter IV, for the endangered mussel *Toxolasma cylindrellus, in vitro* propagated individuals were significantly larger and had lower $\delta^{13}C$ values than fish propagated mussels. Otherwise, there were no differences found between *in vitro* propagated, and fish propagated mussels for nitrogen stable isotopes, total carbon, and nitrogen, lipids, polyunsaturated fatty acids, or glycogen content. The results of this study indicate that *in vitro* propagation is a viable method for *T. cylindrellus* conservation and displays minimal differences in juvenile health and nutrient uptake between propagation methods.

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LITERATURE REVIEW

Section 1: Aquatic Subsidy

Organisms that have biphasic life cycles and undergo metamorphosis, such as aquatic emergent insects, provide direct pathways for the transfer of energy between habitats (Baxter *et al.,* 2005). These insects hatch from eggs deposited in the water and then begin life as fully aquatic larvae, part of an aquatic food web and available to aquatic consumers. Aquatic emergent insects then undergo metamorphosis and emerge from the water as flying adults; at this point in their life cycle, they become a part of the terrestrial food web and are now available to a completely different set of consumers on land. This transition into another food web is unique in that aquatic-based energy and nutrients gained by the insects during their larval stages can be transferred to terrestrial food webs via terrestrial consumers' predation of these insects. This energetic pathway provided by aquatic emergent insects is one of the ways in which aquatic and terrestrial ecosystems interact and exchange energy (Walters *et al.* 2008, Kraus *et al.* 2014).

Aquatic and terrestrial ecosystems are interconnected, with energy flowing between the two, referred to as subsidies (Baxter *et al*., 2005; Polis *et al*., 1997). The flow of energy between aquatic and terrestrial ecosystems is often depicted from terrestrial to aquatic; however, much less studied is the flow from aquatic to terrestrial ecosystems, hereafter referred to as an aquatic subsidy. It has been shown that terrestrial riparian communities benefit from aquatic-based nutritional subsidies, and some consumers depend on aquatic subsidies to survive and gain the proper nutrition for development, with anywhere from 25% to 100% of their energy or carbon coming from the aquatic subsidy (Baxter *et al*., 2005, Twining *et al*., 2019, Twining *et al*., 2021).

Although aquatic subsidy to terrestrial habitats can be beneficial to riparian consumers, there is a "dark side" to this process in which bioaccumulative contaminants can also be transferred between aquatic and terrestrial habitats (Walters *et al.,* 2008; Kraus *et al.,* 2014). Insect-mediated contaminant flux has been identified as a mode of bioaccumulative contaminant transfer that directly connects emergent aquatic insects with the transfer of bioaccumulative contaminants from aquatic to terrestrial ecosystems (Otter *et al.,* 2020). The transfer of bioaccumulative contaminants puts predators at higher trophic levels within riparian areas, such as songbirds and bats, at risk for harmful effects due to the biomagnification of transferred contaminants. Although a small amount may be transferred by aquatic insects individually, biomagnifying contaminants increase as a lower-level predator is consumed by a higher-level predator, becoming more concentrated with each trophic interaction, potentially leading to harmful high concentrations in those higher trophic level predators (Maul *et al*., 2006; Cristol *et al.,* 2008; Walters *et al*., 2008; Beaubien *et al*., 2020; Bundschuh *et al*., 2022).

Estimating the amount of aquatic subsidy occurring between ecosystems is important in determining the amount of bioavailable contaminants available to riparian predators that could be put at risk. Riparian spiders, specifically those in the family *Tetragnathidae* (hereafter referred to as tetragnathids), have been identified as bioindicators of any aquatic subsidy occurring and sentinels of aquatic bioavailable contaminants (Chumchal *et al.,* 2022). Tetragnathid spiders are web-building spiders that reside on low hanging vegetation over water sources and specialize in catching aquatic insect prey for consumption (Levi 1981, Gillespie 1987). Their localized habitats and specialized aquatic insect diet make them an effective indicator of any energy, nutrients, or bioavailable contaminants that become available to terrestrial food webs via the emergent aquatic insect pathway (Chumchal *et al*., 2022). Measurements of contaminants from

these spider bioindicators can also be used in risk assessments to predict exposure rates to high trophic level predators such as birds and bats in the area. The development of spider-based avian wildlife values for some contaminants has helped to advance these risk assessments by determining concentrations at which birds will experience adverse health effects from contaminated spiders. Studies done by Gann *et al.* (2015), Walters *et al.* (2010), and Beaubien *et al.* (2020) were able to determine spider-based avian wildlife values for mercury, polychlorinated biphenyls (PCBs), and various metals, respectively.

Tetragnathid spiders have been successfully used as sentinels of bioavailable contaminants for the past several decades (Chumchal *et al.,* 2022). However, some aspects of their use have not been fully investigated. A sentinel species is defined by Beeby (2001) as "biological monitors that accumulate a pollutant in their tissues without significant adverse effects". Various species, such as these spiders, meet these criteria but are often not properly assessed to ensure that they are an ideal candidate. The characteristics of an ideal sentinel species described in Beeby (2001) include having a large body of knowledge on the species' physiology, such as the effects of age, sex, size, and season. In addition, the validation of a sentinel requires assessment of temporal and spatial variation in the assimilation of contaminants. Stream conditions vary throughout the year, such as temperatures, stream flow, and foliage density changing between seasons (Corbet 1966; Sumner and Fisher 1979; Nakano and Murakami 2000). The benthic macroinvertebrate community is also known to fluctuate over time, with different species of emergent insects depositing eggs and emerging at different times within a year (Corbet 1966; Pinder *et al.* 1993; Nakano and Murakami 2001). A study by Laws *et al.* (2016) found that this was true with emergent chironomids available to riparian spiders, finding that they unequally contributed to spider biomass at different times of the year. This, along with

other environmental factors, can impact the amount and type of insects available to spiders and, subsequently, the amount of nutrients and potential contaminants entering the terrestrial food web (Paetzold, Bernet, and Tockner 2006, Burdon and Harding 2008, Ivković *et al.,* 2013, Chari *et al.,* 2020, Nash *et al.,* 2023).

Section 2: Carbon and Nitrogen Stable Isotopes

Carbon Stable Isotopes

Carbon stable isotopes are widely used tracers of primary dietary sources in organisms (Peterson and Fry, 1987; Wada *et al.,* 1991; Post, 2002). Carbon has naturally occurring stable isotopes of carbon-12 and carbon-13, with carbon-13 having one additional neutron than carbon-12 and, therefore, an increased atomic weight. Carbon-12 is more abundant naturally, and carbon-13 is produced through various mechanisms, such as photosynthesis. This change in atomic weight and production of different isotopes is called fractionation (Schoeller, 1999). Isotope fractionation amounts are reported in delta notation (δ) or permil (‰), calculated as the ratio of the heavy isotope to the light isotope of a sample, divided by a ratio of the heavy isotope to the light isotope of a known standard (for carbon this standard is Vienna Peedee Belemnite Limestone), subtracting one, and multiplying the result by 1000 (McKinney *et al.,* 1950; Ehleringer *et al.,* 1986; Schoeller, 1999). Values for carbon isotopes are estimated to change only by a small amount, 1 permil or less, with each trophic interaction (DeNiro and Epstein, 1978; Post, 2002). This minimal change with trophic interaction enables the estimation of the types of primary producers at the base of an organism's food chain.

The ratio of carbon-12 to carbon-13 isotopes differs between types of primary producers. During the photosynthetic process, plants differ in the way that they fix carbon dioxide (Craig

1953, Bender 1968; Smith and Epstein 1971). The different types of photosynthetic pathways primary producers use have an impact on carbon stable isotope fractionation. C3, C4, CAM, and aquatic primary producers all preferentially retain carbon-12 at different amounts during the fixation of carbon dioxide in the photosynthetic process (Smith and Epstein, 1971; Ehleringer *et al.,* 1986). For example, in terrestrial plants, C3 plants have carbon isotopes ranging from -20 to -35 permil, C4 plants range from -7 to -15 permil, CAM plants range from -10 to -22 permil, and freshwater primary producers range from -11 to -50 permil (Ehleringer *et al.,* 1986; Keeley and Sandquist, 1992). Differing isotopic fractionation leads to unique signatures of the carbon-12 to carbon-13 ratio related to each of these types of plants. This can help determine the primary producers at the base of an organism's food chain, and in cases where an organism may be consuming different types of prey (i.e., aquatic and terrestrial), this can also be useful in determining the amount of influence of each type of prey on their diet. Generally, the more negative a carbon signature is, the more aquatic influence in an organism's diet, and the less negative, the more terrestrial influence.

Nitrogen Stable Isotopes

Nitrogen stable isotopes have also been widely used as tracers in food web studies to determine the trophic levels of organisms within a food web (Peterson and Fry, 1987; Wada *et al.,* 1991; Post, 2002). Nitrogen has naturally occurring isotopes of nitrogen-15 and nitrogen-14 that fractionate with various biological mechanisms, with nitrogen-14 being more abundant in the atmosphere. Nitrogen stable isotope values are reported in delta notation, like carbon stable isotopes mentioned previously, and use a standard of atmospheric air for calculation. Trophic levels of organisms in a food web can be determined because nitrogen stable isotope values increase between trophic levels at an average of about 3.4 permil (Minagawa and Wada, 1984;

Post, 2002). It is unknown the exact mechanisms that cause the isotope of nitrogen-15 to be preferentially retained in an organism from its prey. It is assumed that various metabolic processes taking place within organisms are the source of nitrogen fractionation and that amino acid utilization may play a role (Schoeller, 1999; Vanderklift and Ponsard, 2003). It is also found that the preferential removal of amine groups that contain nitrogen-14 during peptide bond hydrolysis and transamination can also lead to an increased nitrogen-15 value in consumer tissues (Macko *et al.,* 1986; Gannes *et al.,* 1997; Balter *et al.,* 2006). Hare and Estep (1983) found that there was variation between the nitrogen isotopes incorporated into different amino acids, with nitrogen within threonine being the lightest and nitrogen within leucine being the heaviest, thus causing preferential retention of certain nitrogen isotopes. Various excretion pathways are another process that may impact nitrogen fractionation and preferential isotope retention. Steele and Daniel (1978) investigated the pathways of excretion in cattle and found that nitrogen-14 was preferentially excreted in the form of urea, causing nitrogen-15 to be retained in the body.

Combined Use of Carbon and Nitrogen Stable Isotopes

Stable isotope analysis of carbon and nitrogen are typically combined when tracing and reconstructing food webs. Measuring both values provides a clearer picture of an organism's diet and where it falls within the food web in terms of trophic level. The resulting values are plotted with carbon-13 on the x-axis and nitrogen-15 on the y-axis, as seen in Figure 1. As previously mentioned, an organism's trophic level and dietary influence can be predicted because it is thought that carbon-13 changes by 1 permil or less and nitrogen-15 changes by about 3.4 permil with each trophic interaction. This allows for the reconstruction of food webs and the tracing of trophic interactions between organisms.

These values have been assumed to be true and have been applied widely in food web studies. However, there are some factors that can influence fractionation rates of carbon and nitrogen stable isotopes and, therefore, impact these assumed values. This includes environmental factors, sampling factors, dietary factors, and individual metabolic factors. One environmental factor that can influence isotopic values is seasonality. Ecosystems change throughout a year and do not have a set rate of production, amount of light, etc., that stays constant with every season. It has been shown that these seasonal changes can impact the isotopic values of primary producers in aquatic habitats, thus changing the stable isotopes at the base of a food web (Gustafson *et al.,* 2007; Van den Meersche, 2009). Sampling factors impacting the stable isotope measurements include the area or tissue of an animal sampled (Sutoh *et al.,* 1997, Schmidt *et al.,* 2004, Deudero *et al.,* 2008; Reich *et al.,* 2008, Mohan *et al.,* 2016, Wang *et al.,* 2016). Dietary factors may also impact the incorporation of stable isotopes, with diet quality, protein, and lipid content of diet items playing a role in the incorporation of stable isotopes in an organism (Oelbermann and Scheu, 2002; Poupin *et al.,* 2011; Mohan *et al.,* 2016; Hughes *et al.,* 2017).

Individual metabolic factors can also have impacts on stable isotopic values; some organisms have complex life cycles and may go through metabolic processes such as metamorphosis, which has been shown to impact isotopic values, particularly nitrogen (Miller, 2000; Pecquirie *et al.* 2010; Alp *et al.,* 2013). Due to these influences on stable isotope measurements in organisms, it may not always be best to assume a constant amount of change with each trophic interaction. More work in the area of isotopic variation factors is needed to fully understand isotopic differences between trophic levels.

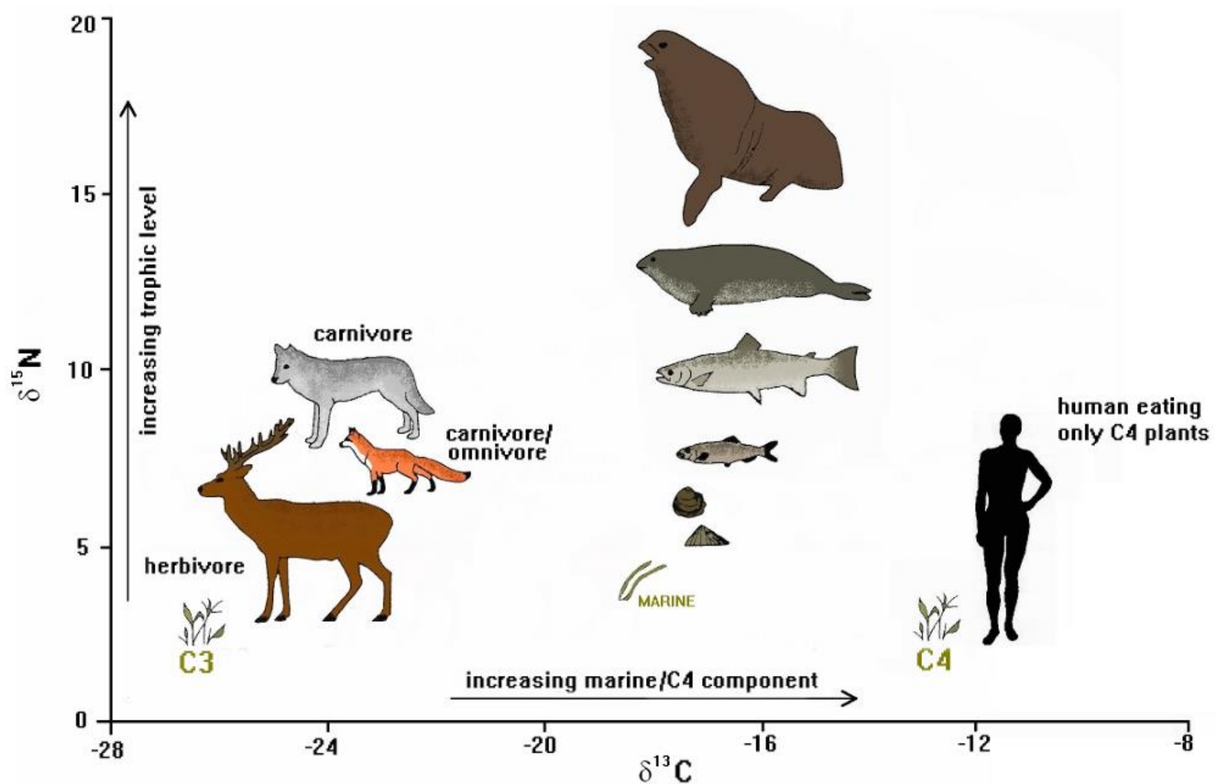

Figure 1. An example of a carbon and nitrogen stable isotope biplot. A typical food chain in an aquatic ecosystem is shown with estimates of carbon and nitrogen stable isotope increases with each trophic level. From Schulting, 1998.

Section 3: Polyunsaturated Fatty Acid Biomarkers

Fatty acids are essential in all organisms and are necessary for cellular functioning. An important type of fatty acid is long-chain polyunsaturated fatty acids (PUFAs); they consist of a hydrocarbon tail of at least 20 carbons in length, which contains at least 2 double-bonded carbons and a carboxylic acid (Vagelos, 1974). The common nomenclature for fatty acids is as follows: # of C: # of double bonds ω (or n) position of first double bond from the omega carbon (3 or 6). Organisms are able to obtain long-chain PUFAs through one of two ways: consuming

the necessary fatty acid in their diet or by obtaining the precursor to the necessary fatty acid and then synthesizing the longer-chain fatty acids needed in the body. If an organism is to synthesize the necessary lipid *de novo*, it must have the required enzymes. They are, however, able to be synthesized *de novo* in aquatic primary producers in the cytoplasm of cells from Acetyl-CoA. From Acetyl-CoA, 16:0 (palmitic acid) is synthesized, and then longer chain fatty acids are synthesized through desaturation and elongation steps requiring specific enzymes (Gurr *et al.* 1971; Vagelos 1974). Certain enzymes, such as Δ^{12} and Δ^{15} desaturase, are mainly found in primary producers, and therefore, those longer chain fatty acids they produce are required to be taken in by a majority of organisms through diet (Dalsgaard *et al.,* 2003; Kelley and Scheibling, 2012; Hixson *et al.,* 2015). There are two precursors for long-chain polyunsaturated fatty acids: linoleic acid (18:2ω6) for ω6 PUFAs and alpha linoleic acid (18:2ω3) for ω3 PUFAs. ω3 PUFAs are those that have their first double bond on the third bond from the omega carbon (the carbon at the methyl end of the fatty acid), and ω6 PUFAs have their first double bond at the sixth bond from the omega carbon (Vagelos, 1974). Both ω3 and ω6 long-chain PUFAs are required for an organism to function (Hixson *et al.,* 2015; Kelley and Scheibling, 2012).

ω3 long-chain PUFAs are produced by aquatic primary producers, such as algae, and are associated with an aquatic-based dietary source. ω6 long-chain PUFAs are associated with terrestrial primary producers and a terrestrial-based dietary source (Hixson *et al*., 2015). It has been found that certain ω3 long-chain PUFAs are useful for determining aquatic influence on an organism's diet due to them being highly conserved during trophic interactions. These fatty acids are also not able to be synthesized by terrestrial organisms on their own, so they must be supplemented through food items (Dalsgaard *et al.* 2003; Kelley and Scheibling 2012; Hixson *et al.* 2015). This enables the ω3 fatty acid amount to be indicative of the amount of aquatic subsidy

to a diet. The ω3 to ω6 fatty acid ratio is used to estimate an organism's ratio of aquatic to terrestrially derived dietary items (Dalsgaard *et al.,* 2003; Kelley and Scheibling, 2012; Taipale *et al.,* 2014). Alternatively, the presence of certain ω6 PUFAs, such as 18:2ω6, in an aquatic environment can indicate terrestrial subsidy to a water source (Dalsgaard *et al.* 2003).

In addition to being the producers of ω3 long-chain PUFAs, algae can also produce higher amounts of certain PUFAs depending on taxa (Dalsgaard *et al.,* 2003; Galloway and Winder, 2015). For example, diatoms are associated with higher amounts of eicosapentaenoic acid (EPA) (20:5ω3) and 16:4ω3; dinoflagellates are associated with higher amounts of 18:4ω3, 18:5ω3, and 22:6ω3 (docosahexaenoic acid or DHA) (Dalsgaard *et al.,* 2003; Galloway and Winder, 2015). Also, bacteria can influence algae sources' fatty acid profiles (Dalsgaard *et al.* 2003). It has also been suggested that the ω3 to ω6 fatty acid ratio is indicative of diet quality and that certain algae taxa provide a better quality diet than others because of their difference in fatty acid production, with an increased ω3 to ω6 fatty acid ratio indicative of a higher quality diet (Galloway and Winder, 2015; Taipale *et al.,* 2015).

Certain ω3 long-chain PUFAs have been assessed as potential tracers of aquatic to terrestrial subsidy, in particular 20:5ω3 EPA. This was identified as a potential biomarker of aquatic dietary influence in a novel study done by Chari *et al.* (2020) in which multiple fatty acids were assessed over various seasons and at distances from the Kowie River in South Africa. EPA was found to be the most effective tracer of aquatic dietary influence, indicating the potential to be developed as a biomarker of aquatic subsidy. EPA, along with arachidonic acid and DHA, have been observed in much higher amounts in aquatic insects when compared to terrestrial insects due to their algae-based diet (Hanson *et al.* 1985; Scharnweber *et al.* 2019; Twining *et al.* 2021). It has also been shown that aquatic insect fatty acid profiles closely match a

river's basal resources (Moyo and Richoux, 2022). However, it has been found that different aquatic insect species, even within the same order, can vary in their amounts of ω3 long-chain PUFAs (Gladyshev *et al.,* 2011; Moyo and Richoux, 2022; Scharnweber *et al.,* 2019). Other variables, such as time of year and location within a water source (i.e., upstream vs downstream in a river), can also impact the type and amount of ω3 long-chain PUFAs being exported by aquatic emergent insects by altering the algal community (Chari *et al.,* 2020; Moyo and Richoux, 2022).

Section 4: Mercury: A Contaminant and Tracer

Mercury is a widespread global contaminant, present even in remote, generally undisturbed areas (Driscoll *et al*., 2013). Mercury can cause adverse health effects in humans and in wildlife that become exposed to it, mainly through ingestion (Clarkson, 1997). Mercury has three main forms: elemental mercury (Hg^0), inorganic mercury (Hg^{2+}), and organic mercury or methylmercury (MeHg) (Clarkson, 1997; Morel, 1998). Mercury in the environment can originate from natural sources, such as volcanic eruptions, or manmade sources, such as industrial activities. Mercury, once in the environment, can alternate between its different forms through various reactions. Elemental mercury can become inorganic mercury through photooxidation reactions in the environment, and inorganic mercury can become elemental mercury through photoreduction reactions (Morel, 1998). Methylmercury forms when inorganic mercury combines with carbon in the environment, which is thought to be facilitated by sulfur-reducing bacteria in anoxic sediments (Ullrich *et al*., 2001). While exposure to elemental and inorganic mercury can cause damage to organisms, it is methylmercury that poses the greatest threat. This is because of its ability to cross the blood-brain barrier and the placental barrier due to being able to bind to thiol groups (-SH) in the amino acid cysteine; this can cause immense

damage to the nervous system and can cause mercury to be passed to offspring (Clarkson, 1997). Another cause for concern is the ability of methylmercury to bioaccumulate and biomagnify through a food chain. This means that organisms can absorb organic mercury through environmental exposure and have it build up over time and also that the amount of organic mercury in higher trophic level organisms can be much higher than at lower trophic levels due to incorporating the mercury load of the prey consumed with each trophic interaction (Morel 1998).

