RESOURCE-MEDIATED INTRASPECIFIC COMPETITION AND ADAPTIVE DIVERGENCE OF PUMPKINSEED SUNFISH (*Lepomis gibbosus*)

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ABSTRACT

RESOURCE-MEDIATED INTRASPECIFIC COMPETITION AND ADAPTIVE DIVERGENCE OF PUMPKINSEED SUNFISH (*Lepomis gibbosus*)

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Intraspecific competition in pumpkinseed sunfish may have caused adaptive diversification between 'ancestral' littoral and 'derived' pelagic ecomorphs that coexist in single lake populations. I used comparative studies and experimental manipulations to investigate, 1) how resource use, resource availability and intraspecific competition influence polyphenism, and 2) the utility of stable isotope methods for inferring resource use in the wild. An enclosure experiment in the field tested the effects of competition and resource availability. Intense resource limitation had a stronger negative effect on littoral compared to pelagic ecomorphs. Wild zooplankton prey increased mortality of both ecomorphs indicating a previously unknown cost to using this resource that may limit diversification. Variation in $\delta^{13}C$ and $\delta^{15}N$ stable isotope values among wild ecomorphs indicated dissimilar diets and greater dietary specialization by pelagic ecomorphs consistent with prior findings. A variety of factors contributed to variation including tissue type, loss of body condition and sample site.

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The mediocre teacher tells. The good teacher explains. The superior teacher demonstrates. The great teacher inspires. ~William A. Ward

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General Introduction

Adaptive diversification of fish species can arise from a variety of species interactions including competition, predation, and parasitism. In many post-glacial lake systems there is repeated diversification of a single species into distinct ecomorphs with associated changes in morphology, behaviour, and feeding strategies (Schluter 1995, Robinson and Schluter 2000). There is evidence of repeated divergence occurring between the littoral and pelagic habitats of lakes, perhaps because there are distinct environmental conditions in each environment with unique challenges associated with predation and gathering resources (Schluter 1995, Schindler and Scheuerell 2002). It is the mechanisms that both promote and limit adaptive diversification of species that will be examined in this thesis.

One of the most commonly studied mechanisms of diversification is competition. The role of competition in the diversification of species is often associated with interactions occurring between individuals of different species leading to ecological character displacement (interspecific competition; eg. Mittelbach 1984,Garvey et al. 2002, Gray et al. 2005). However, there is also much interest in competitive interactions between individuals of a single species that may also contribute to polyphenism (intraspecific competition; eg. Smith and Skulason 1996, Bolnick 2001, Jastrebski and Robinson 2004, Pfennig et al. 2007).

Another component to adaptive diversification of species is related to the spectrum of resources available in an environment. Resource-mediated competition can promote the diversification of a species only when an alternate food source is available. However, the nature of replaceable resources may also limit diversification if they are

sufficiently costly due to the way they are gathered, where and when they are available, associated consequences of predation risk, and the ratio of energy return to energy expended.

For this thesis, I examined a polymorphic population of pumpkinseed sunfish (*Lepomis gibbosus*) from Ashby Lake (45˚05'N, 77˚21'W), a post-glacial Canadian Shield lake. Polymorphic pumpkinseed sunfish have been well studied in many post-glacial lakes (Robinson et al. 1993, Robinson et al. 2000, Gillespie and Fox 2003, McCairns and Fox 2004), including the one used for my study (Jastrebski and Robinson 2004). The ancestral form of these fish is thought to inhabit the littoral zone consuming a variety of benthic macroinvertebrate prey groups (Keast 1978, Werner and Hall 1979). In some lakes there is also a pelagic form that appears somewhat specialized to feed on zooplankton (Gillespie and Fox 2003, Robinson et al. 1993, Robinson et al. 2000, Jastrebski and Robinson 2004). This polymorphism is thought to have arisen through resource-mediated intraspecific competition, which is also thought to drive the diversification of species. My goal was to further examine the resource use of both the littoral and pelagic ecomorphs of pumpkinseed sunfish and to test the role of both competition and resource availability in adaptive diversification between ecomorphs.

The first part of my thesis involves examination of resource use by littoral and pelagic sunfish ecomorphs in the field using stable isotopes to infer long term resource use of sunfish. The Ashby Lake population of sunfish was previously studied by Jastrebski and Robinson (2004) using stomach contents, and they found the littoral ecomorph consuming benthic macroinvertebrates and the pelagic ecomorph eating zooplankton. These stomach content results are consistent with dietary studies for other

polymorphic pumpkinseed sunfish populations (Gillespie and Fox 2003, Robinson et al. 1993). However, stomach contents provide only a limited picture of what was eaten very recently. I used stable isotope analysis of $\delta^{13}C$ and $\delta^{15}N$ to establish if there was a difference in the diets of each sunfish ecomorph over a longer time scale. It is important to establish patterns of resource use by the sunfish ecomorphs as one of the main components of the adaptive diversification model is that they have evolved to use pelagic zooplankton instead of littoral benthic macroinvertebrates.