Because of methylmercury's ability to biomagnify through a food web, it is important to understand its trophic dynamics. The estimation of the increase between trophic levels is referred to as the trophic enrichment factor or biomagnification factor. It is estimated that in planktivorous fish, the methylmercury concentration increases by about 0.5 log units per trophic level (Watras and Bloom, 1992). In a study done by Mason *et al*. (1996), they were able to use this estimate to construct a model that can be used to predict methylmercury concentrations in fish. A study by Watras *et al*. (1998) later found that methylmercury increased 3, 5, and 20 times in relation to background levels of DOC for microseston, microplankton, and fish with the bioaccumulation factor increasing 2-4 times with each trophic level, giving a biomagnifying factor of 1.6 - 4. Chumchal *et al*. (2009; 2011) found a slightly higher value, with methylmercury increasing by a factor of 5-6 and 4.8 with each trophic level in Texas freshwater food webs. In a meta-analysis done by Lavoie *et al*. (2013), it was determined that the global average trophic magnification slope (slope of the linear regression between $\log_{10}$ mercury concentration and stable nitrogen isotope values) for methylmercury was 0.24 ± 0.08, however, it was found that variation in latitude, dissolved organic carbon, total phosphorus, and atmospherically deposited mercury impacted values.

Because of the ability to determine differences between trophic levels of methylmercury, the concentration of methylmercury within an organism can, in turn, be used to help determine the trophic level of that organism. It is this concept that allows for methylmercury to be potentially used as a food web tracer. The constant rate of biomagnification between consumers and food items allows for a factor that increases stepwise, similar to nitrogen stable isotopes, and tracing methylmercury contamination back in the food web to the source can allow for the determination of primary producers or lower trophic level sources much like the use of carbon stable isotopes. The total and organic mercury concentrations have been found to positively correlate to nitrogen stable isotope values (Yoshinaga *et al*., 1992; Chumchal *et al*. 2011; Seco *et al*., 2021) and inversely correlate with carbon stable isotope values (Power *et al*., 2002; Gorsky *et al*., 2003; Kidd *et al*., 2003). Higher levels of methylmercury in riparian habitats are also associated with carbon stable isotopes indicative of aquatic-based dietary influence (Wu *et al*., 2022) and polyunsaturated fatty acids derived from aquatic sources (Twining *et al*., 2021). This gives potential for further research into using methylmercury or other bioaccumulative contaminants to enhance food web tracing.

Section 5: Freshwater Mussels

Freshwater mussels serve as an essential part of our ecosystems. They are a part of important processes such as nutrient recycling, providing structural habitat, water filtration, and food web modification (Vaughn, 2017). They are also used as bioindicators of water quality and have been used in ecotoxicological research (Augspurger *et al.,* 2007). Because of their unique position in aquatic ecosystems as filter feeders that remain stationary in sediments, freshwater mussels give insight into water quality and its impacts on a specific area and provide a means to monitor quality over time (Grabarkiewicz and Davis, 2008; Asif *et al.,* 2017). There are over 300

species of freshwater mussels in the United States. However, they have experienced a multitude of population declines and extinctions (Neves *et al.* 1997; Grabarkiewicz and Davis 2008). Because of their importance to our aquatic ecosystems and ecological research, it is imperative to better understand freshwater mussel life cycles and push conservation efforts forward.

Freshwater mussels have experienced declines globally in the last century, resulting in a high rate of extinctions. These declines have resulted in small, isolated populations of mussels, many of which are endangered, threatened, or vulnerable (Neves *et al.,* 1997; Haag and Williams, 2014; Freshwater Mollusk Biology and Conservation, 2016). There is no one known cause of the mass extinction of freshwater mussels, but instead is attributed to a multitude of causes, including habitat loss and alteration, invasive species, and catastrophic events (Haag and Williams, 2014). North America is home to the most freshwater mussel biodiversity, with the Southeastern United States being a biodiversity hotspot, and recognition of the major species declines in the United States led to the National Strategy for the Conservation of Native Mussels being developed in the late 1990s (Neves *et al.,* 1997; Haag and Williams, 2014; Freshwater Mollusk Biology and Conservation, 2016). This strategy outlined specific goals related to conserving freshwater biodiversity, such as the development of large-scale propagation techniques, reducing the threat of invasive zebra mussels, and improving knowledge of native mussel populations. Since the introduction of the National Strategy for the Conservation of Native Mussels, major work has taken place to fulfill the goals proposed; this includes increased funding for mussel conservation, propagation and reintroduction of endangered populations, and public awareness of mussel declines (Haag and Williams, 2014; Freshwater Mollusk Biology and Conservation, 2016). The National Strategy has since been updated in 2016 to include freshwater snails and update conservation goals in response to progress made (Freshwater Mollusk Biology

and Conservation, 2016). Much more research is needed to fulfill these conservation goals and is necessary to preserve the biodiversity of North America's freshwater mussels.

Mussels have a unique life cycle during which there are 3 stages: glochidia/ larvae, juvenile, and adult. During the larval stage of the life cycle, the mussel is dependent on a fish host to which they attach and live within the epidermis and have a parasitic relationship. The mussels then undergo metamorphosis and exit the fish host as juveniles. At the juvenile stage, the mussels drop to the substrate and are free-living (Lefevre and Curtis, 1912; Coker *et al.,* 1921). It has been shown that a significant amount of nutrients in the glochidia is derived from the fish host, and this relationship is indeed parasitic (Fritts *et al*., 2013; Denic *et al*., 2015). Once independent of their fish host, newly transformed juvenile mussels initially engage in pedal feeding and later feed on a diet of algae and organic matter through filtration, similar to adult mussels (Lefevre and Curtis, 1912; Coker *et al.,* 1921; Gatenby *et al*., 1997).

Conservation efforts have largely focused on artificial propagation of freshwater mussels and reintroducing them to native areas where there have been population declines (Freshwater Mollusk Biology and Conservation, 2016). The propagation involves breeding adult mussels and providing the fish host for the parasitic glochidia stage, then providing adequate conditions for free-living juveniles to grow into adult mussels. Although there has been success in this method of conservation and the propagation of mussels has improved greatly over time, there are still some limiting factors to the success of propagation. During the process of attachment of the glochidia to a suitable fish host from the female adult mussel, many individuals will not attach correctly and will perish if attachment does not occur (Coker *et al.,* 1921). It is also difficult to monitor glochidia development during this process, which can provide insight into more of the developmental stages of mussels (Gąsienica-Staszeczek *et al.,* 2018).

In vitro culturing of freshwater mussels has been developed to combat some of the issues with traditional methods of mussel propagation. This technique involves the use of a nutrient medium to emulate that of the fish host within a petri dish where the glochidia metamorphose into their juvenile stage. This technique not only improves the survival rate of the mussels into their juvenile life stage but also provides a unique insight into glochidia development by having them exposed in petri dishes to observe under a microscope. Because this technique is relatively new, it comes with unique challenges. Because some mussel species require specific fish hosts for successful attachment and metamorphosis, it is necessary for variations of nutrient media to be made to match the nutrient differences that exist in the various fish hosts. Much work has been done to create methods for various mussel species (i.e., Kovitvadhi and Kovitvadhi, 2012; Gąsienica-Staszeczek *et al.,* 2018; Wen *et al.,* 2018), but more work in this area is needed to determine media formulations for remaining species (Lima *et al.,* 2012).

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INTRODUCTION

Tracing food web interactions and knowledge of food web dynamics are an essential part of estimating risk to organisms that may be exposed to bioavailable contaminants through their diet because these contaminants may biomagnify to harmful levels in upper trophic level predators. Previously, researchers have traced these interactions using carbon and nitrogen stable isotopes, with carbon-13 values indicating primary producers in a food web and nitrogen-15 values indicating trophic position of an organism (Wada *et al.,* 1991; Post, 2002). However, it has been suggested that various environmental and individual metabolic factors can impact the fractionation rates of these stable isotopes (Vanderklift *et al*., 2003). Additionally, ω3 polyunsaturated long-chain fatty acid (PUFA) biomarkers, eicosapentaenoic acid (EPA) in particular, have recently been identified as effective aquatic to terrestrial food web tracers (Chari *et al.,* 2020). ω3 PUFAs are synthesized by aquatic primary producers and are highly conserved through food web interactions, with their presence and amount indicating aquatic dietary influence in an organism (Dalsgaard *et al.,* 2003; Iverson *et al.,* 2004; Taipale *et al.,* 2014). Fatty acid tracers are newly being identified and need further assessment. In addition, bioaccumulative contaminants, such as methylmercury, which biomagnify a certain amount with each trophic interaction, can also provide more clarity to trophic interactions.

This dissertation introduces a conceptual framework for tracing trophic interactions and providing more clearly defined food web reconstructions, the Tracer Trichotomy (Figure 1). This framework involves the use of some or all of three separate trophic tracing methods to improve trophic interaction estimates: stable isotopes, polyunsaturated fatty acid biomarkers, and bioaccumulative contaminants. If combined, the use of stable isotopes, PUFA tracers, and bioaccumulative contaminants provide a more clearly defined food web and provide insight into

the pathways in which bioaccumulative contaminants can be transported and by what amount. Accurately assessing these values and pathways is essential in estimating risk to higher level predators in a food web that may become exposed to greatly biomagnified and potentially harmful concentrations of contaminants, and through this multi-biomarker approach, we aim to provide a more accurate determination of risk.

This dissertation describes four separate projects, each assessing environmental or individual life cycle variables that could impact the values of food web tracers. In the first chapter, the impact of seasonality on size, carbon and nitrogen stable isotopes, polyunsaturated fatty acid tracers, and mercury (total and methyl) is determined in a spider used as a sentinel of bioavailable contamination and aquatic to terrestrial transport is investigated. The second chapter determines the impact of metamorphosis in the field-caught mayflies on values of carbon and nitrogen stable isotopes, PUFAs, and mercury (total and methyl). In the third chapter, the impact of metamorphosis on laboratory reared mayfly species on values of carbon and nitrogen stable isotopes and polyunsaturated fatty acid tracers is investigated. In the fourth chapter, the impacts of metamorphosis, as well as *in vitro* culturing techniques on carbon and nitrogen stable isotopes, polyunsaturated fatty acid tracers, and glycogen levels, are determined in freshwater mussels. Through further assessing the variation with the use of each of these tracers, their use can be better informed and improved.

OBJECTIVES

The objectives of this dissertation are:

1) To determine changes in size, carbon and nitrogen stable isotopes, PUFA biomarkers, and mercury (total and methyl) in tetragnathid spiders, an indicator of aquatic to terrestrial subsidies, over multiple seasons in a year.
2) To determine changes in carbon and nitrogen stable isotopes, PUFA biomarkers, and mercury (total and methyl) in larval vs emergent mayflies in the East Fork Stones River
3) To determine changes in carbon and nitrogen stable isotopes and PUFA biomarkers throughout the life cycle of laboratory mayflies.
4) To determine the differences in carbon and nitrogen stable isotopes, PUFAs, and glycogen levels between freshwater mussels cultured traditionally vs. *in vitro*.

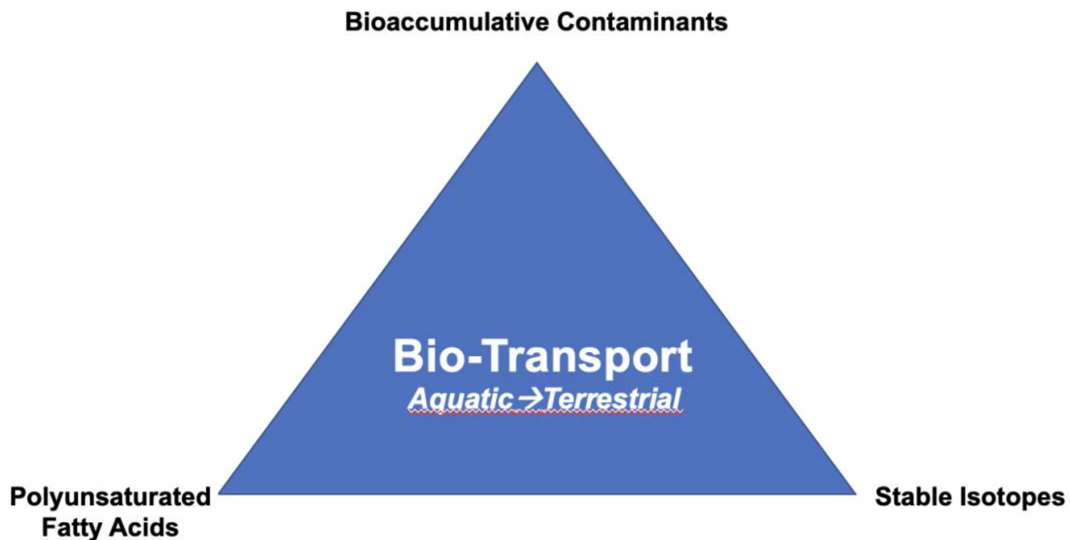

Figure 1. Visual representation of the Tracer Trichotomy framework.

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CHAPTER I: IMPACTS OF SEASONALITY ON MERCURY CONCENTRATIONS, POLYUNSATURATED FATTY ACIDS, AND STABLE ISOTOPES: IMPLICATIONS FOR THE USE OF TETRAGNATHID SPIDERS AS SENTINELS

Introduction

Riparian spiders within the family *Tetragnathidae* are increasingly used as sentinels of bioavailable contamination in aquatic ecosystems (Chumchal *et al*., 2022). Tetragnathid spiders have had success as sentinels, in part because they have a specialized diet consisting primarily of emergent aquatic insects, about 79% on average, as estimated in a study done by Akamatsu *et al*. (2004) (Akamatsu *et al*., 2004; Gillespie 1987; Collier *et al*. 2002). These aquatic insects, which spend most of their lives underwater, complete metamorphosis and transform into terrestrial adults and contribute nutrients, energy, and biomagnify contaminants originating from aquatic primary producers to these spiders (Baxter *et al*. 2005; Walters *et al*. 2008; Schulz *et al*. 2015; Kraus 2019; Bundschuh *et al*. 2022; Otter *et al*., 2024). Based on their unique position as a part of both the terrestrial and aquatic food webs, these spiders play a critical role in understanding the flux and potential risk of aquatic contaminants to terrestrial riparian organisms (Cristol *et al*. 2008; Walters *et al*. 2008; Beaubien *et al*., 2020). These spiders, in addition to being used to determine the risk to riparian predators, are also used to monitor current aquatic conditions of water bodies; because of this, evaluation of these spider biosentinels is needed to determine if aquatic condition changes are reflected over time throughout their active period of a year.

Freshwater stream conditions vary throughout a year, with many environmental factors such as temperatures, stream flow, and foliage density changing between seasons (Corbet, 1966;

Sumner and Fisher, 1979; Nakano and Murakami, 2000). Benthic macroinvertebrate communities are known to fluctuate over time, with different species of emergent insects depositing eggs and emerging at various times throughout the year (Corbet, 1966; Pinder *et al*., 1993; Nakano and Murakami, 2000). Laws *et al*. (2016) found that emergent chironomids unequally contributed to spider biomass at different times of the year, with the greatest amounts contributed from April to July, followed by a decrease in October. The timing of aquatic insect emergence, along with other environmental factors (e.g., temperature), can impact the amount and type of insects available to spiders and, subsequently, the amount of nutrients and contaminants entering the terrestrial food web (Paetzold, Bernet, and Tockner, 2006; Burdon and Harding, 2008; Ivković *et al*., 2013; Chari *et al*., 2020; Nash *et al*., 2023).

This change in subsidy, along with natural changes in stable isotopes in aquatic systems throughout the year, may impact stable isotopes; as a more terrestrial or aquatic supplement in a riparian consumer diet occurs, carbon-13 will change as the food web base producers are changing, with aquatic food webs typically having a more negative carbon isotopic signature than terrestrial (Rounick and Winterbourne, 1986; Post, 2002). It has also been found that aquatic insects serve as a primary source of long-chain ω3 polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) to riparian consumers due to these fatty acids only being produced by aquatic primary producers and having to be acquired by diet in terrestrial consumers. This makes fatty acids an excellent tracer of aquatic dietary input and can vary by season and nutritional needs (Hixson *et al*., 2015; Twining *et al*., 2019). Given the use of stable isotopes and polyunsaturated fatty acids (PUFAs) as food web tracers, it is imperative to understand their variability over time in sentinel species.

Despite the diverse application of riparian spiders as sentinels in environmental studies, most studies utilizing tetragnathid spiders have only used collections within a single season or compositing spiders from multiple sampling events (Collier *et al*., 2002; Walters *et al*., 2008; Otter *et al*., 2013; Gann *et al*. 2015; Beaubien *et al*., 2020; Hannappel *et al*., 2021; Otter *et al*., 2024). Proposed life histories of tetragnathid spiders suggest that differently aged cohorts of spiders may make up a population present at a site depending on the time of active period as it is believed that these spiders mature and reproduce in spring and summer, with older spiders dying off and young spiders growing until fall, after which they overwinter as immature juveniles (Schaefer, 1977; LeSar and Unzicker, 1978). The impact of seasonality on the use of tetragnathids as sentinels for aquatic bioavailable contaminants has yet to be investigated. Chumchal *et al*. (2022) emphasized this gap in knowledge in a recent literature review, finding that no literature existed at the time of publication that investigated the impact of seasonality on contaminant concentrations in tetragnathid spider sentinels, aside from Laws *et al*. (2016) which investigated organotin levels over a year. Laws *et al*. (2016) found that tetragnathid spiders reflected chironomid organotin levels at a contaminated site and that the contribution of chironomids to spider biomass changed with season. Since this literature review, only one other study done by Bollinger *et al*. (2023) has investigated the impact of season on stable isotopes of riparian spiders, including those in *Tetragnathidae*. Bollinger *et al*. (2023) found that the hunting mode of riparian spiders and season impacted energy flow between aquatic and terrestrial ecosystems, with the highest consumption of aquatic insects being by web-building spiders (in the family *Tetragnathidae*) and occurring in June - August.

In the present study, we collected tetragnathid spiders at a single site on the East Fork Stones River in Tennessee, USA, across the entire spider active period (April – October) to

determine the impact of seasonality on 1) size and body condition; 2) stable isotopes of carbon and nitrogen; 3) PUFAs; and 4) mercury (total and methyl), arsenic, cadmium, selenium, and strontium concentrations.

Methods

Study Area

Spider and macroinvertebrate samples were collected from a 100m reach within the East Fork Stones River in Cannon County, Tennessee (35.823485, -86.091313) between April 22nd and October 31st, 2021. This section of the Stones River is surrounded mainly by agricultural land and has a narrow (~5-7m) vegetated riparian zone.

Sample Collection and Processing

Spiders were collected by hand from low-hanging vegetation approximately fortnightly, with sampling occurring during daylight hours on both banks of the 100m reach (n=15 per sampling event). The largest spiders available were collected during each sampling event to obtain the most biomass for chemical analysis; mass varied considerably and ranged from 0.0024g to 0.1273g per spider during the sampling period. All spiders collected were presumed to be adult females by visual inspection of the pedipalps, with the presence of larger tarsi indicating males (Beaubien *et al*., 2019). After capture, spiders were each put into individual 50mL conical polypropylene tubes for transport to the laboratory. Upon arrival at the laboratory, each live spider had its mass recorded using an analytical balance, frozen, and stored at -20°C in its original tube. Frozen spiders were later (~48 hours) placed under a dissecting microscope to remove the front left leg (L1) by cutting the leg between the femur and the trochanter. Digital calipers were used to record the length of L1 and the femur, as well as spider body measurements

of the prosoma (distance from the clypeus to the pedicel), opisthosoma (distance between the most anterior and posterior points of the opisthosoma), and the total body length (distance between the clypeus and the most anterior end of the opisthosoma) (Beaubien *et al*., 2019). To control for body size when comparing the body mass of spiders, we used a common body condition calculation, the ratio index (Jakob *et al*., 1996). The ratio index was calculated by dividing an individual spider's mass by its total body length using the following equation: mass (g) / length (mm), as described in Jakob *et al*. (1996). The L1 leg and the remaining spider body were then placed into separate, prelabeled aluminum foil packets and stored at -20°C for later analysis.

Stable Isotope Analysis

The front left leg (L1) from ten randomly selected individual spiders at each sampling event was analyzed for carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotopes. Each L1 was dried and homogenized in glass tubes. Individual samples were then combusted to CO^2 and N^2 and analyzed using an NC 2500 elemental analyzer with a Delta Plus isotope ratio mass spectrometer. Vienna Peedee Belemnite and air reference material standards were used for carbon and nitrogen stable isotopes, respectively. A calibration curve was constructed that covered all sample peak sizes, and precision was determined to be lower than 0.1‰ for carbon and nitrogen for all samples. Results were reported in δ-notation in parts per thousand.

Lipid and Fatty Acids Analysis

Five spiders (whole body minus L1) were randomly selected from each sampling event for analysis of various ω3 and ω6 polyunsaturated fatty acids and total lipid amount. The fatty acids identified were 16:0, 20:5ω3,18:3ω3, 18:4ω3 20:4ω3, 20:5ω3, 16:2ω6, 18:2ω6, 18:3ω6,

20:2ω6, 20:3ω6, and 20:4ω6. Briefly, each spider body was flash-frozen in liquid nitrogen, placed into 50mL glass test tubes, and homogenized using a glass rod. 5mL chloroform, 5mL phosphate buffer, and 10mL methanol were added and stored at room temperature for 3-5 hours. 5mL chloroform and 4mL deionized water were added afterward and left overnight for mixture separation. The following day, the lower organic layer was separated into a clean test tube using Pasteur pipettes, and then the liquid was evaporated using nitrogen gas. The weight of the material in the tube after evaporation was defined as the total lipid amount (g). 1mL of toluene and 1mL of boron trifluoride methanol was added and individual test tubes were placed on a heating block at 80°C for 1 hour for transesterification of the fatty acids. Afterward, 1mL of deionized water was added to each tube to stop the reaction. A 1:1 mixture of methyl *tert*-butyl ether (MTBE) and hexane was added to separate the mixture, and the upper MTBE layer was then removed and placed into a clean glass tube. This layer was evaporated using nitrogen gas and transferred to a clean 2mL amber vial using methylene chloride. Samples were evaporated, brought to a final volume of 100µl with methylene chloride, and stored at -20°C for later analysis.

From this extract, 1µl was injected into a Thermo TSQ Quantum GC/MS equipped with a Restek Rxi-5Sil MS column (30 m × 0.25 µm film thickness). The injector was set at 200°C, and the oven's initial temperature was at 50°C, then increased to 300°C with various increments. Peaks were visualized using Thermo Xcalibur ver. 2.1. Individual compounds were compared with external standards (16:0: Supelco 37 Component FAME Mix (Supelco), 18:3ω3: α-Linolenic Acid MaxSpec Standard (Cayman Chemical), 18:4ω3: Stearidonic Acid Standard (Cayman Chemical), 20:4ω3: 15(S)-Hydroperoxy-(5Z,8Z,11Z,13E)-eicosatetraenoic acid (Millipore Sigma), 20:5ω3: Eicosapentanoic Acid MaxSpec Standard (Cayman Chemical),

16:2ω6: cis,cis-9,12-Hexadecadienoic acid (Millipore Sigma), 18:2ω6: Linoleic Acid (Cayman Chemical), 18:3ω6: γ-Linolenic Acid (Cayman Chemical), 20:2ω6: Supelco 37 Component FAME Mix (Supelco), 20:3ω6: Dihomo-γ-Linolenic Acid (Cayman Chemical), and 20:4ω6: Arachidonic Acid MaxSpec Standard (Cayman Chemical)) to determine the amount of each fatty acid relative to all fatty acids in the sample.

Mercury and Metals Analysis

Spiders were analyzed for mercury (Hg), methyl mercury (MeHg), and other trace metals (arsenic, cadmium, selenium, and strontium) at the Center for Environmental Systems Engineering at Syracuse University. For MeHg concentrations, individual spiders were acid digested using metal grade 2M nitric acid (HNO_3) in a 60°C water bath. Extractions were analyzed by aqueous ethylation with sodium tetraethylborate, purge and trap, and gas chromatographic separation using an automated MeHg analyzer (Tekran 2700). Instrument calibration consisted of a 6-point calibration curve (Alfa Aesar; R^2>0.995) and an initial calibration verification (ICV; Brooks and Rand) with recoveries deviation no greater than 10%. Continuing calibration verifications (CCVs) and continuing calibration blanks (CCBs) were run every 10 samples. Additional quality control involved the inclusion of quality control samples (QCS) and laboratory control samples (LCS) every 20 samples, consisting of DORM-3 and Mussel 2976. Average recoveries of CCVs were within 15% of their true value. Average QCS and LCS recoveries were within 25% of their true value.

Total mercury (THg) in spiders was analyzed by thermal decomposition, catalytic reduction, amalgamation, desorption, and atomic absorption spectroscopy following the United States Environmental Protection Agency (USEPA) Method 7473 (USEPA 2007) using a Milestone Direct Mercury Analyzer (DMA-80). The calibration utilized method blanks and 6-

point calibration (Mussel 2976, DORM-3, SRM-1946). All CCVs and CCBs were run in duplicate after 10 samples and at the closing of the analytical run. In addition to the CCVs, a QCS sample was run every 20 samples. Average recoveries of CCVs were within 10% of their true values; Average recoveries of QCSs were within 10% of their true values.

For the other trace metals (As, Cd, Se, Sr), an aliquot of the acid-digested samples was diluted in ultrapure water (50x dilution) and analyzed using inductively coupled plasma mass spectrometry according to EPA method 6020b (USEPA, 2014). The calibration consisted of a 6-point calibration curve (Alfa Aesar; R^2> 0.995), an ICV (Brooks Rand), and CCVs/CCBs run every 10 samples. Average recoveries of ICVs and CCVs were within 15% of their true values. Two samples of each digested SRM were used for methyl mercury analyses and were analyzed alongside collected samples, with average recoveries within 25% of their true values.

Data Analysis

To determine differences over time in spider body and condition measurements, carbon and nitrogen stable isotopes, ω3 and ω6 polyunsaturated fatty acids, total lipids, and metals data, one-way analyses of variance (ANOVA) were performed to compare means of individual sampling dates with each other. Post hoc analysis (Tukey's) was done when necessary to determine differences between groups. Locally estimated scatterplot smoothing (LOESS) modeling was performed to determine the relationship between mercury concentrations and the spider active period. Breakpoint analysis was performed on mass and total length measurements for visualization purposes only using the Python library package pwlf, which fits piecewise linear functions to one-dimensional data (Jekel and Venter 2019). Data analysis was completed using JMP software and Jupyter notebooks. Significance was defined as α=0.05.

Results

Spider Body Size and Condition

Spider body measurements and body condition increased through day 163 and decreased through the end of the spider active period (Figure. 1, Table 1). Mass and total body length were significantly different with sampling event (ANOVA: F (13,212) =7.0264, p<0.0001, F (13,212) =7.6611, p<0.0001) (Figure. 1). The ratio index for spider body condition also significantly differed over time (ANOVA: F (13,212) =7.0526, p<0.0001) (Figure. 2) with values increasing throughout the year until 163 days into the spider active period, after which there was a decrease.

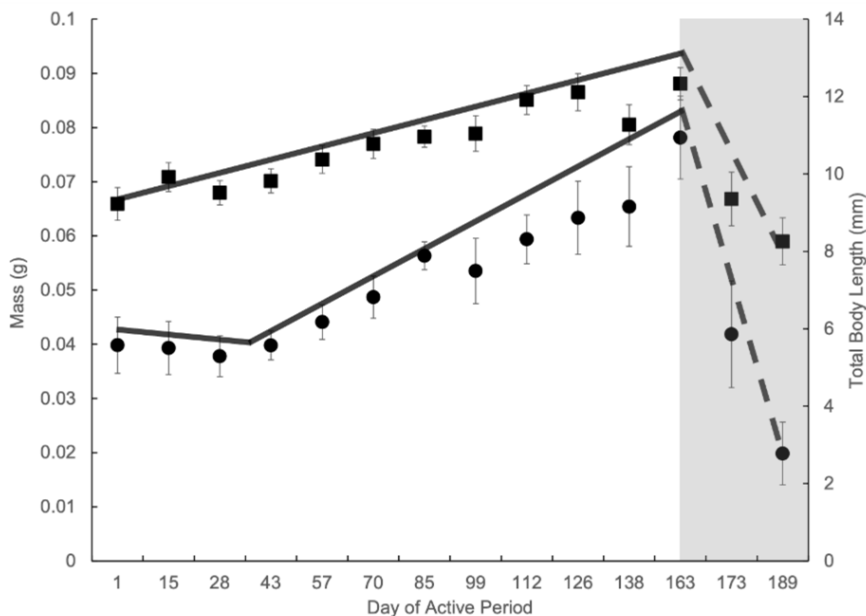

Figure 1. Mean (SE) spider mass and total body length over time with a breakpoint analysis overlayed to show change in correlation with time. Circles represent total length (opisthosoma and prosoma) (mm), and squares represent mass (g). Sampling events starting at day 173 are shaded and connected with a dashed grey line to represent their impact on measurement correlation with time.

Day of Active Period	n	Opisthosoma Length (mm)	Prosoma Length (mm)	L1 Length (mm)	Femur Length (mm)	Total Length (mm)	Mass (g)
0	20	6.88±1.79	2.71±0.49	26.87±4.82	8.39±1.56	9.23±1.95	0.04±0.02
15	15	7.46±1.20	2.68±0.32	32.20±4.16	9.72±1.17	9.92±1.44	0.04±0.02
28	14	6.89±1.07	2.60±0.35	30.25±5.30	9.19±1.59	9.51±1.21	0.04±0.01
43	15	7.26±1.12	2.70±0.29	35.05±3.62	10.37±1.25	9.82±1.17	0.04±0.01
57	15	7.84±1.25	2.84±0.34	37.06±3.81	10.86±1.21	10.37±1.39	0.04±0.01
70	15	8.12±1.21	2.91±0.43	37.52±4.17	11.24±0.99	10.78±1.46	0.05±0.02
85	15	8.05±1.03	3.12±0.35	42.24±3.59	12.20±0.79	10.96±1.07	0.06±0.01
99	15	8.46±1.90	3.03±0.38	40.82±4.90	11.93±1.47	11.05±1.79	0.05±0.02
112	15	9.30±1.28	3.14±0.34	41.83±5.39	12.39±1.32	11.91±1.44	0.06±0.02
126	15	9.54±1.69	3.11±0.34	41.50±5.11	12.53±0.85	12.11±1.87	0.06±0.03
138	15	8.89±1.90	3.00±0.52	41.85±5.71	12.29±1.55	11.27±2.00	0.07±0.03
163	15	10.07±1.47	3.02±0.39	40.25±6.03	11.61±1.71	12.33±1.62	0.08±0.03
173	12	6.67±2.19	2.40±0.46	29.64±11.05	8.92±2.92	9.36±2.40	0.04±0.03
189	15	5.90±1.96	2.31±0.52	25.04±10.41	7.46±2.87	8.26±2.36	0.02±0.02

Table 1. Table of average spider size parameters measured for each individual sampled at different days of the spider active period.

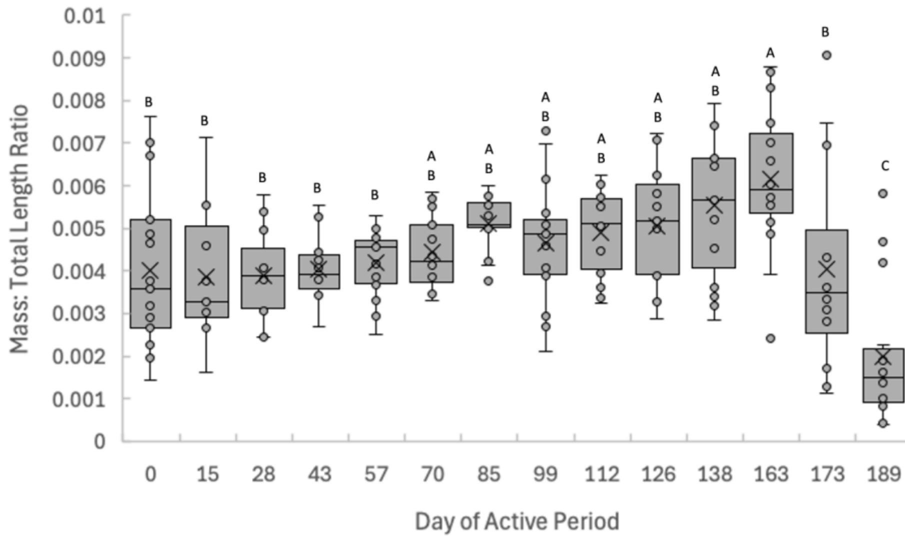

Figure 2. Ratio index of spiders (mass: length) over time. Boxes represent interquartile ranges with the middle line representing the median and "x" representing the mean. Individual data points are represented by circles. Vertical lines extend to minimum and maximums of the data, with outliers shown. Significant differences between days of active period are indicated by different upper-case letters.

Stable Isotopes

Three distinct groups of spiders, based on differences in carbon and nitrogen stable isotopes and day of active period (ANOVA: $\delta^{13}C$: $F(2,126) = 42.026$, $p = < 0.0001$; $\delta^{15}N$: $F(2,126) = 39.247$, $p = < 0.0001$) (Figure. 3). An early, middle, and late active period group emerged with spiders collected between days 1-70, days 85-138, and 173-189, respectively. Days 43 and 163 were not included as they were visual outliers. A more detailed analysis of stable isotope values is included in Figure 4.

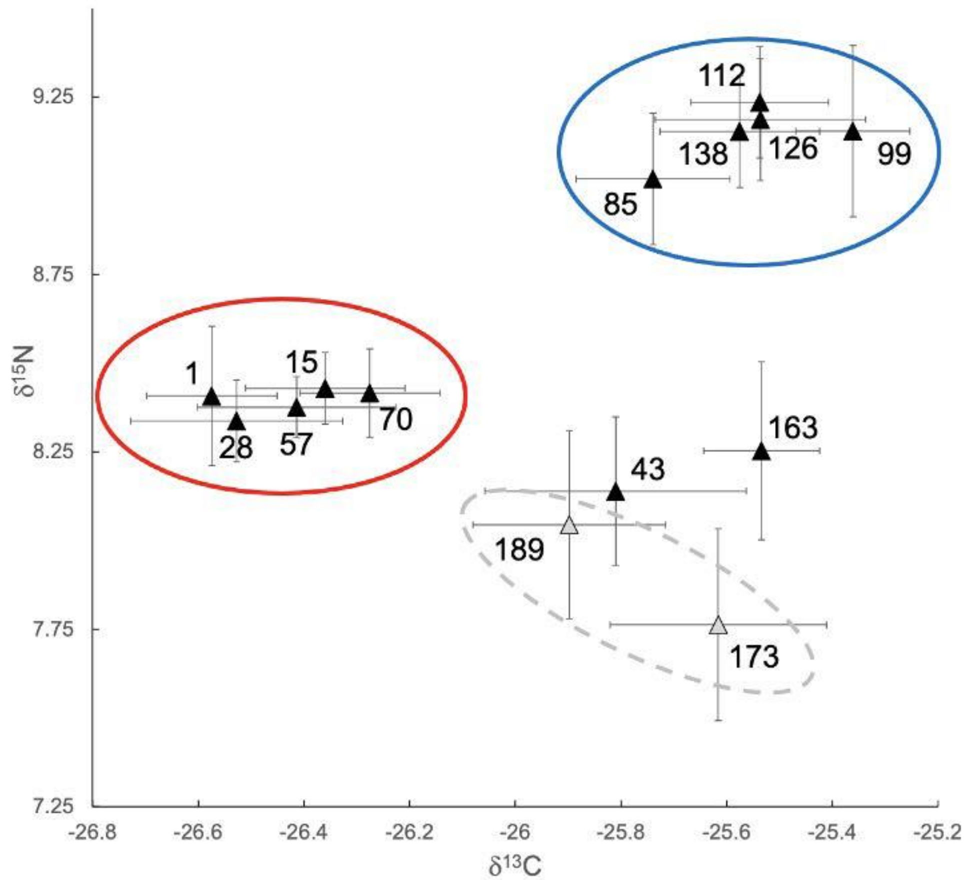

Figure 3. Carbon and nitrogen stable isotope values for average spider values at each sampling event, labeled with the corresponding day of the spider's active period. Different colored circles separate distinct groups with similar isotopic signatures, with red representing earlier days of the active period (1-70), blue representing later days of the active period (85-138), and grey dashes representing events 13 and 14 at the end of the spider's active period (days 173-189).

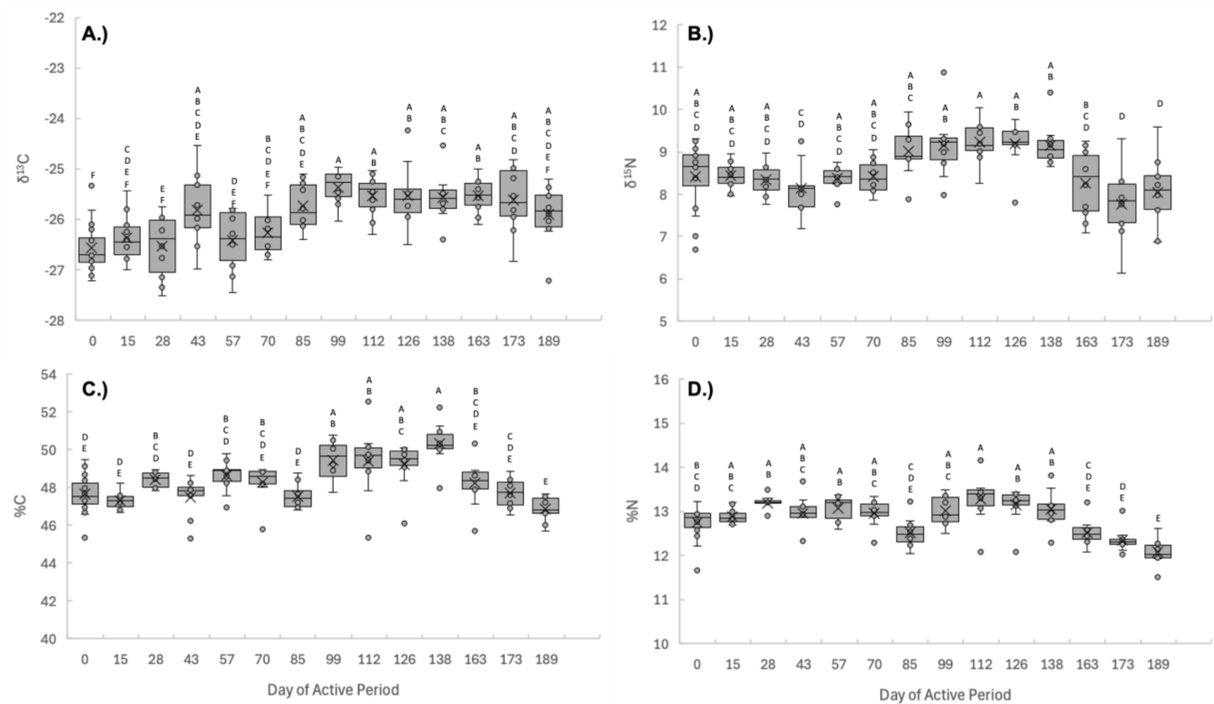

Figure 4. Spider A.) carbon stable isotopes as δ^{13}C, B.) nitrogen stable isotopes as $^{\delta 15}$N, C.) %C, and D.) %N over time. Boxes represent interquartile ranges, with the middle line representing the median and "x" representing the mean. Circles represent individual data points. Vertical lines extend to the minimum and maximum of the data, with outliers shown. Significant differences between days of active period are indicated by different upper-case letters.

Fatty Acid Analysis

The average proportions of ω3 to ω6 polyunsaturated fatty acids and amount of eicosapentaenoic acid (EPA) of total fatty acids were not significantly different throughout the spider active period (ANOVA: ω3: $F_{(13,71)}=1.51$, $p=0.139$; ω6: $F_{(13,71)}=1.27$, $p=0.252$) (Figure. 5A, 5B).

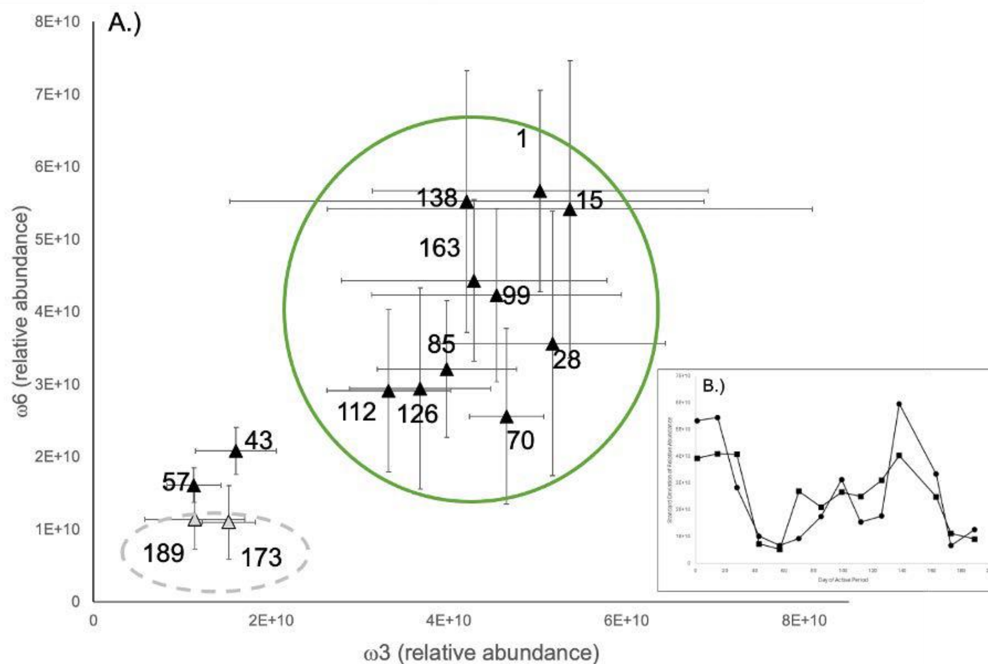

Figure 5. A.) Average values of the ratio of ω3 and ω6 polyunsaturated fatty acids (calculated area under chromatogram curves as relative abundance) plotted for each sampling event, labeled with the corresponding day of the spider active period. A green circle represents similar values over most of the spider active period. Sampling events at the end of the spider active period (days 173-189) are distinguished by a dashed grey circle. B.) Standard deviation values for average ω3 and ω6 polyunsaturated fatty acid ratios (calculated area under chromatogram curves as relative abundance) plotted for each sampling event by day of spider active period.

Mercury and Metals

LOESS modeling showed that average methyl mercury and total mercury were significantly positively correlated with the day of the spider active period from days 0 – 189 (methyl mercury: $R^2(14) = 0.3362$, $p<0.0001$; total mercury: $R^2(14) = 0.2201$, $p=0.0005$) (Figure.

6A, Figure. 6B); however, the % methyl of total mercury relationship was not significantly correlated for days 0 – 189 ($R^2(14)$ =0.4122, p=0.1049) (Figure. 6C). When the end of the spider active period (days 173-189) was excluded the methyl mercury, total mercury, and the methyl:total mercury ratio was all significantly positively correlated with the spider active period (methyl mercury: $R^2(12)$ =0. 5234, p=<0.0001; total mercury: $R^2(12)$ =0. 2202, p=0.0018; methyl:total ratio: R^2 =0.5870, p=0.0235) (Figure. 6A, 6B, 6C).

Concentrations of selenium and arsenic varied significantly over time (ANOVA: F (11,56) = 3.2124, p=0.0027, F (11,57) = 4.4820, p=0.0001), while cadmium and strontium concentrations did not (ANOVA: F (10,29) = 0.4005, p=0.9300, F (11,56) = 2.6367, p=0.0108) For strontium, which showed significance, a post hoc test showed no differences when comparing means (Figure 7).