In this section I also examined several non-diet related factors that can cause variation in stable isotope signatures of wild populations, such as spatial variation (Hobson 1999, Vander Zanden and Rasmussen 2001), temporal variation (Zohary et al. 1994, Post 2002, Matthews and Mazumder 2003, 2004), tissue-related differences (MacAvoy et al. 2001, Bearhop 2002, Perga and Gerdeaux 2005), and starvation (Hobson et al. 1993, Adams and Sterner 2000, Haubert et al. 2005). In both the first chapter and appendix of this thesis I assessed the ability of stable isotopes to accurately infer information about resource use in the light of other factors that may contribute to variation in $\delta^{13}C$ and $\delta^{15}N$ in pumpkinseed sunfish.

The second part of my thesis was a manipulative experiment designed to test two factors involved in adaptive diversification of sunfish. I placed enclosures in the littoral habitat of Ashby Lake and manipulated the density of fish and prey resources to test the role of both intraspecific competition and resource availability. The intensity of competition should be influenced by consumer density such that at low densities there is little competition for food, but at high densities food becomes scarce causing natural selection that favours phenotypes that use alternate resources. However, such

diversifying selection is only possible when alternate resources are available for use, and also resources may be unavailable if they are too costly to use. Experimental manipulations of both sunfish density and resource availability allowed me to evaluate the role of both factors on the fitnesses of alternate sunfish ecomorphs in a single study.

In the general discussion I will relate what these studies mean to both our understanding of the evolution of polymorphic pumpkinseed sunfish and the use of stable isotopes in studies of resource use by wild fish populations.

Chapter 1

The use of $\delta^{13}C$ and $\delta^{15}N$ stable isotopes to infer resource use in a population of polymorphic pumpkinseed sunfish (*Lepomis gibbosus*)

Abstract

One of the key elements in the adaptive divergence of pumpkinseed sunfish (*Lepomis gibbosus*) is the differential use of resources by the ecomorphs. Typically, pumpkinseed sunfish consume macroinvertebrates in the littoral habitat, but in some lakes a pelagic ecomorph coexists by consuming zooplankton. Previous analyses used stomach contents to infer resource use, although this only reflects short term resource use. In this study I applied stable isotope analysis in order to test for differential resource use of sunfish ecomorphs over longer periods. I analyzed variation in stable isotopes of sunfish in order to, 1) examine resource use of both sunfish ecomorphs, 2) examine possible non-diet related sources of variation in stable isotopes, and 3) trophic specialization of each ecomorph. I sampled sunfish from both habitats and reference prey from each lake habitat, analyzed $\delta^{13}C$ and $\delta^{15}N$ from reference prey and white muscle and liver tissues in sunfish. I found subtle differences in the enrichment of $\delta^{13}C$ and $\delta^{15}N$ between sunfish ecomorphs, that the littoral ecomorph was most similar to littoral prey, and that my prey reference group may not include all types of prey consumed by pelagic sunfish. Stable isotope values in sunfish were also influenced by sampling variation in space and time, type of tissues analyzed, but not body size. Trophic width analysis indicated that the diets of pelagic ecomorphs were more specialized than littoral ecomorphs. My results are consistent with prior analyses of resource use by polymorphic

sunfish populations. However, the analysis of stable isotopes requires careful consideration of other sources of variation that may act in wild populations.

Introduction

The ecology of organisms in their natural habitat is often understood by their trophic relations with other organisms. One common way to characterize the ecology of an organism is by analyzing which resources it consumes. This can be challenging because we often cannot follow long-lived individuals over extended periods of time to observe diet, and so instead rely on short term estimates of diet, such as stomach contents. Such 'snapshot' methods of resource use, if not made over the lifetime of an organism, can be easily biased by limitations in time frame, stomach capacity (especially in small species), and patchy distributions of resources in space (Bearhop et al. 2004, Araújo et al. 2007, Svanback and Bolnick 2007). Stable isotopes may provide the ability to infer dietary and habitat use information about organisms drawn from their natural habitat over a longer time scale than can be achieved by short term dietary studies (Araújo et al. 2007, Svanback and Bolnick 2007).

Stable isotopes of $\delta^{13}C$ and $\delta^{15}N$ have been used to establish the ecological characteristics of a population related to diet. $\delta^{13}C$ and $\delta^{15}N$ can be used to examine the diets of different species sharing a habitat (Hobson et al. 1996, Paterson et al. 2006), members of the same species in different locations (Hobson 1999, Hobson and Bairlein 2003), and members of a single species within a single habitat (MacAvoy et al. 2001, Perga and Gerdeaux 2005) over different spatial and temporal scales.

Many studies have established the relationship of $\delta^{13}C$ and $\delta^{15}N$ between consumers and their resources. The utility of $\delta^{13}C$ values is that they vary little from a resource to the consumer, usually by less than 1‰ (Harrigan et al. 1989, Post 2002, Bearhop et al. 2004), making it a good isotope to establish direct links in dietary studies when prey vary in $\delta^{13}C$ values. On the other hand, $\delta^{15}N$ enriches 3-5‰ as it transfers from one trophic level to the next and so can provide an estimate of the trophic position of an organism (Vander Zanden et al. 1997, Bearhop et al. 2002).

Understanding the ecology of a focal population requires placing it correctly in the context of its local community and ecosystem. The use of $\delta^{13}C$ and $\delta^{15}N$ stable isotopes to infer diet or habitat use depends on accurate information from the focal consumer species and an array of possible resource taxa. In addition, a range of samples of both consumers and resources may be required when stable isotopes vary over spatial (Hobson 1999, Vander Zanden and Rasmussen 2001) and temporal scales (Zohary et al. 1994, Post 2002, Matthews and Mazumder 2003, 2004).

Previous stable isotope studies of organisms from lakes have found general differences in stable isotope values of $\delta^{13}C$ between samples from littoral and pelagic habitats (Schindler and Scheuerell 2002) suggesting that stable isotopes can signal habitat-specific resource use. Differences in stable isotop signatures of consumers from different habitats is the result of general difference in the $\delta^{13}C$ signatures of littoral macrophyte primary producers and pelagic phytoplankton (France 1995). These differences in the primary producers are then transmitted up their respective food chains to all consumer groups. Pelagic phytoplankton discriminates between the different isotopes of carbon and incorporates less ^{13}C than the macrophyte producers of the littoral

habitat resulting in baseline differences in the isotope ratios of ^{13}C and ^{14}C and more enriched $\delta^{13}C$ signatures of both littoral primary producers and their consumers (France 1995, Post 2002, Vander Zanden et al. 2003, Paterson et al. 2006). If the primary production in these two habitats fix different enrichment values of carbon, then a consumer population's connection to the littoral or pelagic food chain may be indicated by an analysis of the $\delta^{13}C$ even when consumers move between habitats.

For this study, I examined resource use of a polymorphic pumpkinseed sunfish (*Lepomis gibbosus*) population found in both the littoral and pelagic habitats of a lake. Past work has indicated that individuals inhabit either the littoral or pelagic habitat (Robinson et al. 1993, Robinson et al. 2000, McCairns and Fox 2004), exhibit predictable variation in external body form, and consume different prey (Robinson et al. 1993, Robinson et al. 2000, Gillespie and Fox 2003, Jastrebski and Robinson 2004). Pumpkinseed sunfish are one of the most abundant sunfish species to be found in North America, occurring at the northern limits of most sunfish species (Scott and Crossman 1998). In most lakes they only occur in the littoral habitat where they primarily feed on a variety of benthic macroinvertebrates, but also have the ability to specialize on gastropods and bivalves (Keast 1978, Werner and Hall 1979). However, in some northern postglacial lakes they have diversified into the pelagic habitat where they have been observed to feed extensively on zooplankton (Robinson et al. 1993, Robinson et al. 2000, Gillespie and Fox 2003, Jastrebski and Robinson 2004). Analyses of parasites (Robinson et al. 2000) and mark-recapture studies (McCairns and Fox 2004) both indicate that ecomorphs show strong habitat fidelity. The use of stable isotopes in this

population allows me to examine one of the key components of the sunfish diversification model, the use of different resources by the ecomorphs (see Chapter 2).

If littoral and pelagic food chains vary in stable isotope values due to different $\delta^{13}C$ and $\delta^{15}N$ signatures of their respective primary producers, then the reference prey taxa should vary between lake habitats. Littoral pumpkinseed sunfish consume benthic macroinvertebrates (gastropoda, amphipoda, bivalvia, ephemeroptera, and odonata), while the pelagic sunfish ecomorph is thought to consume pelagic zooplankton (copepoda and cladocera) which are predicted to differ in $\delta^{13}C$ and $\delta^{15}N$ signatures. Thus, if stable isotope signatures of sunfish consumers are primarily determined by the prey they consume, then I predicted that (1) sunfish $\delta^{13}C$ should be similar to that of their prey reference values for both ecomorphs and (2) $\delta^{15}N$ should show an enrichment of 3-5‰ over that of the prey values.