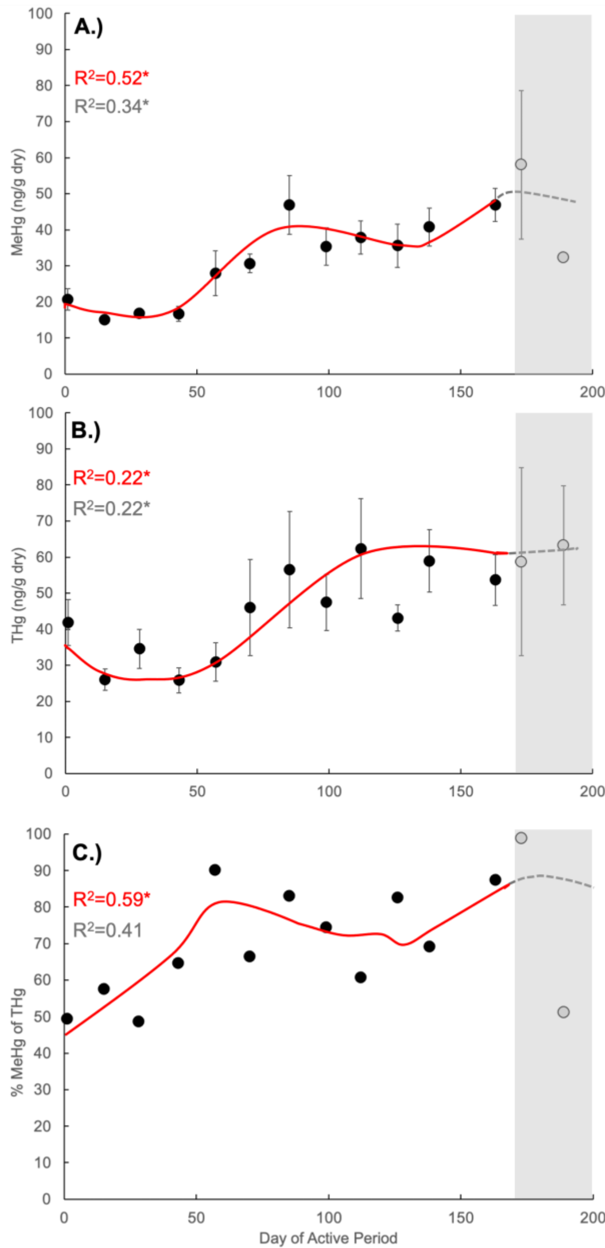

Figure 6. Concentrations of A.) methyl mercury, B.) total mercury, and C.) % methyl mercury of total mercury in spider bodies over time. LOESS modeling for the entire spider active period is represented by a line going through data points. Red portions of the line represent active period days 1 – 163 and the dashed grey portion within the grey shaded area represents sampling done after day 173 of the spider active period. R^2 values for each are displayed in corresponding red and grey. Statistical significance is represented by an asterisk (*).

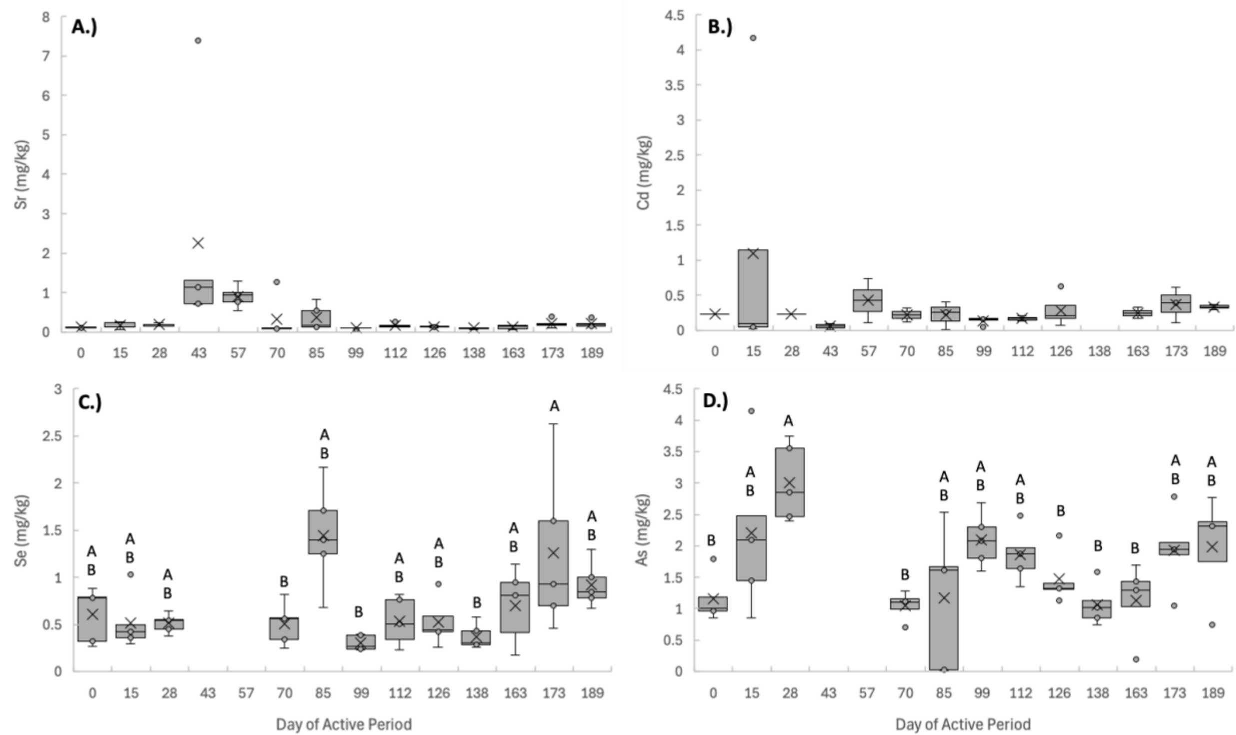

Figure 7. Spider A.) strontium, B.) cadmium, C.) selenium, and D.) arsenic concentrations in spiders over time in mg/kg. Boxes represent interquartile ranges, with the middle line representing the median and "x" representing the mean. Circles represent individual data points. Vertical lines extend to the minimum and maximum of the data, with outliers shown. Significant differences between days of active period are indicated by different upper-case letters.

Discussion

Aquatic ecosystems across nearly all biomes undergo seasonal changes. In temperate river systems, benthic macroinvertebrate and emergent aquatic insect communities change over time, and therefore, the prey availability of riparian predators changes as well (Paetzold, Bernet, and Tockner, 2006; Burdon and Harding, 2008; Chari *et al*., 2020). Fluctuations in insect emergence events play a prominent role in food web dynamics, impacting the proportion of diet coming from aquatic sources and potentially the amount of energy and contamination exposure

in riparian predators (Laws *et al*., 2016; Ortega-Rodriguez *et al*., 2019). In addition, spider life cycles can impact the age, size, and body condition of available spiders at a site (Schaefer, 1977; LeSar and Unzicker, 1978). In this study, we investigated the impact of sampling time (seasonality) during the spider active period on the largest caught female tetragnathid spiders at a single site in an attempt to improve our understanding of riparian spiders as sentential species of bioavailable contaminants in aquatic systems and their movement from aquatic to terrestrial ecosystems (Chumchal *et al*., 2022).

Spider Body Measurements

In the present study, spider mass and total length, as well as body condition and other body measurements, experienced a decrease toward the end of the sampling period (Figure. 1, Table 1). This data is consistent with the proposed life history of spiders within the family *Tetragnathidae* described in LeSar and Unzicker (1978) and Schaefer (1977). Both studies describe tetragnathids as stenochronus, with mating occurring throughout spring and summer and resulting offspring maturing through autumn. The offspring grow rapidly until around October when growth slows due to decreased daylight. The immature juveniles overwinter and then undergo their final molts in the spring of the following year. The findings of this study suggest that the spider species selected within the family *Tetragnathidae* at this field site follow this life history pattern. The life history of these spiders can impact the age and, therefore, the size of available individuals at various times of the year.

Carbon and Nitrogen Stable Isotopes

Although there were significant differences in nitrogen stable isotopes between sampling events in the present study, notably a decrease after active period day 173, the average values for

spiders during each sampling event only ranged 1.4‰ (Figure. 3). This is similar to the findings of Laws *et al*. (2016), where observed variation between months did not exceed 3‰. Laws *et al*. (2016) found this was due to differing contributions of chironomids, an order of aquatic emergent insects, to spider diet. We hypothesize that our variation in nitrogen stable isotopes is due to variation in available dietary items to tetragnathid spiders. Carbon stable isotope averages in spiders for each sampling event were found to span over a range of 1.21‰ in the present study, with a significant decrease after day 173. In a study by Belivanov and Hamback (2015), it was found that dietary shifts of prey items of spiders were reflected in carbon stable isotopes of spider leg tissue over time, similar to findings in our study. The fractionation that occurs between trophic interactions is commonly estimated to be approximately 3.4‰ for nitrogen-15 and less than 1‰ for carbon-13 in a food web tracing study (DeNiro and Epstein, 1978; Minagawa and Wada, 1984; Post, 2002). However, it has been found that dietary quality can impact these fractionation rates between spiders and dietary items (Belivanov and Hambeck, 2015). Because the largest differences in average nitrogen stable isotopes or carbon stable isotopes did not exceed these amounts, the impact of seasonality on a typical trophic level calculation used by environmental risk assessors would likely be minimal. This finding is significant since the primary utilization of riparian spiders in risk assessments is tied to the trophic exchange of contaminants between aquatic and terrestrial environments (Otter *et al*., 2020; Chumchal *et al*., 2022).

Fatty Acids

The ratio of ω3 to ω6 polyunsaturated fatty acids is used to estimate the dietary input of aquatic vs. terrestrial subsidy into a consumer's diet. This is due to aquatic insects having fundamentally different signatures from terrestrial insects, namely the presence of long-chain

polyunsaturated fatty acids such as 20:5ω3, which can be used as tracers of aquatic input themselves (Hixson *et al*., 2015). In the present study, the proportion of ω3 to ω6 polyunsaturated fatty acid concentrations and the percent of eicosapentaenoic acid of total fatty acids did not differ during the spider active period (Figure. 5A, 5B). These results are similar to those of Chari *et al*. (2020), who observed no significant differences in ω3:ω6 with season in riparian spiders collected along the Kowie River in South Africa. However, there was a significant difference in site along the river, suggesting that this result may not hold true at all field sites depending on aquatic insect emergence rates. The present results differ from that of Kowarik *et al*. (2021), who observed the highest ω3:ω6 ratio occurring in spring and decreasing throughout the year in riparian spiders collected in northern Switzerland. The differences in results found in the present study may be due to shorter periods of time between sampling events or the restriction of only using riparian spider species within the family *Tetragnathidae*, as well as regional differences in aquatic insect availability and emergence times.

Mercury and Metals

In the present study, average methyl mercury and total mercury values were significantly correlated to the day of the spider active period, but the methyl:total mercury ratio was not significantly correlated (Figure. 6A-C). Our results suggest that seasonality does have an impact on the use of spiders as sentinels of insect-mediated contaminant flux since the time of year samples were collected significantly affects the Hg concentrations observed. Because the spider population changes over time as a function of aging and reproduction, changes in mercury could be due to aging and growth. Hannappel *et al*. (2021) found that body size in shoreline spiders, including tetragnathids, was positively correlated with mercury concentration. As spiders age and, therefore, increase in body size, their overall concentration of methyl and total mercury

could increase as a function of their growth and aging over a year (Hannappel *et al.*, 2021). Ortiz *et al*. (2015) is the only other published study to examine the effect of season on mercury spider concentrations when they found that total mercury concentrations increased in wolf spiders from March through August and then decreased in October around Monterey Bay, California (USA), which was associated with the number of days those months had foggy conditions. Foggy conditions were hypothesized to be connected to Hg deposition. The pattern observed by Ortiz *et al*. is similar to that observed in the present study; however, Ortiz *et al*. also found significant increases in total mercury, which was not observed in our spiders (Figure. 8).

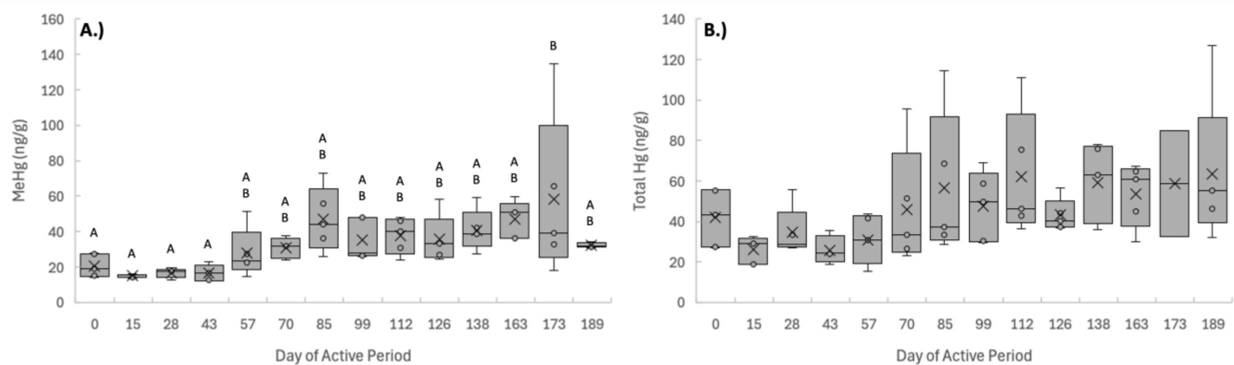

Figure 8. Spider A.) methyl mercury and B.) total mercury concentrations in spiders over time in ng/g. Boxes represent interquartile ranges, with the middle line representing the median and "x" representing the mean. Circles represent individual data points. Vertical lines extend to the minimum and maximum of the data, with outliers shown. Significant differences between days of active period are indicated by different upper-case letters.

In the present study, methyl mercury concentrations, spider size, and body condition each increased throughout the spider active period, with a decrease near the end after day 173. A

positive relationship between spider mass and methyl mercury concentrations was also found in Hannappel *et al*. (2021) within 4 of 6 shoreline spider taxa, including *Tetragnathidae*.

The mercury (methyl, total, and methyl:total) patterns observed in the present study raise the question of mercury dynamics during the non-active season (i.e., winter). Our results showing consistent concentrations of mercury in the very first sampling event compared to other time points indicate that spiders that overwintered retained, at least to some degree, the mercury they accumulated the previous year. Although the mercury dynamics of overwintering spiders would likely not impact field risk assessments using tetragnathid spiders, as sampling is done in warmer months due to spider life history and active periods, this raises questions about how mercury and other contaminant concentrations vary with overwintering in tetragnathid spiders. This question was not originally part of our study design, so a detailed analysis is outside the scope of this study. Still, our results highlight a knowledge gap that warrants further investigation.

In this study, we found that selenium and arsenic varied over time, while cadmium and strontium concentrations did not (Figure. S4). The variation of selenium concentrations in spiders peaked at the end of the active period at day 173 and was lowest at days 70 and 38-99 (Figure. S4). This is the first study to investigate the temporal variation of metals in sentinel riparian spiders, so no other studies directly compare our results. Given that tetragnathids are known to align well with aquatic food webs we would expect a similar temporal pattern as seen in other aquatic organisms, but more detailed and focused studies are needed to understand these dynamics.

Seasonality in Prey Availability

Emergent aquatic insects serve as vectors of transport for nutrients and energy as well as bioaccumulative contaminants to riparian predators in the process of insect-mediated contaminant flux (Otter *et al*., 2020). As these insects are a crucial link between aquatic and terrestrial ecosystems, it is important to understand the variation in their emergence patterns and take this into consideration when using sentinel spiders for risk assessment. When a recent large emergence event occurs, the incorporation of a concentrated amount of a single food item may not be reflected immediately in the stable isotopes, polyunsaturated fatty acids, and contaminant concentrations of spiders. Belivanov and Hamback (2015) showed that the legs and abdomen of a spider were reflective of the recent dietary items being consumed, with a turnover rate of 20 days and 8 days, respectively. With these turnover times being reflective of relatively current diet conditions, seasonal patterns in the emergence of aquatic insects that may impact diet and, therefore, polyunsaturated fatty acids or stable isotopes as food web tracing endpoints in risk assessment should be considered. This study did not collect data on seasonal emergent insect community patterns and their relationship with spider bioindicators, and this warrants further investigation for a better understanding of potential seasonally impacted endpoints in risk assessment.

Conclusion

Spider size, methyl mercury, selenium, and arsenic concentrations changed during the active period of tetragnathid spiders, particularly during the final weeks before overwintering. The functions of seasonality, such as spider life history impact on community structure and, therefore, age and size, as well as aquatic emergent insect emergence patterns, need to be considered when using riparian spiders as sentinels of bioavailable contaminants in aquatic

ecosystems or transfer of contaminants into terrestrial food webs from water sources. When taken collectively, the results of this study indicate that, at our sampling location, the most consistent data was gathered during the first 163 days (~86%) of the active period of tetragnathid spiders. Our findings suggest that the timing of spider collection is an important factor to consider when designing studies assessing the contents of spider tissue. In order to avoid any potential variation in endpoints used in this study, the collection of spiders during the first 86% of their active period is recommended to avoid any variation due to seasonality at a field site.

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CHAPTER II: INTER-YEAR VARIATIONS IN MERCURY CONCENTRATIONS, POLYUNSATURATED FATTY ACIDS, AND STABLE ISOTOPES OF TETRAGNATHID SPIDER SENTINELS

Introduction

Ecosystems are energetically connected, with resources constantly being transferred between compartments, such as a river and a riparian terrestrial environment (Polis, 1997). Aquatic to terrestrial subsidies (aquatic subsidies) are a less commonly considered type of ecological energetic connection. They occur when organisms with biphasic lifecycles, such as aquatic emergent insects, bring accumulated energy from the aquatic food web in their larval phase to predators in the terrestrial food web after metamorphosis occurs. Resources like energy and nutrients can be very beneficial between ecosystems, such as when the transfer of polyunsaturated fatty acids from aquatic to terrestrial ecosystems aids in the growth of bird nestlings (Twining *et al*., 2016). Although aquatic to terrestrial subsidies benefit many riparian consumers, there is a risk for harmful subsidies, such as bioaccumulative and biomagnifying contaminants. Contaminants such as methylmercury and PCBs that are present in bodies of water have been shown to be retained through the process of metamorphosis and can then potentially be transferred to predators, such as songbirds and bats in the area near the body of water (Baxter *et* al., 2005; Walters *et* al., 2008; Kraus *et al*., 2014; Otter *et al*., 2020). It is important to recognize this pathway of exposure since these contaminants may biomagnify within the riparian food web and expose predators to high concentrations that can cause adverse effects (Walters *et al*., 2008).

Spiders that live in riparian areas alongside lakes and rivers are used in the field of ecotoxicology for ecological risk assessments. Riparian spiders, particularly those in the family *Tetragnathidae*, have a uniquely high proportion of aquatic emergent insects in their diet (Akamatsu *et al.*, 2004; Gillespie, 1987; Collier *et al.,* 2002). It has been found that this percentage of aquatic insects in tetragnathids' diet is around 79% on average (Akamatsu *et al.* 2004). This makes tetragnathid spiders excellent sentinels of bioavailable contaminants that would be transferred out of a body of water through aquatic emergent insects (Chumchal *et al*., 2022). In terms of ecological risk assessment use, tetragnathid spiders have been used to develop spider based avian wildlife values, values that estimate the risk to birds using their dietary preferences and specific contaminants for various contaminants such as methyl mercury, metals, and PCBs (Walters *et al*., 2010; Gann *et al*., 2015; Beaubien *et al*., 2020). This makes it possible to determine the risk to riparian birds without analyzing their tissues.

Although these spiders have been used increasingly often in ecotoxicological studies throughout the past few decades, some aspects of their use have yet to be investigated. Only two studies to date have investigated seasonal variation in tetragnathid contaminant concentrations (organotin - Laws *et al*., 2016; mercury - Landaverde *et al.,* 2025), and only one study has investigated annual variation (PCBs - Otter *et al*., 2024) at the time of this publication. In addition, only two studies have investigated seasonal changes in food web tracers of carbon and nitrogen isotopes in tetragnathid spiders (Bollinger *et al.*, 2023; Landaverde *et al*., 2025), with both studies showing significant variation within a single year for both contaminants and isotopic tracers.

In this study, tetragnathid spiders were collected during June, July, and August at a single site on the East Fork Stones River in Tennessee, USA, and compared to spiders previously collected at this site over an entire active period the previous year. We aimed to determine inter-year differences in these sentinel spiders for 1) size and body condition, 2) stable isotopes of carbon and nitrogen, 3) polyunsaturated fatty acids, and 4) total mercury concentrations.

Methods

Field Site

Spider collections in both 2021 and 2022 occurred in the East Fork Stones River in Cannon County, Tennessee (35.823485, -86.091313). This section of the Stones River is surrounded by agricultural land. Spiders were collected within a 100m reach, including different river features such as riffles and pools.

Sample Collection and Processing

Spiders were collected from hanging branches and vegetation along the river's banks from standing within the river. Spiders were visually identified to be within the family *Tetragnathidae* and to be female through visual inspection of the pedipalps, with the presence of swollen tarsus indicating males (Beaubien *et al.*, 2019). Spiders were captured by hand through grasping of the front legs and transferred into 50mL conical tubes for transport and storage. Largest sized females were selected for analysis biomass availability. Conical tubes containing collected spiders were transported in a cooler with ice to Middle Tennessee State University, where their live mass was recorded. They were then euthanized by freezing in a -20°C freezer for at least 24 hours. Frozen spiders had their front left leg, L1, removed where the femur meets the trochanter.

Digital calipers were used to take measurements of the L1 length, prosoma, opisthosoma, and total body length. L1 and spider bodies were labeled and stored separately for further analysis at -20°C.

Stable Isotope Analysis

Spider's legs were analyzed for stable isotopes of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) at the University of Arkansas Stable Isotope Laboratory. Spider legs were homogenized and then analyzed using an NC 2500 elemental analyzer with a Delta Plus isotope ratio mass spectrometer. The reference materials of Vienna Peedee Belemnite and atmospheric air were used for carbon and nitrogen stable isotopes, respectively. Using a calibration curve covering all peak sizes, the precision was determined to be lower than 0.1‰ for carbon and nitrogen samples. Results were reported in δ-notation in parts per thousand.