However, variation in stable isotope signatures of consumers may also be influenced by a variety of other factors, including the type of tissue analyzed (eg. Bearhop 2002, Pearson et al. 2003, Perga and Gerdeaux 2005), spatial variability in stable isotope signatures (Hobson 1999, Vander Zanden and Rasmussen 2001), and an effect of body size on consumer diet (Osenberg and Mittelbach 1989, Svanbäck and Eklöv 2002, Hjelm et al. 2003). We are just beginning to understand the significance of these sources of variation, which when overlooked may bias inferences about the ecology of a natural population.

The type of tissue sampled is one possible source of variation because stable isotope signatures are incorporated into body tissues at different rates based on tissue-specific metabolic activity (Hobson et al. 1996, MacAvoy et al. 2001, Bearhop 2002,

Pearson et al. 2003, Perga and Gerdeaux 2005). For example, when an individual shifts its diet, tissues like white muscle will take a longer period to change stable isotope signatures (Fry and Parker 1979, Cabana and Rasmussen 1994) than liver (Hesselin et al. 1993, Perga and Gerdeaux 2005) because the muscle is less metabolically active. Tissue-specific differences in $\delta^{13}C$ and $\delta^{15}N$ then are expected to be a function of two factors: 1) Variation in tissue metabolic activity (so that one tissue may better reflect dietary values over hours or days while another type may better reflect diets over a period of weeks to months); and 2) Variation in diet over time or space (e.g., winter diets differing from summer diets, or variation in prey diversity and abundance from one locale to another). As a result when there is dietary variation stable isotope values can differ between tissues of high and low metabolic activity within the same individual. If tissue-specific metabolic rate influences stable isotope signatures, then I expected there to be differences in the stable isotope signatures between highly active tissues (liver) and less active tissues (white muscle) in both sunfish ecomorphs.

Another potential source of variation in stable isotope signatures is related to spatial variation. Many studies have found variation in $\delta^{13}C$ and $\delta^{15}N$ between different sample sites, such as when comparing one lake to another (Cabana and Rasmussen 1996, del Giorgio and France 1996, Hobson 1999). As a result of this spatial variation it is necessary to establish baseline stable isotope values of potential prey groups from each site being sampled. However, there is also evidence of variation on smaller spatial scales (eg. differences in stable isotope signatures between littoral and pelagic habitats; France 1995, Post 2002, Paterson et al. 2006). It is also conceivable that there may be spatial

differences within each of the general lake habitats (i.e. littoral and pelagic) which were examined as a possible source of variation in this study.

For many consumer species, the type of prey in the diet is limited by the consumer's body size, and so diets change with ontogeny (eg. Rosenzweig 1973, Werner and Gilliam 1984, Werner 1988, Osenberg and Mittelbach 1989, Svanbäck and Eklöv 2002, Hjelm et al. 2003). If diet varies ontogenetically, then body size may contribute to variation in stable isotope values.

The body size of an individual may influence diet, mainly by excluding prey that exceed the oral gape-width which is strongly correlated with body size. If this is true, then I expected that stable isotope values would be related to body size in these sunfish. I also predicted that the relationship between diet and body size would be more pronounced in littoral compared to pelagic ecomorphs because pelagic sunfish are thought to consume significant amounts of zooplankton throughout their lives and so do not experience as strong an ontogenetic niche shift as littoral sunfish.

Variation in diets among individuals from a population can not only be used to estimate average resource use of a population but also to address similarities or differences in resource use among individuals, referred to as the trophic width of a population (Bolnick et al. 2003, Bearhop et al. 2004, Matthews and Mazumder 2004, Layman et al. 2007). For example, a generalist population can be composed of individuals that eat a wide diversity of prey (ecological generalists) or of individuals that each specialize on different prey, both yielding a wide average trophic width for the population. While a population of specialists is expected to be composed of individuals that are all consuming the same limited variety of resource. Variation in isotope values

among individuals would be high in the generalist case and low in the specialist case. Variation in both $\delta^{13}C$ and $\delta^{15}N$ values is more likely in generalist populations because consumers may be eating from both multiple food chains and trophic levels.

A second way to infer trophic width involves comparing variation in stable isotope signatures among different tissues within each individual. Stable isotope values are expected to vary among body tissues when the tissues vary in metabolic rate and so integrate changing dietary signals over different time scales. For example, white muscle is expected to reflect a longer term dietary signal than the liver. If a population is made up of individual generalists whose diets vary over time, then the stable isotope signal will vary between tissues of low and high metabolic rates (e.g., white muscle vs. liver respectively) within each individual. If individuals specialize on particular prey items, then the stable isotope signal should not vary much between different tissues of an individual.

Prior dietary analyses using stomach contents indicated that littoral pumpkinseed sunfish consume a variety of benthic macroinvertebrate prey while pelagic sunfish consume zooplankton (Jastrebski and Robinson 2004). Based on this information, I expected greater variation in stable isotope values among littoral individuals compared to pelagic individuals. As well, if there is greater variation between the tissue types of a generalist compared to specialist population then I expected that the littoral sunfish ecomorph would show greater variation than pelagic sunfish.

My goal was to use stable isotopes to infer the trophic ecology of polymorphic pumpkinseed sunfish that inhabit different lake habitats and are thought to consume distinct diets. By doing this I was able to examine the long term use of a species whose

model of divergence is based on differences in resource use between the ecomorphs. As well, I evaluated general patterns of variation in stable isotope values to perform a preliminary analysis of the effect of tissue type, sample site, and body size on variation in stable isotope signals. Finally, I also tested if the trophic widths of different ecomorph populations vary in predictable ways. It is only by testing diet along with other possible factors that influence $\delta^{13}C$ and $\delta^{15}N$ stable isotope signatures, that we will be able to reliably use stable isotopes to study resource use in a wild population.

Materials and Methods

Study Site

Ashby Lake (45˚05'N, 77˚21'W) is located in the Mazinaw region of Ontario. This region has multiple polymorphic pumpkinseed populations (Gillespie and Fox 2003) including a previously studied population in Ashby Lake (Jastrebski and Robinson 2004). As part of the Canadian Shield, this lake is characterized by extensive rocky shoals rising to just below the surface of the pelagic habitat where pumpkinseed sunfish congregate and feed on zooplankton. The extent of the littoral habitat is relatively minor, representing less than 10% of the total lake area, due to a shoreline that rapidly drops off into pelagic habitat, although several shallow bays contain vegetation that provides littoral habitat.

Pumpkinseed Sunfish Collection

Samples of five pumpkinseed sunfish were collected from littoral and pelagic habitats each week between July 17th and August 18th, 2007 for a total of 25 littoral and

25 pelagic sunfish. Sample sites varied from week to week (Fig. 1.1) and some sites were sampled more than others. All fish were collected by angling yielding fish between 15g and 80g.

Sunfish were euthanized in benzocaine solution (stock solution created by adding ethyl-*p*-aminobenzoate to ethanol for a 2.5g/L solution, then diluted in 10L of water to a concentration of 0.03mL/mL water) for 10 minutes. Blotted wet-weight of each fish was determined, the liver was removed, and a sample of white muscle tissues was taken from the base of the dorsal fin on the right side of each fish. Both tissues were then frozen at -20˚C.

Prey Sampling

Samples of benthic macroinvertebrates and zooplankton were collected weekly to provide reference measurements of the most common prey species available in the littoral and pelagic habitats. Littoral benthic macroinvertebrates were collected by sweeping a D-net through the macrophytes and upper sediment layers. Samples were then hand-sorted into orders using a nested sieve system (openings of 12.7, 4, 2, 1 and 0.5 mm). Stable isotopes of $\delta^{13}C$ and $\delta^{15}N$ were analyzed from benthic macroinvertebrate groups: gastropoda, bivalvia, odonata, amphipoda, and ephemeroptera because they represent the most commonly collected littoral prey species. Pelagic zooplankton (copepoda and cladocera) were collected between 2 and 4pm by vertical tows from a depth of 15m with a mesh size of 0.5µm. Both pelagic zooplankton and littoral benthic macroinvertebrate samples were frozen at -20˚C.

Stable Isotope Preparation and Measurement

Sunfish tissue samples and prey samples were freeze dried at -50˚C for 24 hours and ground into a fine powder using a ThermoSavant FastPrep (FP120) tissue grinder. Lipids were then removed from the tissues following Bligh and Dyer (1959) in which a 2:1 chloroform-methanol solution was mixed into the tissue, spun, and the lipids extracted. This procedure was repeated three times. All tissue samples were weighed to the nearest 0.001mg to obtain a final sample weight of 0.25 - 0.30mg. Prior to freeze drying the benthic macroinvertebrate prey samples had carbonates removed with a 10% HCl solution, except for snails and bivalves whose outer shells were manually removed.