Lipid and Fatty Acid Analysis

Spider bodies minus the L1 leg were analyzed for total lipids and select polyunsaturated fatty acids. The fatty acids identified were 16:0, 20:5ω3,18:3ω3, 18:4ω3 20:4ω3, 20:5ω3, 16:2ω6, 18:2ω6, 18:3ω6, 20:2ω6, 20:3ω6, and 20:4ω6. Each spider body was frozen using liquid nitrogen, then placed into 50mL glass test tubes and homogenized using a glass stir rod. The homogenized spiders separated their lipid content using a two-phase extraction using methanol, chloroform, and phosphate buffer. The resulting extract was separated and dried using a stream of nitrogen gas. The weight of dried material was recorded as the lipid content by weight. 1mL toluene and 1mL of boron trifluoride methanol were added to the tubes with the lipid material. The tubes were placed on a heating block at 80°C for 1 hour for transesterification of the fatty acids. Afterward, 1mL of deionized water was added to each tube to stop the reaction. A 1:1

mixture of methyl *tert*-butyl ether (MTBE) and hexane was added to separate the mixture, and the upper MTBE layer was then removed and placed into a clean glass tube, which was then dried using a stream of nitrogen gas. Tube contents were transferred into clean 2mL amber GCMS vials using methylene chloride. Samples were evaporated using nitrogen gas and then brought to a final volume of 100µl with methylene chloride. Samples were stored at -20°C for later analysis.

Analysis was done using a Thermo TSQ Quantum GC/MS equipped with a Restek Rxi-5Sil MS column (30 m × 0.25 µm film thickness). 1µl of extract from each sample was injected directly into the injector port of the instrument. Peaks were visualized using the program Thermo Xcalibur ver. 2.1. Individual compounds were compared with external standards (16:0: Supelco 37 Component FAME Mix (Supelco), 18:3ω3: α-Linolenic Acid MaxSpec Standard (Cayman Chemical), 18:4ω3: Stearidonic Acid Standard (Cayman Chemical), 20:4ω3: 15(S)-Hydroperoxy-(5Z,8Z,11Z,13E)-eicosatetraenoic acid (Millipore Sigma), 20:5ω3: Eicosapentanoic Acid MaxSpec Standard (Cayman Chemical), 16:2ω6: *cis*,*cis*-9,12-Hexadecadienoic acid (Millipore Sigma), 18:2ω6: Linoleic Acid (Cayman Chemical), 18:3ω6: γ-Linolenic Acid (Cayman Chemical), 20:2ω6: Supelco 37 Component FAME Mix (Supelco), 20:3ω6: Dihomo-γ-Linolenic Acid (Cayman Chemical), and 20:4ω6: Arachidonic Acid MaxSpec Standard (Cayman Chemical)) to determine the amount of each fatty acid relative to all fatty acids in the sample and recorded.

Mercury analysis

Spider bodies minus L1 were analyzed for total mercury (THg) at Syracuse University Center for Environmental Systems Engineering for 2021 samples and at Grand Valley State University Annis Water Center for 2022 samples. In both locations, THg in spiders was analyzed by thermal

decomposition, catalytic reduction, amalgamation, desorption, and atomic absorption spectroscopy following United States Environmental Protection Agency (USEPA) Method 7473 (USEPA 2007) using a Milestone Direct Mercury Analyzer (DMA-80)

Data Analysis

To determine differences over time between months and years in spider measurements, carbon and nitrogen stable isotopes, ω3 and ω6 polyunsaturated fatty acids, total lipids, and mercury data, one-way analyses of variance (ANOVA) were performed to compare means of each time point with each other. Post hoc analysis (Tukey's) was done when necessary to determine differences between groups. Data analysis was done using JMP software and Jupyter notebooks. Significance was defined as $\alpha=0.05$.

Results

Stable Isotopes

Spiders collected in 2022 significantly differed by month in carbon and nitrogen stable isotopes, with carbon and nitrogen stable isotopes becoming more enriched with each month from June to August ($\delta^{13}C$ ANOVA: $F_{(2,40)}=5.6130$, $p=0.0074$; $\delta^{15}N$ ANOVA: $F_{(2,40)}=28.9337$, $p<0.0001$). When comparing the values for carbon and nitrogen stable isotopes from the months of June, July, and August of 2022 to those of 2021, there were significant differences in June, with $\delta^{13}C$ and $\delta^{15}N$ being less enriched in 2022 ($\delta^{13}C$ ANOVA: $F_{(1,39)}=23.8558$, $p<0.0001$; $\delta^{15}N$ ANOVA: $F_{(1,39)}=25.5206$, $p<0.0001$). In July, $\delta^{15}N$ was less enriched in 2022, but not $\delta^{13}C$ ($\delta^{13}C$ ANOVA: $F_{(1,30)}=0.4852$, $p=0.4918$; $\delta^{15}N$ ANOVA: $F_{(1,30)}=13.3290$, $p=0.0011$). In August, $\delta^{13}C$ was less

enriched in 2022 but not $\delta^{15}N$ ($\delta^{13}C$ ANOVA: $F_{(1,40)}=4.1263$, $p=0.0493$; $\delta^{15}N$ ANOVA: $F_{(1,40)}=1.8102$, $p=0.1865$) (Figure 1A, 1B).

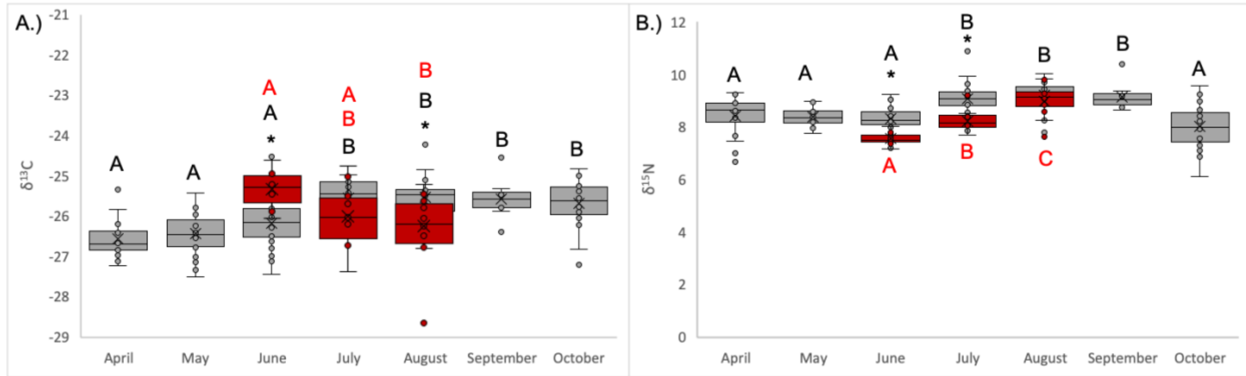

Figure 1. Spider A.) carbon stable isotopes as $\delta^{13}C$ and B.) nitrogen stable isotopes as $\delta^{15}N$ over time. Grey and black boxes and letters represent 2021 values, and red boxes and letters represent 2022 values. Significant differences between monthly values between years are represented by an asterisk (*). Significant differences between monthly values within year are represented by different upper-case letters. Boxes represent interquartile ranges, with the middle line representing the median and "x" representing the mean. Circles represent individual data points. Vertical lines extend to the minimum and maximum of the data, with outliers shown.

Fatty Acid Analysis

The ω3:ω6 ratio and the %EPA of total fatty acids (TFA) did not significantly differ between the months of June, July, and August for spiders collected in 2022 (ω3: ω6 ANOVA: $F_{(2,20)}=0.6103$, $p=0.5547$; %EPA of TFA ANOVA: $F_{(2,20)}=0.6272$, $p=0.5460$). When compared by month between 2021 and 2022 spider values, there was no difference in June for %EPA of TFA, but

there was a difference in ω3:ω6, with 2022 values being lower (ω3: ω6 ANOVA: $F_{(2,20)}$ =432.4695, $p<0.0001$; %EPA of TFA ANOVA: $F_{(2,20)}$ =0.2156, p=0.6480), in July there was a lower ω3:ω6 ratio in 2022, but not %EPA of TFA (ω3: ω6 ANOVA: $F_{(2,15)}$ =123.4298, $p<0.0001$; %EPA of TFA ANOVA: $F_{(2,15)}$ =2.9986, p=0.1070), and for August there was also a lower value for in 2022 ω3:ω6 ratios, but not % EPA of TFA (ω3: ω6 ANOVA: $F_{(2,20)}$ =70.3617, $p<0.0001$; %EPA of TFA ANOVA: $F_{(2,20)}$ =1.2328, p=0.2815) (Figure 2A, 2B).

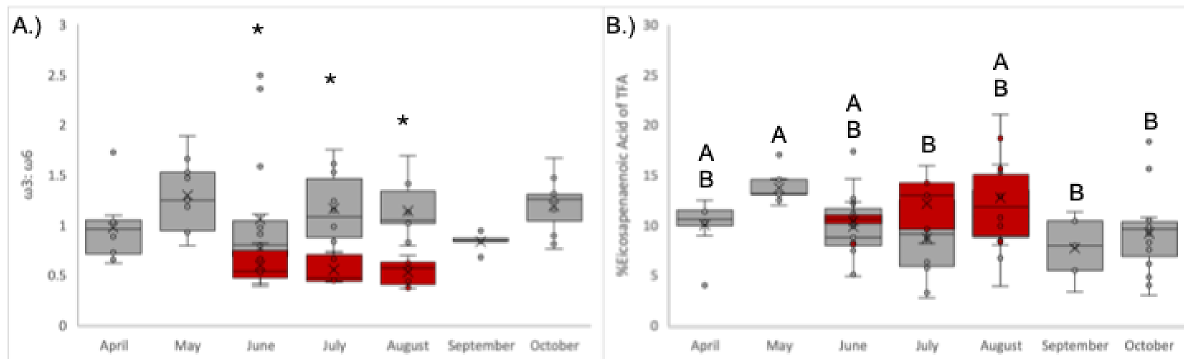

Figure 2. A.) ω3: ω6 ratio of polyunsaturated fatty and B.) eicosapentaenoic acid (EPA), 20:5ω3, of total fatty acids in spiders over time. Grey and black boxes and letters represent 2021 values, and red boxes and letters represent 2022 values. Significant differences between monthly values between years are represented by an asterisk (*). Significant differences between monthly values within year are represented by different upper-case letters. Boxes represent interquartile ranges, with the middle line representing the median and "x" representing the mean. Circles represent individual data points. Vertical lines extend to the minimum and maximum of the data, with outliers shown.

Mercury

Spiders collected in 2022 did not significantly differ in total mercury values between the months of June, July, and August (ANOVA: $F_{(2,38)}$ =0.4607, p=0.6346). When compared to 2021 total mercury values, there was a significant increase in 2022 for the months of June and August, but not July (June THg ANOVA: $F_{(1,22)}$ =15.8319, p=0.0007; July THg ANOVA: $F_{(1,20)}$ =3.4730, p=0.0788; August THg ANOVA: $F_{(1,30)}$ =6.8008, p=0.0144) (Figure 3).

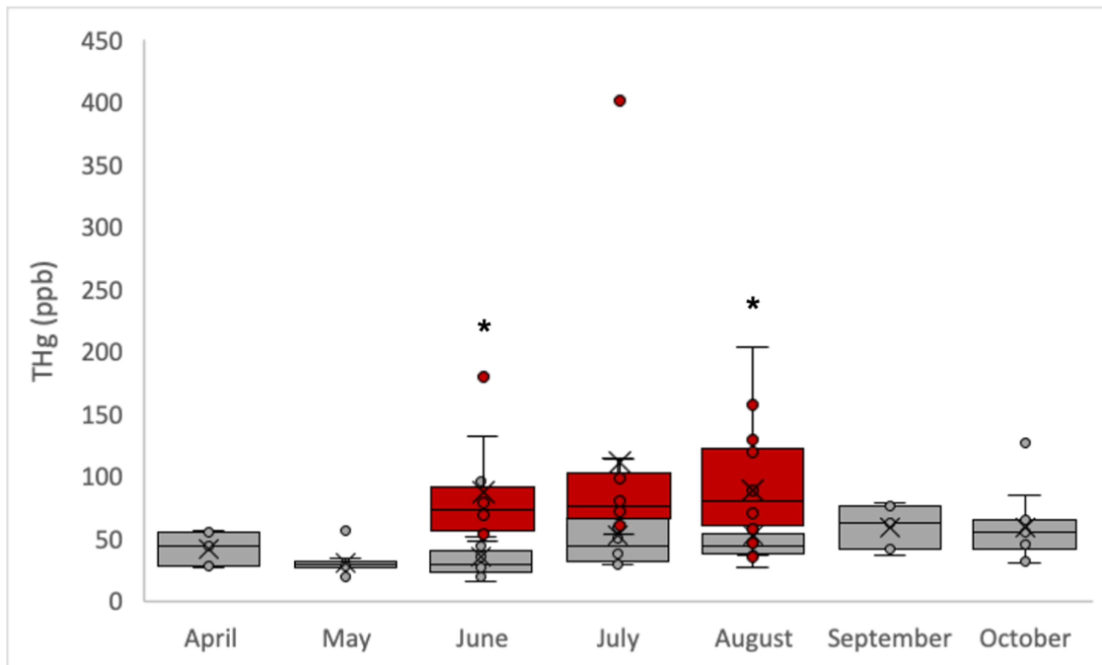

Figure 3. Spider total mercury concentrations over time. Grey and black boxes and letters represent 2021 values, and red boxes and letters represent 2022 values. Significant differences between monthly values between years are represented by an asterisk (*). Significant differences between monthly values within year are represented by different upper-case letters. Boxes represent interquartile ranges, with the middle line representing the median and "x" representing the mean. Circles represent individual data points. Vertical lines extend to the minimum and maximum of the data, with outliers shown.

Discussion

Aquatic subsidies, while beneficial to riparian organisms, can also deliver harmful contaminants (Walters *et al*., 2008; Twining *et al*., 2024). Tracing these subsidies is essential to predicting risk to riparian birds and bats that may be harmed by exposure to biomagnifying contaminants. Riparian tetragnathid spiders have been useful for ecotoxicological studies in serving as sentinels for the movement of contaminants. However, variation in contaminant concentrations and food web tracers used in these studies has rarely been studied beyond a single sampling time point (Laws *et al.*, 2016; Ortega-Rodriguez *et al.,* 2019; Chumchal *et al*., 2022; Otter *et al*., 2024; Landaverde *et al*., 2025). In this study, we investigated the inter-year variation in carbon and nitrogen stable isotopes, polyunsaturated fatty acids, and total mercury. This will help improve the understanding of the use of tetragnathid spiders as sentinel species in the movement of subsidies from aquatic to terrestrial ecosystems.

Carbon and Nitrogen Stable Isotopes

Previous research has shown that carbon and nitrogen stable isotopes significantly vary over a spider's active period throughout a year (Landaverde *et al*., 2025). In the present study, spiders became significantly enriched in $\delta^{13}C$ and $\delta^{15}N$ throughout the study period (Figure 1A, 1B). Although there were significant differences, the variation between months' mean values in $\delta^{13}C$ and $\delta^{15}N$ did not exceed the ranges reported in the literature. The fractionation that occurs between trophic interactions is estimated to be approximately 3.4‰ for nitrogen-15 and less than 1‰ for carbon-13 in a food web tracing study (DeNiro and Epstein, 1978; Minagawa and Wada, 1984; Post, 2002). This is similar to the findings of Laws *et al.* (2016), where the observed $\delta^{15}N$ variation of spiders between months did not exceed 3‰ and was also within common ranges. Because the main use of carbon and nitrogen stable isotopes in riparian spiders is to estimate

trophic linkages and the annual differences in values do not exceed the estimated variation between trophic levels, it is not expected that the variation found in this study would have a great impact to ecological risk assessment work where food webs are traced with stable isotopes.

Fatty Acids

Previous findings by Landaverde *et al.* (2025) found that within a single year, there was no significant variation in ω3:ω6 ratios and the % eicosapentaenoic acid of total fatty acids over the active period of tetragnathid spiders. In the present study, spider ω3:ω6 ratios and the % eicosapentaenoic acid of total fatty acids (TFA) did not significantly differ between the months of June, July, and August in 2022. This is indicative of annual differences in the amount of ω3 polyunsaturated fatty acids (PUFAs) compared to that of ω6, which can be caused by varying amounts of terrestrial subsidy to an aquatic ecosystem, as increased ω6 PUFAs are associated with terrestrial food webs/primary producers (Hixson, 2015). The findings of this study also suggest that eicosapentaenoic acid may be a sufficient food web tracer to be used with riparian spiders in aquatic subsidy research since it has not been found to vary in tetragnathid spiders, as previously suggested in a study done by Chari *et al*. (2020).

Mercury

A previous study by Landaverde *et al.* (2025) found no differences in total mercury concentrations during the active season of tetragnathid spiders. However, the results of Landaverde *et al*. (2025) differed from those previously found by Ortiz *et al.* (2015), where total mercury concentrations significantly increased in wolf spiders from March through August and then decreased in October around Monterey Bay, California (USA), which was associated with the number of days those months had foggy conditions. These previous findings suggest that

there may be differences in species or environmental conditions contributing to mercury concentrations over a year in spiders. In the present study, tetragnathid spiders collected did not significantly differ in total mercury values between the months of June, July, and August (Figure 3). These results are similar to those observed by found in Landaverde *et al.* (2025), which utilized the same sampling locations as the present study.

When the present results are compared to 2021 total mercury values (Landaverde *et al*., 2025), there were significant increases between years for the months of June and August, but not July. Ultimately, these results show that total mercury concentrations at the same location can vary significantly year-to-year. This has implications for ecological risk assessments, where data collected from a single year may not be applicable for the following years. A study done by Otter *et al*. (2024) investigated PCB concentrations in tetragnathid spiders at a remediation site across multiple years and found that spider concentrations differed between years but were significantly positively correlated with PCB concentrations in macroinvertebrates and water samples. The present study is similar. It found differences in contaminant concentrations across years. However, further investigation is needed to determine whether the differing concentrations correlate with mercury concentrations in macroinvertebrates or environmental samples.

Conclusion

Carbon and nitrogen stable isotopes, ω3:ω6 ratios, and mercury concentrations differed significantly in tetragnathid spiders between consecutive years at the same site location, implying that various factors (ecological, toxicological, etc.) cause enough variation annually that the data of one year cannot be applied to following years.

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CHAPTER III: EFFECTS OF MAYFLY METAMORPHOSIS ON FOOD WEB TRACERS: STABLE ISOTOPES, BODY COMPOSITION, AND FATTY ACIDS

Introduction

Metamorphosis creates a pathway for the transfer of energy between aquatic and terrestrial food webs through predator-prey dynamics (Baxter, 2005; Polis *et al.,* 1997). The subsidy from aquatic to terrestrial food webs, or aquatic subsidy, serves as an important source of essential nutrients to riparian organisms. Some riparian consumers depend on aquatic subsidies to survive and gain the proper nutrition for development, with up to 100% of their energy or carbon coming from aquatic subsidies (Baxter *et al*., 2005; Twining *et al.,* 2019; Twining *et al.,* 2021). Although beneficial to terrestrial food webs, aquatic subsidies can also transfer bioaccumulative and biomagnifying contaminants to consumers (Baxter, 2005; Walters *et al.,* 2008). The transfer of contaminants that biomagnify (e.g., methylmercury, polychlorinated biphenyls) poses a risk to high trophic level predators as they can reach potentially harmful concentrations as they increase through the lower trophic levels of the food web (Maul *et al*., 2006; Walters *et al*., 2008; Beaubien *et al*., 2020; Bundschuh *et al*., 2022).

Tracing food web interactions and predicting the movement of contaminants from aquatic to terrestrial ecosystems have typically employed the use of stable isotopes (e.g., carbon, nitrogen) and/or polyunsaturated fatty acids (Akamatsu *et al*., 2004; Raikow *et al*., 2011; Twining *et al*., 2016; Chari *et al*., 2020). To determine trophic connections stable isotopes of carbon and nitrogen are used to estimate the primary producers in a food web and an organism's trophic level, respectively (Post, 2002). Omega-3 polyunsaturated fatty acids (PUFAs), such as

eicosapentaenoic acid, have been used to estimate the amount of aquatic influence in an organism's diet (Hixson *et al*., 2015) because aquatic primary producers, such as algae, produce these long-chained fatty acids while terrestrial primary producers and animals typically cannot. Omega-3 PUFAs have also been shown to be highly conserved through trophic interactions, leading to their use in tracing interactions at higher trophic levels (Kelly and Scheibling, 2012).

Mayflies are ideal for studying metamorphosis's effects because of their biphasic life cycle (Figure 1) and current utilization in the laboratory and field. Mayflies begin life in freshwater as eggs that hatch into fully aquatic larvae that are part of the aquatic food web. Then, after metamorphosis, they emerge out of the water and become part of the terrestrial food web as flying subimago adults. Once they undergo a final molt and reach sexual maturity, they reach their final imago stage. During their adult (terrestrial) phases, mayflies cannot feed because they no longer have functional mouthparts, making them an ideal model for studying metamorphosis by removing the influence of diet shifts post-emergence. In the field, mayflies are a highly sensitive group of aquatic insects used to assess stream health (Wallace *et al*., 1996; Barbour *et al*., 1999). The mayfly species *Neocloeon triangulifer* is used as a model organism in various laboratories across North America for ecotoxicology research and is being developed for use in high-sensitivity toxicity assays (Sweeney *et al*., 1992; Buchwalter *et al*., 2018; Soucek *et al*., 2020) *N. triangulifer* reproduce parthenogenetically in the lab, have a short lifespan at room temperature and have published literature for maintaining laboratory cultures (Sweeney *et al*., 1992; Weaver *et al*., 2014). *N. triangulifer* has already been shown to be highly sensitive in toxicity assays to various metals, perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA), and major ions, among other contaminants (Kim *et al*., 2012; Kunz *et al*., 2013; Wesner

et al., 2014; Johnson *et al*., 2015; Soucek and Dickinson, 2015; Soucek *et al*., 2018; Soucek *et al*., 2020; Soucek *et al*., 2023).

In this study, we investigated if the process of metamorphosis caused significant changes to key food web tracers in mayflies. The specific objectives were to determine if significant differences in stable isotopes, polyunsaturated fatty acid profiles, and body composition existed in 1) the model laboratory mayfly *N. triangulifer* and 2) field-collected mayflies within the order *Ephemeroptera* family *Heptageniidae*.