Stable isotope values of all samples were analyzed using the Isochrom spectrophotometer at the University of Waterloo Environmental Isotope Laboratory for ^{15}N, ^{14}N, ^{12}C, and ^{13}C. Samples of an internal standard reference material were analyzed every 5 samples (Pee Dee Belemnite (PDB) was used for carbon, and atmospheric N_2 for nitrogen). Precision of the internal standards are ±0.3‰ for nitrogen and ±0.2‰ for carbon. Isotope ratios are expressed as parts per thousand (‰) differences from the standard reference material:

$$\delta X = (R_{sample}/R_{standard} - 1) \text{ X } 10^3$$

where X is N^{15} or C^{13}, R is the ratio of N^{14}:N^{15} or C^{13}:C^{12} and δ is the measure of heavy to light isotope in the sample.

Statistical Analyses

I first analyzed variation in stable isotope signatures among prey taxa and their lake habitats. The mean $\delta^{13}C$ and $\delta^{15}N$ isotope values of all prey orders from littoral (all

benthic macroinvertebrates combined) and pelagic habitats (all zooplankton) were compared using a two-tailed t-test. Analysis of variance (ANOVA) was then used to compare mean $\delta^{13}C$ and $\delta^{15}N$ values among prey taxa (littoral: gastropoda, amphipoda, bivalvia, ephemeroptera, odonata, and pelagic: combined zooplankton samples). When differences were found between prey groups, pair-wise contrasts were made between reference prey groups using multiple student's t-tests.

I then analyzed variation in stable isotope signatures between sunfish ecomorphs, and compared these to the reference prey values above. The mean $\delta^{13}C$ and $\delta^{15}N$ values of the pumpkinseed sunfish were compared using a three-factor ANOVA model (Factors: pumpkinseed sunfish ecomorph, tissue type, sampling site, and their interactions). I used Dunnett's multiple comparisons to compare pumpkinseed sunfish $\delta^{13}C$ and $\delta^{15}N$ values to prey reference values,

To analyze variation in stable isotope signatures among sunfish sampled from different sites, I compared the mean $\delta^{13}C$ and $\delta^{15}N$ values of pumpkinseed sunfish between sample sites using a one-factor ANOVA. Muscle and liver tissues were combined for this analysis unless they differed significantly from each other, in which case each tissue was analyzed separately. If the ANOVA model indicated significant differences between sample sites, then pair-wise contrasts were made using student's t-tests. If the ANOVA model indicated that sample sites were not significantly different, then Tukey's HSD was used to compare among sites.

The trophic width of pumpkinseed sunfish was assessed by comparing the variation in the stable isotope values between littoral and pelagic ecomorph samples and

between tissue types (muscle and liver). Levene's test for equal variances was used to look for significant differences in the variation of $\delta^{13}C$ and $\delta^{15}N$ stable isotope values.

The relationships between $\delta^{13}C$ and $\delta^{15}N$ values and body size were tested using an ANCOVA model (Factors: ecomorph and tissue type, Covariate: body weight, and their interactions).

All analyses were completed using JMP IN v. 5.1. An α-value < 0.10 was considered as weak statistical evidence, and ≤ 0.05 as strong statistical evidence.

Results

Relationships between Reference Prey and Sunfish Stable Isotopes

Common littoral macroinvertebrates (gastropoda, amphipoda, bivalvia, ephemeroptera, and odonata) and pelagic zooplankton (copepoda and cladocera) prey species were used to establish reference prey stable isotope signatures. When I grouped $\delta^{13}C$ and $\delta^{15}N$ of all littoral benthic macroinvertebrates together and compared them to the pelagic zooplankton signature I found differences in both $\delta^{13}C$ (t=test, $t_{10.79} = 4.23$, $P = 0.002$) and $\delta^{15}N$ values ($t_{15.93} = -5.89$, $P < 0.001$). On average the $\delta^{13}C$ of the littoral resources were more enriched than the pelagic resources (Fig. 1.2). $\delta^{15}N$ values indicated enrichment in the pelagic zooplankton compared to the littoral prey.

The littoral benthic macroinvertebrate reference prey groups analyzed here are composed of different taxa that potentially use a variety of feeding strategies of organic carbon, such as shredders of coarse particulate organic material, grazers of biofilms, and filters of fine particulate organic matter. As a result, I expected and found variation in $\delta^{13}C$ and $\delta^{15}N$ values among littoral reference prey groups (ANOVA, $F_{5,17} = 7.14$, P =

0.003; Fig 1.2). The ephemeroptera differed in $\delta^{13}C$ from all other littoral prey species (gastropoda, amphipoda, odonata, and bivalvia; student's t-test, all $P < 0.013$). Surprisingly, the $\delta^{13}C$ value of ephemeroptera differed little from that of pelagic zooplankton ($P = 0.73$). There was weak evidence of differences in $\delta^{15}N$ values between prey taxa (ANOVA, $F_{5,17} = 2.51$, $P = 0.09$; Fig. 1.2) with pelagic zooplankton more enriched in $\delta^{15}N$ compared to all other benthic macroinvertebrates. The pelagic zooplankton signatures represent combined samples of mostly cladocera and copepoda, therefore further analysis of variation in pelagic reference prey groups was not possible.

Pumpkinseed sunfish ecomorphs are thought to have diets strongly linked to their respective habitats, and so I predicted that there should be differences in stable isotope values between the sunfish ecomorphs. The pelagic ecomorph was more enriched in $\delta^{13}C$ than the littoral ecomorph by an average of 0.79‰ (ANOVA, $F_{1, 80} = 16.91$, $P < 0.001$; Fig. 1.3). No significant differences were found in $\delta^{15}N$ values (ANOVA, $F_{1, 80} = 2.19$, $P = 0.14$) between sunfish ecomorphs (Fig. 1.3).

As expected, the littoral sunfish ecomorphs $\delta^{13}C$ values for both muscle and liver were similar to gastropods (Dunnett's test, $P = 0.99$ for both tissues) and bivalves (Muscle, $P = 0.20$; Liver, $P = 0.24$), and significantly different from all other prey (all $P < 0.03$ for both tissues; Fig. 1.4). Unexpectedly, the $\delta^{13}C$ values of the pelagic ecomorph were also most similar to gastropods in both muscle and liver tissue (muscle, $P = 0.99$; liver, $P = 0.40$), but different from all other prey taxa, including zooplankton (all $P < 0.003$).

As expected, in both sunfish ecomorphs and tissue types there was an increase in the $\delta^{15}N$ values over that of all reference prey taxa (Dunnett's test, all $P < 0.05$). The

mean $\delta^{15}N$ value of pelagic sunfish was enriched by 1.59‰ and 3.20‰ in liver and muscle tissues respectively over the mean $\delta^{15}N$ of zooplankton. This was less than the $\delta^{15}N$ enrichment of the littoral ecomorph by 4.85‰ and 6.50‰ (liver and muscle tissue respectively) over the mean benthic macroinvertebrate prey values.

Variation in Stable Isotope Values between Sunfish Tissues

There were no differences in mean $\delta^{13}C$ values between muscle and liver tissues of pumpkinseed sunfish (ANOVA, $F_{1,80} = 0.65$, $P = 0.42$; Fig. 1.3), but there were differences in mean $\delta^{15}N$ values between tissues: Muscle and liver tissues were 8.67‰, and 7.05‰ respectively (ANOVA, $F_{1,80} = 347.13$, $P < 0.0001$; Fig. 1.3).

Variation in Stable Isotope Values in Response to Fish Body Size

I found no evidence of a relationship between mean $\delta^{13}C$ or $\delta^{15}N$ and body size of sunfish over the size range included here when the two tissue types were combined or were treated separately (Table 2.1).

Spatial Variation in Sunfish Stable Isotopes

Pumpkinseed sunfish were collected from a variety of locations around Ashby Lake in this study (two in the pelagic habitat and three in the littoral habitat, Fig. 1.1). I tested for significant variation in $\delta^{13}C$ and $\delta^{15}N$ signatures between littoral and pelagic habitats and among sites within habitats.

For $\delta^{13}C$, both types of fish tissue were combined as it was previously found that there were non-significant differences between muscle and liver tissues. Significant

differences in $\delta^{13}C$ occurred between sample sites (ANOVA, $F_{4, 95} = 5.96$, $P = 0.0003$, Fig. 1.6). Significant variation was attributed to one littoral site that differed from the other two littoral sites ($P = 0.06$ and 0.02; Fig. 1.7) and no significant variation was found between pelagic sites (student's t-test, $P = 0.27$).

Sampling site variation in $\delta^{15}N$ was analyzed separately for each tissue type due to a general enrichment in muscle compared to liver. Analysis of liver tissue suggested weak evidence of differences between sampling sites in $\delta^{15}N$ when all sites were considered together (ANOVA, $F_{4, 45} = 2.17$, $P = 0.09$; Fig. 1.8). Sunfish sampled from littoral sites were enriched in $\delta^{15}N$ compared to pelagic sites, but when contrasting individual sites there were no significant differences (Tukey's HSD, all $P > 0.05$; Fig. 1.8). Analysis of muscle tissue revealed no evidence of variation in $\delta^{15}N$ signal among sample sites (ANOVA, $F_{4, 45} = 1.32$, $df = 4$, $P = 0.28$; Fig. 1.8).