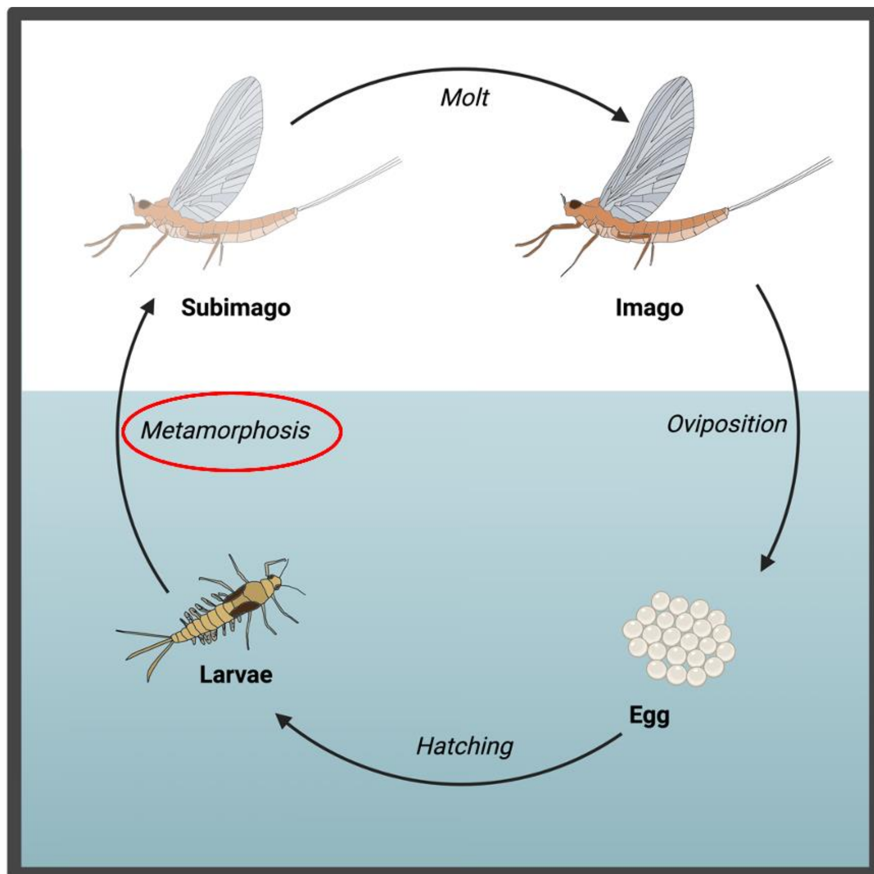

Figure 1. The typical life cycle of a mayfly. Life stages are in bold and processes between life stages are in italics. The red circle indicates the focus on a life cycle process, metamorphosis, for this study.

Methods

Sample Collection

Laboratory Reared Mayflies

Laboratories with existing cultures of *Neocloeon triangulifer* across North America were identified and contacted about participation in this experiment. All laboratories had similar mayfly husbandry protocols derived from Weaver *et al*. (2014), which included a biofilm diet of periphyton (*Navicula* sp.) and a constant temperature between 20-25°C. All participating laboratories contributed approximately 10 individuals from each of the three life stages in the mayfly life cycle: larvae, subimago, and imago. Larval individuals were collected at approximately 20 days after hatching. Prior to sample shipment to Middle Tennessee State University (MTSU), all mayfly samples were stored at -20°C, loaded into coolers, and shipped on wet ice. Upon arrival at MTSU, samples were separated into individual containers (if needed), labeled, and stored at -20°C until further analysis.

Field Collected Mayflies

Larval and adult mayflies were collected from a 100m reach of the East Fork Stones River in Cannon County, Tennessee (35.823485, -86.091313). Mayfly larvae were collected by hand from the underside of partially submerged rocks found in the river and then placed into individual 15mL conical tubes. Adult mayflies were collected via a sheet light trap along the river's shoreline. Individual adults were collected by hand and placed into 15mL conical tubes. All tubes were transported on wet ice in coolers to MTSU and stored at -20°C until further analysis. Mayflies were all identified to the family level and determined to be within *Heptageniidae.*

Elemental and Stable Isotope Analysis

Individual mayfly samples were analyzed for %carbon, %nitrogen, and stable carbon and nitrogen isotopes. Samples were dried, homogenized, and then filtered through a 40-micron mesh screen. The samples were combusted to CO_2 and N_2 and then analyzed using an NC 2500 elemental analyzer with a Delta Plus isotope ratio mass spectrometer. Reference standards of Vienna Peedee Belemnite and ambient air were used for carbon and nitrogen stable isotopes, respectively. Isotope results were reported in δ-notation in parts per thousand.

Lipid and Polyunsaturated Fatty Acid Analysis

Mayflies were composited into samples consisting of approximately 10 individuals and analyzed for various polyunsaturated ω3 and ω6 fatty acid biomarkers and total lipid amount. The fatty acids identified were 16:0, 20:5ω3,18:3ω3, 18:4ω3 20:4ω3, 20:5ω3, 16:2ω6, 18:2ω6, 18:3ω6, 20:2ω6, 20:3ω6, and 20:4ω6. Mayflies were oven dried overnight at 70°C and then placed into 50mL glass test tubes. The samples were homogenized within the tubes using a glass rod. 5mL chloroform, 5mL phosphate buffer, and 10mL methanol were added to the homogenized samples, vortexed, and then stored at room temperature for 3-5 hours. Then, 5mL chloroform and 4mL deionized water were added afterward and left overnight for separation. The following day, the lower organic layer was separated into clean test tubes using Pasteur pipettes, and then the liquid was evaporated with nitrogen gas. The weight of the material in the tube after evaporation was used to calculate the total lipid amount. After evaporation, 1mL of toluene and 1mL of boron trifluoride methanol were added to the samples, and they were then placed on a hot block at 80°C for 1 hour for transesterification of the fatty acids. Afterward, 1mL of deionized water was added to the tubes to stop the reaction. A 1:1 mixture of methyl *tert*-butyl ether (MTBE) and hexanes was added to separate the mixture, and the upper MTBE layer was then removed and placed into

a clean glass tube. This layer was evaporated using nitrogen gas and transferred to a clean 2mL amber vial using methylene chloride. The samples were evaporated, brought to a 50µl volume with methylene chloride, and stored at -20°C for later analysis.

From this extract, 1-2µl was injected into a Thermo TSQ Quantum GC/MS equipped with a Restek Rxi-5Sil MS column (30 m × 0.25 µm film thickness). The injector was set at 200°C, and the oven's initial temperature was at 50°C, then increased to 300°C with various increments. Peaks were visualized using Thermo Xcalibur ver. 2.1 and compared to known standards to determine the relative amount of each fatty acid in the sample.

Data Analysis

Significant differences in carbon and nitrogen stable isotope values, total carbon and nitrogen, and polyunsaturated fatty acid profiles between laboratory groups and mayfly life stages were determined using one-way analysis of variance (ANOVA). Post hoc Tukey's analysis was used to determine differences between groups. Data analysis was completed using JMP software and Jupyter notebooks. Significance was defined as $\alpha=0.05$.

Results

Algal Diet – Laboratory Mayflies

Significant differences were found between laboratory algal samples for carbon and nitrogen isotopes (Figures 2A, 2B) ($\delta^{13}C$ ANOVA: $F_{(5,32)} = 76.0747$, $p<0.0001$; $\delta^{15}N$ ANOVA: $F_{(5,32)} = 120.5666$, $p<0.0001$). These differences led us to present our remaining results using both raw and diet-normalized stable isotope data to highlight the inter-lab difference due to mayfly diet. When mayfly $\delta^{13}C$ and $\delta^{15}N$ values were plotted against each other, a clear separation of values by the laboratory was seen; however, when results were normalized for diet by subtracting the

known isotopic values of that lab's specific algae diet samples, this separation was much less pronounced (Figures 3A, 3B).

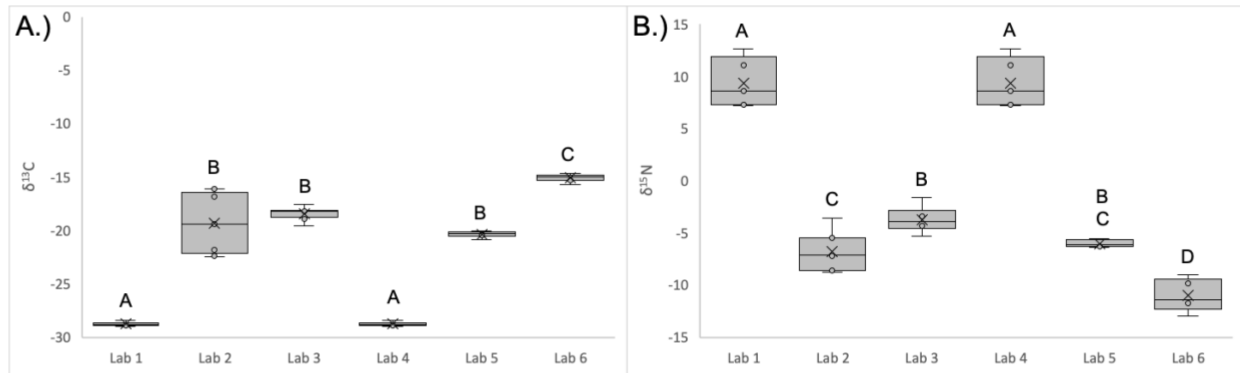

Figure 2. Values of A.) carbon and B.) nitrogen stable isotope values found in algal diet samples of laboratory-reared *N. triangulifer* mayflies used in different laboratories. Boxes represent interquartile ranges, with the middle line representing the median and "x" representing the mean. Circles represent individual data points. Vertical lines extend to the minimum and maximum of the data, with outliers shown. Significant statistical differences are indicated by different letters.

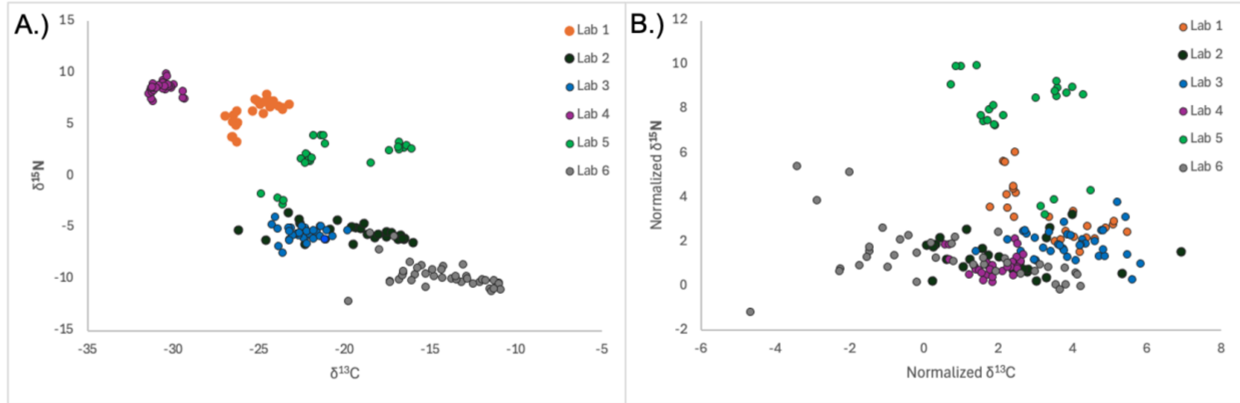

Figure 3. A biplot of carbon and nitrogen stable isotope values of all laboratory-reared *N. triangulifer* mayflies across all life stages from A.) raw data and B.) algae diet normalized data. Different colors indicate different laboratory cultures where mayflies were sourced.

Stable Isotopes

In laboratory mayflies using raw data, a significant increase occurred between the larval and imago stage for $\delta^{13}C$, with subimago not significantly different from any stage; no differences were observed for $\delta^{15}N$ ($\delta^{13}C$ ANOVA: $F_{(2,179)} = 4.7630$, p=0.0097; $\delta^{15}N$ ANOVA: $F_{(2,179)} = 0.8177$, p=0.4431) (Figures 4A, 5A). For diet-normalized data in laboratory mayflies, a significant increase occurred between the larval and imago stage for $\delta^{13}C$, with subimago not significantly different from either stage (normalized $\delta^{13}C$ ANOVA: $F_{(2,179)} = 0.7326$, p=0.4821; normalized $\delta^{15}N$ ANOVA: $F_{(2,179)} = 3.3288$, p=0.0381) (Figures 4A, 5A). In field collected mayflies, no significant differences were found between $\delta^{13}C$ results, but a significant decrease was observed between larval and adult life stages for $\delta^{15}N$ ($\delta^{13}C$ ANOVA: $F_{(1,39)} = 0.8312$, p=0.8312; $\delta^{15}N$ ANOVA: $F_{(1,39)} = 5.9855$, p=0.0192) (Figures 4B, 5B).

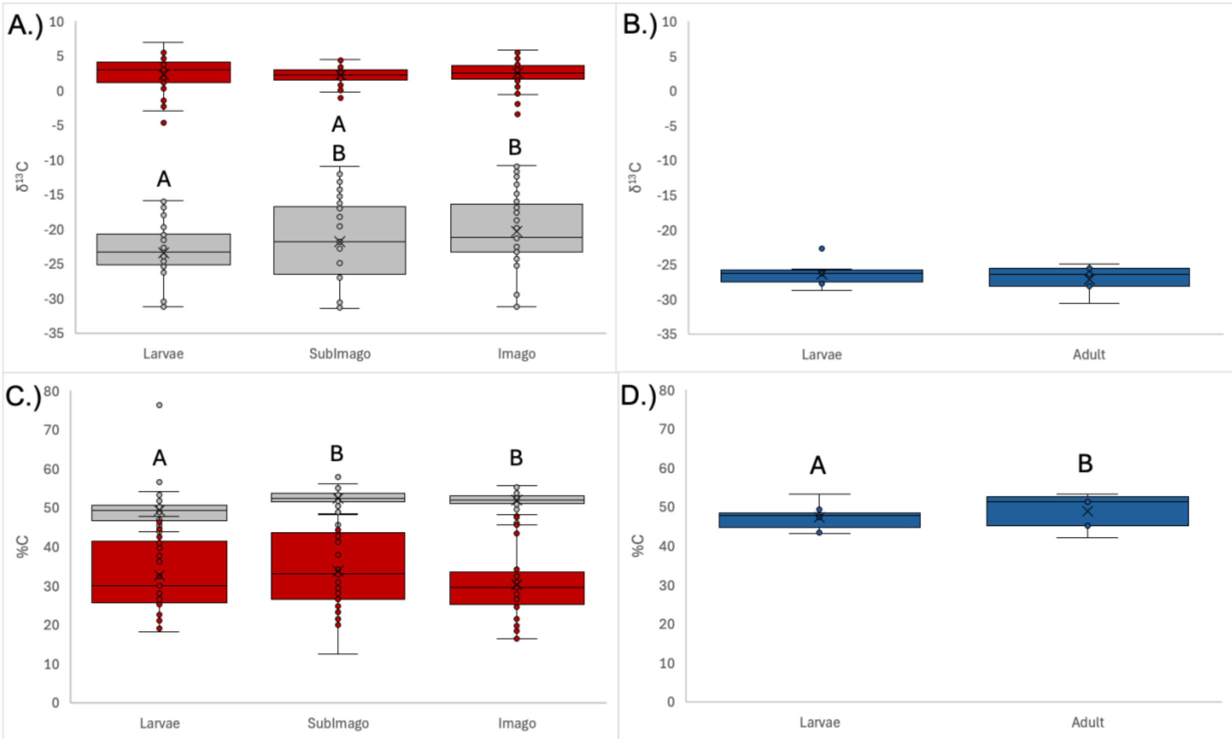

Figure 4. Values of carbon stable isotope values and total body % carbon found in different life stages of mayflies. Grey boxes (represent raw laboratory-reared *N. triangulifer* mayflies for carbon stable isotopes (A) and total % carbon (C), red boxes represent diet normalized raw laboratory-reared *N. triangulifer* mayflies for carbon stable isotopes (A) and total % carbon (C), and blue boxes represent field collected *Heptageniidae* mayflies for carbon stable isotopes (B) and total % carbon (D). Boxes represent interquartile ranges, with the middle line representing the median and "x" representing the mean. Circles represent individual data points. Vertical lines extend to the minimum and maximum of the data, with outliers shown. Significant statistical differences are indicated by different letters.

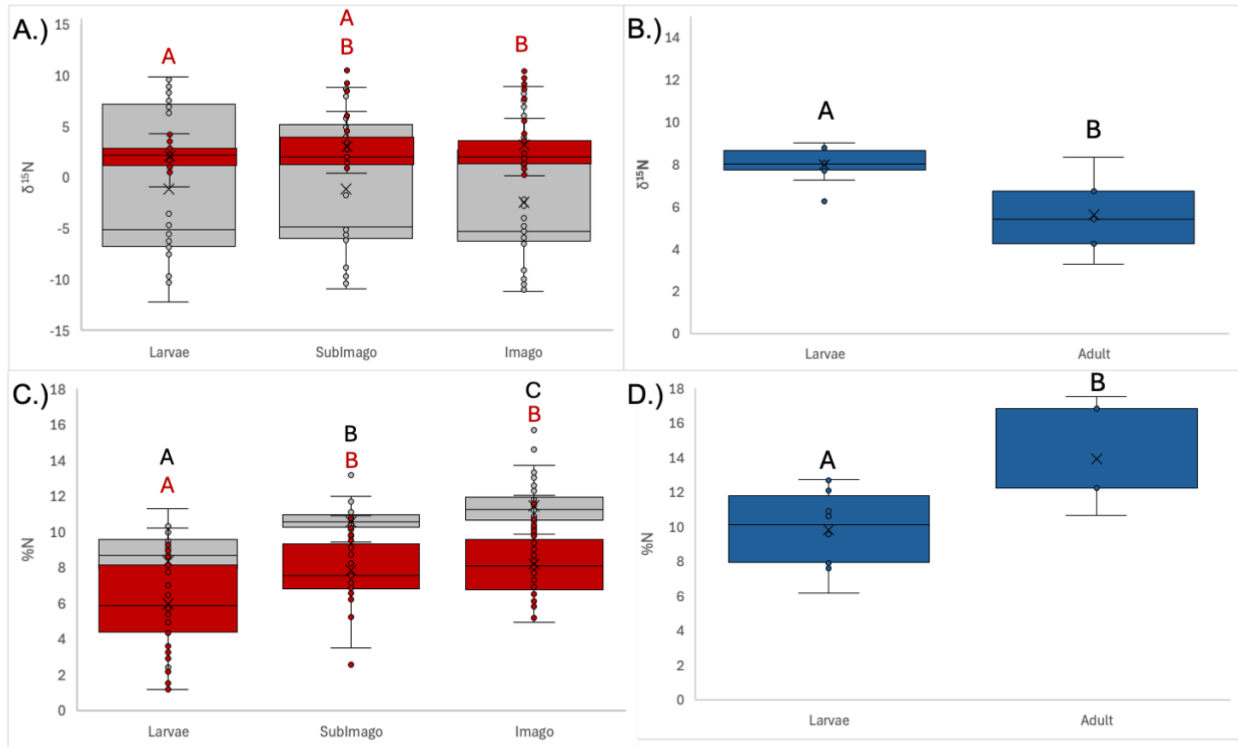

Figure 5. Values of nitrogen stable isotope values and total body % nitrogen found in different life stages of mayflies. Grey boxes (represent raw laboratory-reared *N. triangulifer* mayflies for nitrogen stable isotopes (A) and total % nitrogen (C), red boxes represent diet normalized raw laboratory-reared *N. triangulifer* mayflies for nitrogen stable isotopes (A) and total % nitrogen (C), and blue boxes represent field collected *Heptageniidae* mayflies for nitrogen stable isotopes (B) and total % nitrogen (D). Boxes represent interquartile ranges, with the middle line representing the median and "x" representing the mean. Circles represent individual data points. Vertical lines extend to the minimum and maximum of the data, with outliers shown. Significant statistical differences are indicated by different letters.

Body Composition

A significant increase in %C between larval and adult (both subimago and imago) life stages was observed in raw data for laboratory mayflies (ANOVA: $F_{(2,179)} = 13.2510$, $p<0.0001$), which was not seen when analyzed using diet normalized data (ANOVA: $F_{(2,179)} = 2.7277$, $p=0.0681$) (Figure 4C). There was also a stepwise increase in %N from larvae to adult life stages in both raw and diet normalized data of laboratory mayflies (raw %N ANOVA: $F_{(2,179)} = 97.6966$, $p<0.0001$; diet normalized %N ANOVA: $F_{(2,179)} = 21.4997$, $p<0.0001$) (Figure 5C). The ratio of C:N in laboratory mayflies decreased significantly between aquatic and terrestrial life stages in both raw and diet normalized data (raw C:N ANOVA: $F_{(1,179)} = 24.6022$, $p<0.0001$; diet normalized C:N ANOVA: $F_{(1,179)} = 27.9063$, $p<0.0001$) (Fig 6A). A significant increase in %C and %N between larval and adult mayfly life stages was observed for field collected mayflies (%C ANOVA: $F_{(1,39)} = 13.0415$, $p=0.0009$; %N ANOVA: $F_{(1,39)} = 13.7521$, $p=0.0007$) (Figures 4D,5D). The ratio of C:N of field collected mayflies decreased significantly between larval and adult life stages (C:N ANOVA: $F_{(1,179)} = 5.7183$, $p=0.0218$) (Figure 6).

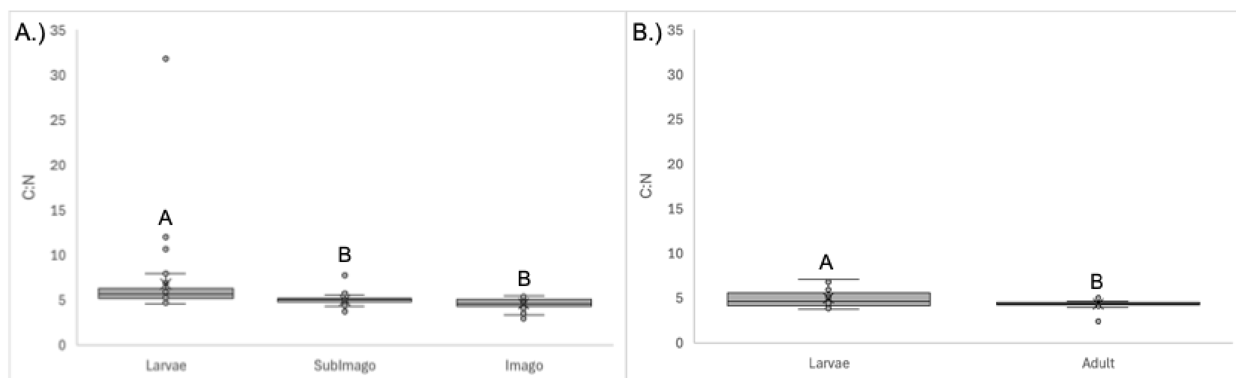

Figure 6. Total carbon to nitrogen ratio of A.) laboratory-reared *N. triangulifer* mayflies and B.) field collected *Heptageniidae* mayflies at different life stages. Boxes represent interquartile ranges, with the middle line representing the median and "x" representing the mean. Circles represent individual data points. Vertical lines extend to the minimum and maximum of the data, with outliers shown. Significant statistical differences are indicated by different letters.

Polyunsaturated Fatty acids

Except for arachidonic acid (ANOVA: $F_{(2,20)} = 4.6049$, p=0.0252), no significant differences were observed between mayfly life stages for individual or aggregate (e.g., total fatty acids) fatty acids (Table 1). Arachidonic acid was significantly increased in subimago and imago life stages when compared to the larval stage. In field collected mayflies, no significant differences in any fatty acid were observed between life stages (Table 1).

Fatty acid	Lab % change with metamorphosis	Field % change with metamorphosis
16:2ω6	-0.37±0.98	0.18±0.8
Linoleic acid (18:2ω6)	-1.87±3.23	-0.14±2.1
γ-Linolenic acid (18:3ω6)	-1.3±2.37	-1.47±3.26
Stearidonic acid (18:4ω3)	0.06±1.58	2.04±2.38
20:2ω6	-0.03±0.66	0.12±0-0.54
Arachidonic acid (20:4ω6)	-1.87±1.69*	1.49±1.98
Eicosapentaenoic acid (20:5ω3)	2.60±2.5	4.69±3.01

Table 1. Change in various polyunsaturated fatty acids during metamorphosis relative to all fatty acids for laboratory-reared *N. triangulifer* mayflies and field collected *Heptageniidae* mayflies. Asterisks (*) represent statistically significant differences.

Discussion

Aquatic emergent insects, like mayflies, have a unique life cycle that bridges two ecosystems, the aquatic and terrestrial, making them key vectors for transferring nutrients between these environments. However, they can also carry bioaccumulative contaminants, which, when biomagnified, may reach concentrations of concern in top trophic level predators (Walters *et al.*, 2008). Understanding the life cycle of these insects and how food web tracers can change during their metamorphosis is crucial for tracking both subsidies and contaminants at the water-land interface.

Stable Isotopes

In the present study, significant differences in $\delta^{15}N$ existed between laboratory and field mayflies' results, where a significant increase was observed between laboratory mayfly life stages (normalized values) and a significant decrease was seen in field-collected *Heptageniidae* (Figures 5B, 5D). The findings for diet normalized values of $\delta^{15}N$ in laboratory mayflies were similar to those observed by Wesner *et al.* (2017), who found $\delta^{15}N$ increased from mayfly larval to adult life stages in field collected *Baetis tricaudatus* in Colorado, USA. Doi *et al.* (2007) had a similar result when investigating chironomid metamorphosis, with enriched $\delta^{15}N$ values in adults compared to larvae. This shift in $\delta^{15}N$ has real-world implications, specifically in ecological risk assessments. $\delta^{15}N$ is used in risk assessments to predict the trophic position of organisms and to help predict biomagnifying contaminant concentrations (Jardine *et al*., 2006). $\delta^{15}N$ values are commonly used to calculate biomagnification factors, which requires an assumption of a consistent isotopic increase between trophic levels. Risk assessments involving emergent aquatic insects can include individuals at either the larval or adult stages, and based on the results of this study, this could skew the isotopic estimate used (Tibbets *et al.*, 2008; Alp *et al*., 2013; Wesner *et al*., 2017). This can also cause a miscalculation in baseline values of stable isotopes for the ecosystem in which the risk assessment is taking place (Jardine *et al*., 2006; Kraus *et al*., 2014).

In the present study, raw isotopic values (not diet normalized) showed significant differences in $\delta^{13}C$ but not $\delta^{15}N$ across laboratories (Fig 3). We speculate this could be due to laboratory-to-laboratory variations in the application of the mayfly rearing protocol (Weaver *et al*., 2014). For example, the recommended rearing temperature is between 20°C and 25°C, and different laboratories used different temperatures within this range. In addition, cultures of algae diet may have varied in quality. Although all laboratories used algae cultures from *Navicula* sp., the

quality may have been different depending on the success of individual algae batches, leading to differing isotopic values. Field-collected *Heptageniidae* mayflies were found to have no difference in $\delta^{13}C$ values between larval and adult life stages (Figure 3B). As they were all collected from the same 100m reach of the same river within 3 months of each other, it can be assumed that their diet did not differ much. The differences observed in carbon isotopic values between lab and field mayflies highlight the need for more work to determine if a predictable relationship exists.

Body Composition

In the present study %N increased significantly between larval and adult life stages; this was similar to the results found by Fogal and Kwain (1974) where total nitrogen content increased with metamorphosis in field collected sawflies, *Neodiprion sertifer,* from Ontario, CA. In a study investigating the effects of metamorphosis in moor frogs (*Rana arvalis*) collected in Russia, the C:N ratio decreased from tadpoles to metamorphs (Doronin *et al*., 2017), which is comparable to the results seen with aquatic insects in the present study. A decrease C:N ratio as an organism goes through metamorphosis could be due to the high usage of carbon-based energy stores during this highly energetic process. Evans (1932) found that *Lucilia sericata* larvae saw a decrease in glycogen over its metamorphosis and, therefore, a decrease in carbon.

Fatty Acids

In the present study, aside from arachidonic acid in lab-reared mayflies, no other significant differences between mayfly life stages for any single fatty acid, %ω3, or %ω6 of total fatty acids were observed. These results differ from that of Pietz *et al.* (2023), who found fatty acid profiles significantly changed with chironomid metamorphosis. These conflicting results indicate species

differences exist for how polyunsaturated fatty acid profiles are influenced by metamorphosis and highlight the need for more investigations across a wider range of insects.

Conclusion

This study's main finding suggests that metamorphosis impacts the isotopic signature of mayflies, notably $\delta^{15}N$. As a commonly used food web tracer, this change should be taken into consideration when using mayflies, or potentially other emergent aquatic insects, in calculations connected to ecological risk assessments as this could result in inaccurate risk values for higher trophic level predators.

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CHAPTER IV: THE EFFECTS OF *IN VITRO* PROPAGATION ON THE ENDANGERED MUSSEL *TOXOLASMA CYLINDRELLUS*: SIZE, BODY COMPOSITION, STABLE ISOTOPES, AND POLYUNSATURATED FATTY ACIDS

Introduction

Freshwater mussels are an essential part of aquatic ecosystems and provide beneficial ecosystem services such as water filtering, nutrient recycling, and structural habitat for other organisms (Vaughn, 2018); they are also among the most endangered groups of animals in the United States (Bogan, 1993). There are over 300 species of freshwater mussels in the United States, with over 70% of these species endangered, threatened, or of special concern (Haag and Williams, 2014). Mussels have experienced a multitude of population declines and extinctions due to various factors, including habitat destruction and invasive species (Neves *et al.,* 1997; Grabarkiewicz and Davis, 2008). The National Strategy for the Conservation of Native Mussels was developed in the late 1990s to conserve freshwater mussels (Neves *et al*., 1997; Haag and Williams, 2014; Freshwater Mollusk Biology and Conservation, 2016). One proposed action was to use propagation, the breeding of native mussels in a facility, as a main way to produce and reintroduce mussels to their natural habitat.

Mussels have a unique life cycle during which there are 3 stages: glochidia/ larvae, juvenile, and adult. During the larval stage of the life cycle, the mussel is dependent on a fish host to which they attach and live in the epidermis and have a parasitic relationship. The mussels then go through metamorphosis and are released from the fish host. The mussels drop to the substrate at the juvenile stage and are free-living (Lefevre and Curtis, 1912). During propagation,

the host fish is provided for attachment of glochidia, juveniles are collected upon dropping off of their host after transformation and fed an algae diet until they reach an appropriate size for release.

While traditional mussel propagation practices using a captive fish host are commonly used in conservation efforts, in recent years, *in vitro* mussel propagation has gained attention as a potential conservation tool (Lima *et al*., 2012; Patterson *et al*., 2018). This technique involves using a combination of nutrient medium, antibiotics/antimycotics, and animal serum within a petri dish where the glochidia can transform into their juvenile stage. Because many freshwater mussel species require specific fish host species for juvenile transformation, variations of nutrients and media must be made to match the nutrient differences that exist in the various fish hosts. Much work has been done to create methods for various mussel species, with over 60 species having been successfully produced via *in vitro* propagation (i.e., Kovitvadhi and Kovitvadhi, 2012; Lima *et al*., 2012; Gąsienica-Staszeczek *et al*., 2018, Wen *et al*., 2018), including federally endangered species of freshwater mussels such as *Cyprogenia stegaria*, *Epioblasma capsaeformis*, *Epioblasma brevidens*, and *Lampsilis* abrupta. (Lima *et al*. 2012; Patterson *et al*., 2022). More *in vitro* propagation research is needed to determine ideal *in vitro* media formulations for other species of interest, optimal nutritional content, juvenile transformation (Lima *et al*., 2012; Patterson *et al*., 2018), and if significant differences exist between fish host and *in vitro* propagated mussels.

This study aimed to determine if size, health, and nutrient uptake differences existed between two laboratory cultured sub-populations of the endangered mussel *Toxolasma cylindrellus*, one propagated using traditional fish host methodology and the other propagated

using *in vitro* methods. Specifically, we investigated if differences existed in 1.) stable isotopes of carbon and nitrogen, 2.) total carbon and nitrogen, 3.) fatty acids, and 4.) glycogen content.

Methods

Experimental Design

Six gravid females were collected from the field and transported to the laboratory, where they were each flushed to collect glochidia. All glochidia were randomly divided into two equal-sized groups. Each group was treated identically, except one was transformed using traditional fish propagation, henceforth known as the fish propagation group, and the other was transformed using *in vitro* methods, henceforth known as the *in vitro* propagation group (details below). After transformation, the two groups of juveniles were placed in separate yet identical grow-out systems and fed identical commercial algae diets until both groups reached an average shell length of 6mm, signifying the experiment's end. Thirty mussels from each group were then euthanized, processed, and analyzed for carbon and nitrogen stable isotopes, total carbon and nitrogen, fatty acids, and glycogen. The experimental design is depicted in Figure 1.

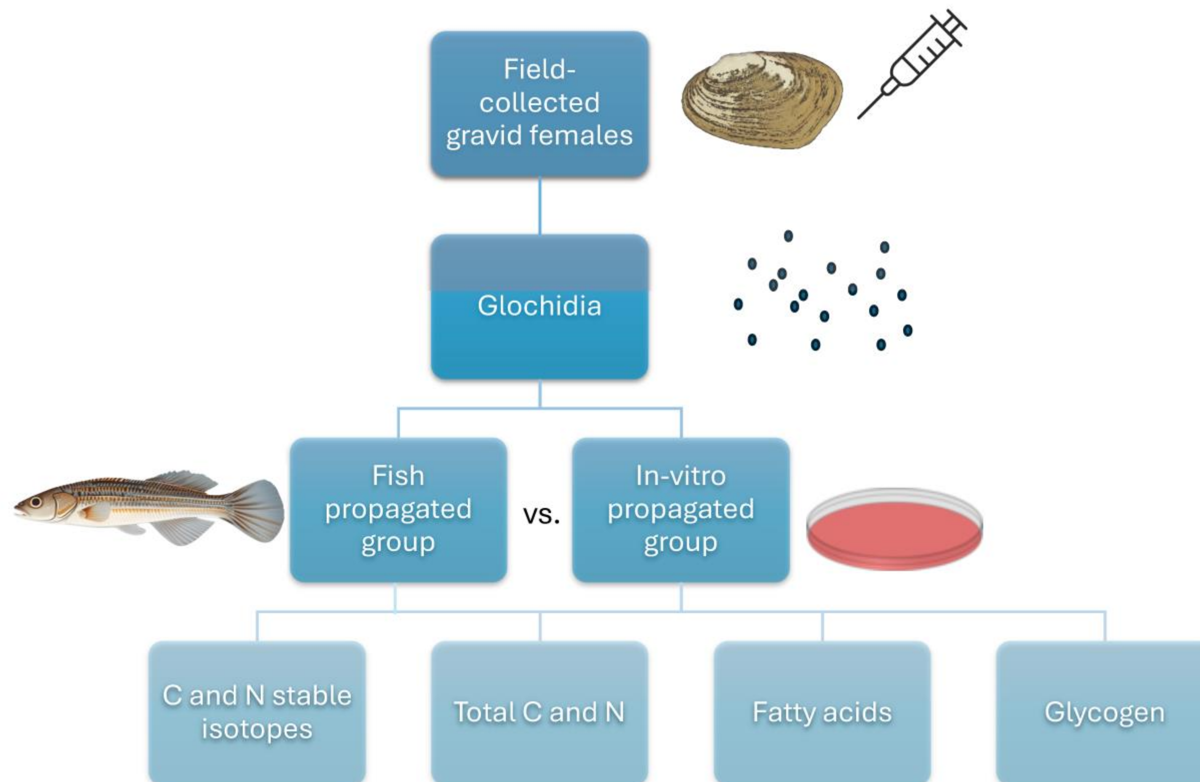

Figure 1. A flowchart displaying the experiment design of this study. Glochidia collected from field collected gravid females are divided into two groups, one to be fish propagated and one to be *in vitro* propagated. The resulting juvenile mussels from both groups were then analyzed for carbon and nitrogen stable isotopes, total carbon and nitrogen, fatty acids, and glycogen content.

Brood Stock Collection

Toxolasma cylindrellus (pale lilliput) gravid females were collected within the Duck River watershed from Lick Creek in Tennessee, USA. Individuals were collected by hand from sediment, visually inspected, and placed into a cooler with river water for transport to the propagation facility. Upon arrival at the propagation facility, mussels were placed into bins with gravel in a flow through system with water from the Cumberland River.

Glochidia Extraction

Collected female gravid mussels were flushed with syringes filled with river water to move glochidia into a petri dish for visual inspection of development under a light microscope. Once glochidia were determined to be developmentally ready to be extracted, reverse pliers were used to gently hold the mussel's shell open wide enough to insert a small syringe, taking care not to damage the adductor muscle. The syringe was then used to flush out the adult mussel's gills, rinsing the glochidia into a petri dish. The glochidia collected were then divided into two groups: one for fish propagation and one for *in vitro* propagation.

Fish Propagation

One half (~5,000) of the total collected glochidia from gravid *T. cylindrellus* were used for infestation of host fish for propagation. The fish used were *Fundulus catenatus* (northern studfish) collected from Lick Creek in Tennessee, USA. The fish were temporarily moved from their holding tanks in the facility and placed into temporary plastic tanks with air stones and water after temperature acclimation. *T. cylindrellus* glochidia were then placed into the plastic tanks with the fish, ensuring the air stones circulated the water and glochidia from the bottom of the tank. Fish remained in these tanks for approximately 30 minutes to allow for glochidia attachment before being placed back into their holding tanks.

The tanks containing the glochidia infested fish were monitored daily for dropped juveniles by collecting the sieved material from siphoning of the bottom of the tank and rinsing catch bags placed over the tank outflows. Any juveniles collected were documented and placed in a juvenile grow out system.

***In vitro* Propagation**

One half (~5,000) of the total collected glochidia from gravid *T. cylindrellus* were used for *in vitro* propagation. The glochidia were taken into a sterilized clean room where they were viewed under a microscope and all mucus and debris were removed from the petri dish, leaving only glochidia. These glochidia were then transferred and placed into a petri dish of nutrient media (L15 or M199). Individuals who did not close in response to the media salts were also removed. A series of petri dishes were then prepared in a hood, all having a ratio of 0.5:1:2 of antibiotic solution (carbenicillin, gentamicin sulfate, rifampin, and the antimycotic amphotericin B): animal serum: nutrient media (L15 or M199). The remaining glochidia were spread evenly into these dishes.

Various combinations of nutrient media M199 or L15 with animal serums, both commercially available and field-collected from fish, were tested for the highest survivorship and transformation of glochidia. Animal serums used were horse, rabbit, sheep, bovine, common carp, silver carp, and bighead carp. None of the combinations using L15 nutrient media or the combination of M199 media and silver carp serum had any surviving individuals. The combination using M199 and horse serum was found to be the most successful, with transformation rates of 68% to 87% of the surviving individuals on the final day of incubation. Only *in vitro* transformed *T. cylindrellus* individuals using horse serum and M199 media were used for the analyses presented in this study.

The petri dishes were placed into an incubator at 24°C and daily monitoring was done to ensure there was no contamination of the *in vitro* mussel cultures. Deceased glochidia were removed daily, and media mixtures were replaced as needed to avoid bacterial contamination. Daily monitoring of glochidia development was done to determine when transformation into juveniles

was complete. When glochidia reached 13 days and were transformed, they were counted and released into a juvenile grow out system.

Juvenile Grow Out

When glochidia were transformed into juveniles, they were collected, counted, and released into a flow-through river/RO water system into a prepared modified plastic bin with 100mm sieved sediment collected from Venable Spring in Marshall Co., Tennessee, USA. They were fed a commercial algae diet mixture daily (1:1:1 mixture of Shellfish Diet 1800: TP 1800: Nanno 3600, Reed Mariculture). Juveniles were kept in the flow-through system until both groups reached about 6mm shell length on average so that they would be large enough for analysis. Juvenile flow through systems were monitored daily for water quality and had RO water added if needed to ensure low ammonia levels. Both systems used were determined to have water quality parameters not significantly different from each other.

Sample Preparation

Once juvenile mussels in both propagation groups reached the appropriate size (average shell length of 6mm), 30 individuals from each group (60 total) were transported to Middle Tennessee State University. There, all mussels were euthanized by freezing. Dissection of the visceral mass from the shell of each juvenile mussel was done using a scalpel and a pair of forceps. Forceps were used to open and hold the mussel shell, while the scalpel was used to scrape out tissue. Visceral mass was stored at -80°C in 2mL cryotubes until needed for analyses.

Stable Isotope Analysis

The visceral mass from 10 juvenile mussels from each group (20 total), fish and *in vitro* propagated, were analyzed for carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotopes. Each individual

sample was dried and homogenized in glass tubes. Individual samples were then combusted to CO_2 and N_2 and analyzed using an NC 2500 elemental analyzer with a Delta Plus isotope ratio mass spectrometer. Vienna Peedee Belemnite and air reference material standards were used for carbon and nitrogen stable isotopes, respectively. A calibration curve was constructed that covered all sample peak sizes, and precision was determined to be lower than 0.1‰ for carbon and nitrogen for all samples. Results were reported in δ-notation in parts per thousand.

Lipid and Fatty Acid Analysis

The visceral mass of 10 mussels from the group of fish propagated juveniles, and 10 from the group of *in vitro* propagated juveniles (20 total) were analyzed for various ω3 and ω6 polyunsaturated fatty acids and total lipid amount. The fatty acids identified were 16:0, 20:5ω3,18:3ω3, 18:4ω3 20:4ω3, 20:5ω3, 16:2ω6, 18:2ω6, 18:3ω6, 20:2ω6, 20:3ω6, and 20:4ω6. Each mussel visceral mass tissue was oven dried at 70°C overnight. They were placed into 50mL glass test tubes and homogenized using a glass rod the next day. 5mL chloroform, 5mL phosphate buffer, and 10mL methanol were added into the tube and stored at room temperature for 3-5 hours. 5mL chloroform and 4mL deionized water were added later and left overnight for mixture separation. The following day, the lower organic layer was separated into a clean test tube using Pasteur pipettes, and then the liquid was evaporated using nitrogen gas. The weight of the material in the tube after evaporation was used to calculate the total lipid amount (g). 1mL of toluene and mL of boron trifluoride methanol were added, and individual test tubes were placed on a heating block at 80°C for 1 hour to transesterify the fatty acids. Afterward, 1mL of deionized water was added to each tube to stop the reaction. A 1:1 mixture of methyl *tert*-butyl ether (MTBE) and hexane was added to separate the mixture, and the upper MTBE layer was then removed and placed into a clean glass tube. This layer was evaporated using nitrogen gas

and transferred to a clean 2mL amber vial using methylene chloride. Samples were evaporated, brought to a final volume of 100µl with methylene chloride, and stored at -20°C for later analysis.

From this sample, 1µl was injected into a Thermo TSQ Quantum GC/MS equipped with a Restek Rxi-5Sil MS column (30 m × 0.25 µm film thickness). The injector was set at 200°C, and the oven's initial temperature was at 50°C, then increased to 300°C with various temperature increments. Peaks were visualized using Thermo Xcalibur ver. 2.1. Individual compounds were compared with external standards (16:0: Supelco 37 component FAME mix (Supelco), 18:3ω3: α-linolenic acid MaxSpec standard (Cayman Chemical), 18:4ω3: stearidonic acid standard (Cayman Chemical), 20:4ω3: 15(S)-hydroperoxy-(5Z,8Z,11Z,13E)-eicosatetraenoic acid (Millipore Sigma), 20:5ω3: eicosapentaenoic acid MaxSpec standard (Cayman Chemical), 16:2ω6: cis, cis-9,12-hexadecanoic acid (Millipore Sigma), 18:2ω6: linoleic acid (Cayman Chemical), 18:3ω6: γ-linolenic acid (Cayman Chemical), 20:2ω6: Supelco 37 component FAME mix (Supelco), 20:3ω6: dihomo-γ-linolenic Acid (Cayman Chemical), and 20:4ω6: arachidonic acid MaxSpec standard (Cayman Chemical)) to determine the amount of each fatty acid relative to all fatty acids in the sample.

Glycogen Analysis

Glycogen analysis was done on the visceral mass of individual juvenile mussels, 10 from the fish propagated group and 10 from the *in vitro* propagated group, following the methods of Vodáková *et al*. (2019). Briefly, tissue was digested, and glycogen was extracted by adding 30% KOH, then boiling in a water bath for homogenization for 20 minutes, followed by adding 96% ethyl alcohol and boiling again for 15 minutes. The solution was then diluted with deionized water. For spectrophotometry to quantify glycogen amounts, 40ul of 80% phenol and 2180ul of 96%

sulfuric acid were added to sample solutions, and 250ul aliquots were added into appropriate wells on a 96-well plate along with prepared calibration standards (Glycogen from Mytilus edulis (Blue mussel), Sigma Aldrich) and sample spikes. The plate was immediately read on a spectrophotometer.

Data Analysis

One-way analysis of variance tests (ANOVA) was done to compare individual size measurements, stable isotope signatures, fatty acid values, and glycogen amounts between the fish host propagated and the *in vitro* propagated mussel groups. Post hoc Tukey's tests were done when necessary to compare means of groups and create connected letters reports. Statistics were run using JMP software.

Results

Size

The *in vitro* propagation group was significantly greater in size for shell length (ANOVA: $F_{(1,60)} = 19.1118$, $p<0.0001$) and mass (mass ANOVA: $F_{(1,60)} = 49.6037$, $p<0.0001$) when compared to fish propagated mussels (Figure 2A, 2B).

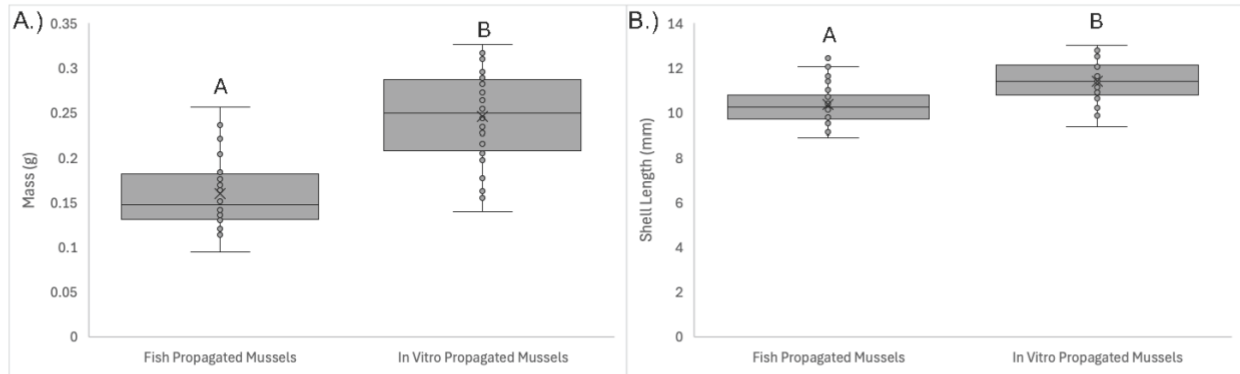

Figure 2. A.) mass and B.) shell length of *T. cylindrellus* juveniles from the fish propagation and *in vitro* propagation groups. Boxes represent interquartile ranges, with the middle line representing the median and "x" representing the mean. Circles represent individual data points. Vertical lines extend to the minimum and maximum of the data, with outliers shown. Significant statistical differences are indicated by different letters.

Stable Isotopes and Body Composition

For $\delta^{13}C$, fish host propagated mussels were significantly more enriched than *in vitro* propagated mussels (ANOVA: $F_{(1,20)} = 4.5690$, p=0.04W65). However, for $\delta^{15}N$, %C, and %N, no significant differences were observed between individuals in the different propagation groups ($\delta^{15}N$ ANOVA: $F_{(1,20)} = 1.5834$, $p=0.2244$, %C ANOVA: $F_{(1,20)} = 0.6704$, $p=0.4236$; %N ANOVA: $F_{(1,20)} = 1.6569$, $p=0.2143$) (Figure 3 A-D).

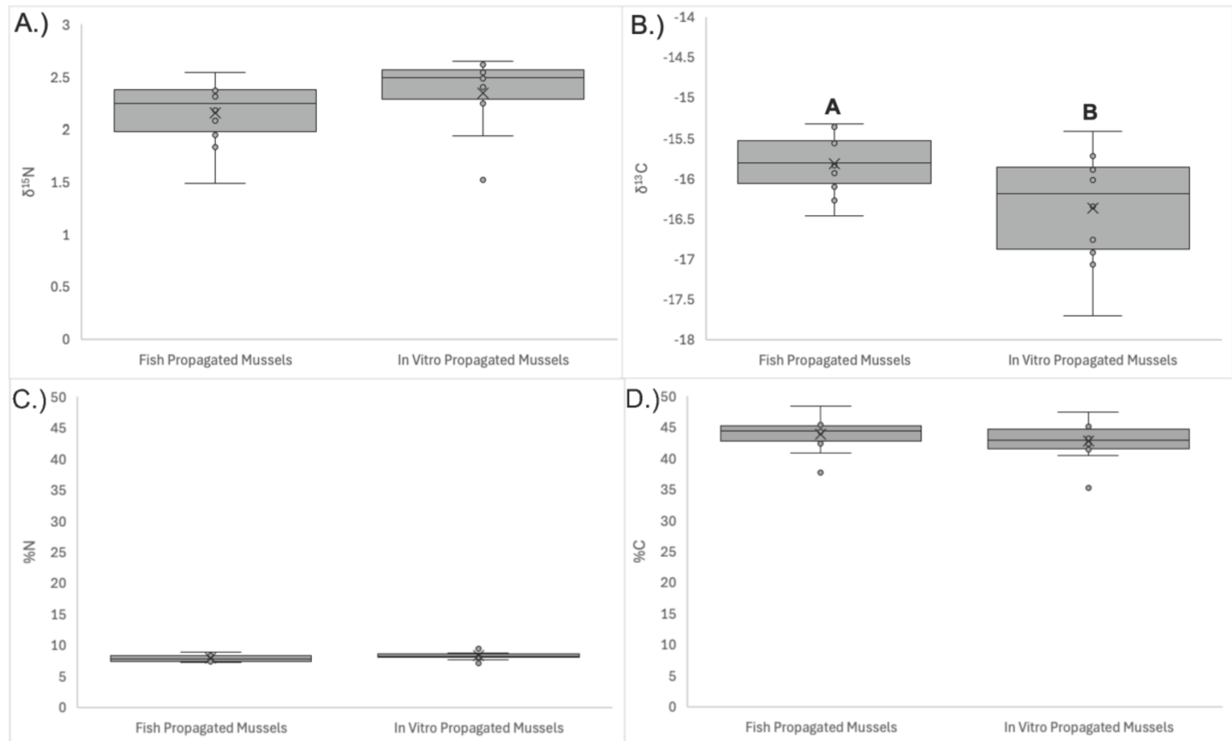

Figure 3. A.) nitrogen stable isotopes, B.) carbon stable isotopes, C.) total % nitrogen, and D.) total % carbon of the visceral mass of *T. cylindrellus* juveniles from the fish propagation and *in vitro* propagation groups. Boxes represent interquartile ranges, with the middle line representing the median and "x" representing the mean. Circles represent individual data points. Vertical lines extend to the minimum and maximum of the data, with outliers shown. Significant statistical differences are indicated by different letters.

Lipids and Fatty Acids

No significant differences in total lipids by weight, %ω3, %ω6, %eicosapentaenoic acid, %linoleic acid, or %stearidonic acid of total fatty acids (TFA) were observed between fish host propagation and *in vitro* propagation mussels (total lipids by weight ANOVA: $F_{(1,20)} = 0.0009$, p=0.9768; % ω3 of TFA ANOVA: $F_{(1,20)} = 1.7336$, p=0.2077; % ω6 of TFA ANOVA: $F_{(1,20)} =$

0.6536, p=0.4294; ω3:ω6 ANOVA: $F_{(1,20)} = 0.2731$, p=0.6089; % eicosapentaenoic acid of TFA ANOVA: $F_{(1,20)} = 0.9797$, p=0.3370; % linoleic of TFA ANOVA: $F_{(1,20)} = 0.3019$, p=0.5894; %steridonic of TFA ANOVA: $F_{(1,20)} = 1.1208$, p=0.3106) (Figure 4A, 4B).

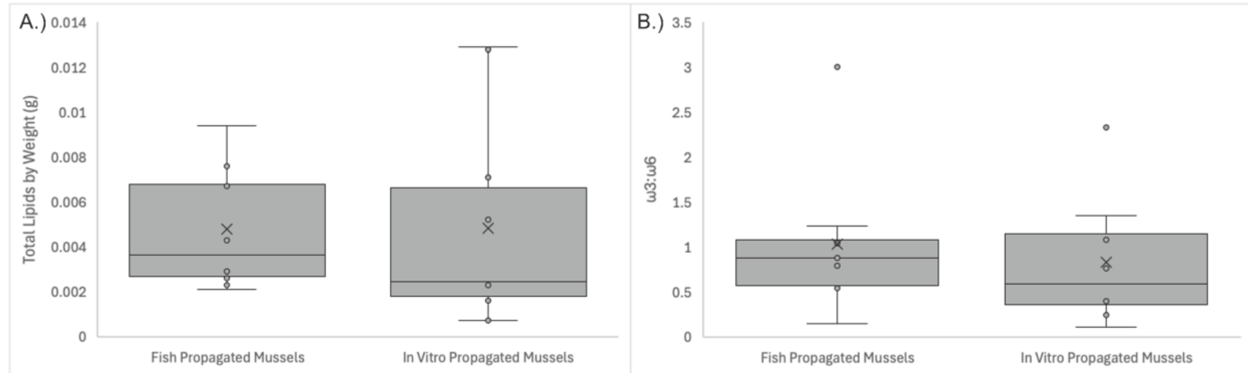

Figure 4. A.) total lipids by weight and B.) ω3:ω6 ratio of polyunsaturated fatty acids of the visceral mass of *T. cylindrellus* juveniles from the fish propagation and *in vitro* propagation groups. Boxes represent interquartile ranges, with the middle line representing the median and "x" representing the mean. Circles represent individual data points. Vertical lines extend to the minimum and maximum of the data, with outliers shown. Significant statistical differences are indicated by different letters.

Glycogen

No significant difference in glycogen concentration was observed between individuals within the fish host and the *in vitro* propagation group of mussels (ANOVA: $F_{(1,20)} = 2.2577$, p=0.1503) (Figure 5).

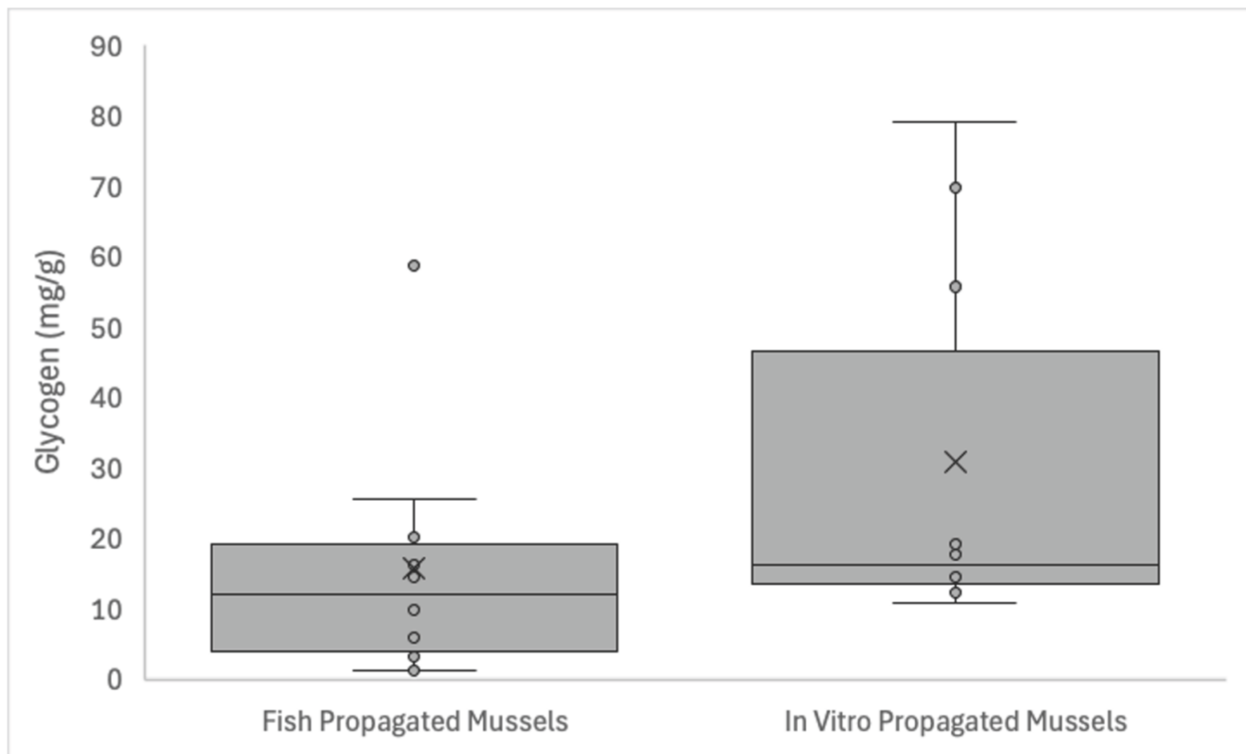

Figure 5. Glycogen content of the visceral mass of *T. cylindrellus* juveniles from the fish propagation and *in vitro* propagation groups. Boxes represent interquartile ranges, with the middle line representing the median and "x" representing the mean. Circles represent individual data points. Vertical lines extend to the minimum and maximum of the data, with outliers shown. Significant statistical differences are indicated by different letters.

Discussion

Propagation serves a role in the reintroduction of declining freshwater mussel populations to their native habitat. Although traditional methods of mussel propagation utilizing fish hosts for glochidia transformation have been successful, limitations exist. The benefits of *in vitro* propagation techniques include improved juvenile survivorship, reduced labor and space needs, and improved control of the mussel transformation environment (Lima *et al.*, 2012). The

differences that exist in freshwater mussels propagated using fish host methods, and *in vitro,* methods have not been thoroughly investigated. To date, only two studies have directly compared these propagation methods; one focuses on contaminant sensitivity (Popp *et al*., 2018), and the other focuses on reproduction (Douda *et al*., 2021). In this study, we aimed to determine if the endangered mussel, *Toxolasma cylindrellus*, could be successfully propagated with *in vitro* methods and if significant differences exist between *in vitro* propagated mussels compared to those propagated using traditional fish host methods.

Size

In the present study, *in vitro* propagated *Toxolasma cylindrellus* mussels had significantly higher mass and shell lengths than fish host propagated mussels (Figure 2A, 2B). These results are in contrast to Douda *et al*. (2021), who found that *Lampsilis cardium* juvenile mussels propagated using *in vitro* methods had a lower mass than those propagated with fish hosts and no significant difference in shell length 5 months post-transformation. The present study and Douda *et al*. (2021) are the only two studies comparing *in vitro* and fish propagated mussels directly in terms of size. The difference in results highlights the need for more investigation into growth rate differences between these propagation methods and into potential inter-species differences.

Stable Isotopes

It has been found that freshwater mussels derive the majority of their nutrients from their host fish during the glochidia phase and that the carbon and nitrogen stable isotope signatures change significantly when mussels transition between glochidia and their juvenile life stage (Fritts *et al*., 2013). In the present study, no significant differences were observed between *in vitro* and fish host propagated groups of mussels for $\delta^{15}N$; however, fish propagated mussels were more

enriched in $\delta^{13}C$ compared to *in vitro* propagated mussels (Figure 3A, 3B). The *in vitro* group in the present study was found to have a larger average shell length and mass, and it has previously been shown that respiration in mussels is inversely proportional to shell length (Lomte and Nagabhushanam, 1971; Hamburger *et al*., 1983; Zotin and Vladimirova, 2001; Tyner *et al*., 2015). In addition, carbonate used for mussel shell precipitation can come from metabolic CO_2 rather than environmental dissolved inorganic carbon and can vary between individuals, also causing a difference in carbon isotopic ratios (Geist *et al*., 2005). With the juvenile stage mussels in this study being fed the same diet under similar conditions, it would be likely that these signatures would not differ between groups due to dietary differences or environmental conditions. The difference in $\delta^{13}C$ values could potentially be attributed to differing size and growth rates. The respiration rate impacts the carbon isotope ratio in an animal through the excretion of CO_2 (DeNiro and Epstein, 1978).

Body Composition

In organisms that undergo major ontogenetic shifts, such as metamorphosis, it can be expected that the body composition would go through major changes (Sheridan and Kao, 1998). When these changes occur, the ecological stoichiometry, the balance of major elements like carbon and nitrogen, of a food web may be impacted as there is a difference in the composition of prey being consumed (Sterner and Elser, 2003). In the present study, no significant differences were observed between *in vitro* and fish host propagated groups of mussels for total %C and %N (Figure 3C, 3D). These results indicate that the retained carbon and nitrogen in the tissues of both groups of propagated mussels are similar, and they are utilizing carbon and nitrogen rich biomolecules at a similar amount. This is an important finding because, when viewed from an ecological conservation perspective, the question of how released organisms may impact native

habitats and food webs is valuable (Atkinson *et al*., 2010; Vaughn and Hoellein, 2018), and no significant difference was found between *vitro* and fish host propagation techniques.

Lipids and Fatty Acids

It has been shown that the use of different host fish species by freshwater mussels can result in a difference in lipid reserves (Douda, 2015). Douda (2015) found that post-transformation juvenile *Unio crassus* propagated using different host fish species and, therefore, different nutrient sources, had significantly different lipid reserves between groups cultured using *Phoxinus phoxinus, Cottus gobio,* and *Chondrostoma nasus*. The same was found with *Anodonta anatine*, with juveniles differing in lipid reserves between groups cultured using the host fish species *Scardinius erythrophthalmus*, *Perca fluviatilis*, *Squalius cephalus*, and *Rutilus rutilus* (Douda, 2015). In the present study, no significant differences between *in vitro* and fish host propagated mussels were observed for total lipids by weight, %ω3, %ω6, %eicosapentaenoic acid, %linoleic acid, or %stearidonic acid of total fatty acids (TFA) (Figure 4A, 4B). These results indicate that the nutrient uptake and lipid reserves for post-transformation juveniles were similar, giving both mussels similar body conditions if they were introduced into their natural habitat. Our results also suggest that both groups of mussels received and incorporated similar amounts of beneficial polyunsaturated fatty acids and lipids, which have been shown to be essential for the optimized growth of freshwater mussels (Gatenby *et al*., 1997; Bartsch *et al*., 2017).

Glycogen

Glycogen is the main form of carbohydrates in freshwater mussels, with the content indicative of energy usage and stress (Naimo *et al*., 1998). In the present study, no significant difference was found in glycogen content between *in vitro* and fish host propagated groups of mussels (Figure

5), indicating that the propagation method had no impact on overall energy reserves and stress. At the time of this publication, no other study compares glycogen content between *in vitro* and fish host propagated mussels. However, it had previously been seen in larvae of *Utterbackia imbecillis* that *in vitro* propagated individuals had fewer lipid droplets and glycogen in the bases of the cells of the mushroom body compared to fish host propagated individuals (Fisher and Dimock, 2002).

Conclusion

Results of this study demonstrate that *Toxolasma cylindrellus*, an endangered freshwater mussel, can successfully transform and propagate using *in vitro* methods. No significant differences between propagation groups existed for nitrogen stable isotopes, total carbon and nitrogen, lipids, polyunsaturated fatty acids, or glycogen content. Significant differences were found between propagation groups in shell length, mass, and carbon stable isotopes, where it was found that *in vitro* propagated mussels had increased shell length and mass and had lower $\delta^{13}C$ values than fish host propagated mussels. These results suggest that *in vitro* propagated mussels had a faster growth rate than those propagated using fish hosts and could reduce the time needed in a juvenile grow out system before reaching the appropriate size for release. In addition, upon release of *in vitro* propagated mussels into their suitable habitat, impacts on the ecological stoichiometry of the food web would be minimal compared to the release of fish host propagated mussels.

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DISSERTATION CONCLUSION

The studies done for this dissertation examined the environmental and individual organism factors that can impact the traditional food web tracers of carbon and nitrogen stable isotopes. They showed how the use of multiple biomarkers, as shown in the Tracer Trichotomy, can help minimize the impacts of the variation due to these factors by providing a more complete and accurate understanding of trophic pathways and contaminant transport than the use of any individual method.

In chapter I, the impact of seasonality on size, carbon and nitrogen stable isotopes, PUFA biomarkers, metals, and mercury (total and methyl) in tetragnathid spider sentinels was assessed. It was found that spider size, methyl mercury, selenium, and arsenic concentrations changed during the active period of tetragnathid spiders, particularly during the final weeks before overwintering. The results suggest that spider sentinels are to be collected in the first 86% of their active period to avoid seasonal variation in food web tracers and contaminants.

In chapter II, it was found that carbon and nitrogen stable isotopes, ω3:ω6 ratios, and mercury concentrations differed significantly in tetragnathid spiders between consecutive years at the same site location. The results indicated that enough variation occurs annually that the data of one year cannot be applied to following years at the same sampling site using tetragnathid spider sentinels.

In chapter III, the individual life cycle variable of metamorphosis on the food web tracers of carbon and nitrogen stable isotopes and polyunsaturated fatty acids in mayflies was assessed in laboratory-reared *N. triangulifer* and field-caught *Heptageniidae* mayflies. There were no significant differences in polyunsaturated fatty acid profiles between larval and adult

stages of field-collected mayflies; however, there was a significant increase in % arachidonic acid in laboratory-reared mayflies. These results indicate that metamorphosis has a significant impact on food web tracers in laboratory and field mayflies, which should be considered when using mayflies or potentially other emergent aquatic insects in ecological risk assessments.

In chapter IV, the differences in size, carbon and nitrogen stable isotopes, and polyunsaturated fatty acids were assessed between groups of juveniles of the endangered mussel *Toxolasma cylindrellus* that were propagated using *in vitro* vs. traditional fish host propagation methods. *In vitro* propagated juveniles were significantly larger and had lower $\delta^{13}C$ values than fish propagated mussels. Otherwise, no differences were found between *in vitro* propagated, and fish propagated mussels for nitrogen stable isotopes, total carbon, nitrogen, lipids, polyunsaturated fatty acids, or glycogen content. The results of this study demonstrate that *in vitro* propagation is a viable method for *T. cylindrellus* conservation and displays minimal differences in juvenile health and nutrient uptake between propagation methods.