Comparing Trophic Width of Sunfish Ecomorphs Using Stable Isotopes

The final component of this study was to test the ability of stable isotopes to infer trophic width of a population. First, I compared the variation in stable isotope signatures ($\delta^{13}C$ or $\delta^{15}N$) between the presumed generalist form (littoral sunfish ecomorph feeding on benthic macroinvertebrates) to a specialist (pelagic ecomorph feeding on zooplankton). I predicted greater variation in stable isotopes of generalist populations to specialists because of the greater diversity of prey contributing to the $\delta^{13}C$ and $\delta^{15}N$ values of the littoral sunfish ecomorph compared to the pelagic ecomorph. As expected, there was greater variation in the $\delta^{13}C$ values among littoral compared to pelagic ecomorphs for liver ($F_{1, 48} = 8.28$, $P = 0.006$) and muscle tissues ($F_{1, 48} = 4.45$, $P = 0.04$;

Fig. 1.3). There was no evidence of differences in the variation in $\delta^{15}N$ values between ecomorphs for either liver (Levene's test, $F_{1, 48}= 1.93$, $P = 0.17$) or muscle ($F_{1, 48} = 0.77$, $P = 0.38$) tissues (Fig. 1.3).

I also analyzed variation in $\delta^{13}C$ or $\delta^{15}N$ between slow (muscle) and fast (liver) metabolism tissues thought to respectively integrate diet over a longer and shorter time scales within individuals. In a population composed of individual generalists (littoral ecomorph here), variation in stable isotope signal should arise between tissue types because tissues effectively indicate change in diet with time. A population composed of individual specialists (pelagic ecomorph) will not have as much variation between tissue types. I found no evidence of significant differences between tissue types for either $\delta^{13}C$ or $\delta^{15}N$ and either sunfish ecomorph (littoral $\delta^{15}N$ Levene's test, $F_{1, 48} = 1.35$, $P = 0.25$; pelagic $\delta^{15}N$: $F_{1, 48} = 0.34$, $P = 0.56$; littoral $\delta^{13}C$, $F_{1, 48} = 0.25$, $P = 0.62$; pelagic $\delta^{13}C$, $F_{1, 48} = 0.001$, $P = 0.97$; see Fig. 1.3).

Discussion

In this study I examined a polymorphic population of pumpkinseed sunfish using stable isotopes to examine their long term resource use. However, stable isotopes may also be affected by other factors that need to be evaluated in order to effectively apply them. I examined two possible sources of variation, spatial variation and consumer body size, which may decrease the ability to accurately infer resource use of a wild population from reference prey signatures alone. As well, I tested if stable isotopes can be used to determine the trophic width of a natural population and provide details about the ecology of individuals within a population.

Relationships between Reference Prey and Sunfish Stable Isotopes

I was able to detect significant variation among potential prey groups from littoral and pelagic habitats which may be transmitted upwards to higher trophic levels. Variation in $\delta^{13}C$ between littoral and pelagic food chains is thought to reflect fundamental differences in the fractions of each isotope fixed by primary producers at the base of each food chain (France 1995, Post 2002, Vander Zanden et al. 2003). Of five littoral benthic macroinvertebrates groups tested here, only ephemeroptera had $\delta^{13}C$ values similar to that of pelagic zooplankton resources. I also found a general difference in the $\delta^{15}N$ signatures of the prey resources between littoral and pelagic habitats. These fundamental differences between prey signals should make it possible to detect variation in stable isotope values between sunfish ecomorphs if they exclusively use resources of either the littoral or pelagic habitat, but not both.

Ideally, $\delta^{13}C$ should vary minimally between resources and consumers (Harrigan et al. 1989, Post 2002, Bearhop et al. 2004), allowing for a direct comparison of the resources to the sunfish. The littoral sunfish ecomorph muscle and liver tissue $\delta^{13}C$ values were similar to gastropods and bivalves, consistent with the idea that these are a common prey of littoral pumpkinseed sunfish (Jastrebski and Robinson 2004). Surprisingly, the $\delta^{13}C$ values of pelagic sunfish were also most similar to littoral gastropods and were significantly enriched from all other prey types, including zooplankton. This suggests that both ecomorphs of pumpkinseed sunfish preferentially consume gastropods, which is inconsistent with previous analyses of stomach contents of pelagic pumpkinseed sunfish from Ashby Lake, where benthic macroinvertebrates,

including gastropods, constituted only 2% of the diet compared to 98% for zooplankton (% by count of prey items; Jastrebski and Robinson 2004). Snails are likely available on rocky shoals in the pelagic habitat where they graze on the biofilm and so may contribute to the stable isotope signatures of the pelagic sunfish ecomorph, but it seems unlikely that they would make up a great enough proportion of the diet to significantly affect $\delta^{13}C$ values. Another possibility is that sunfish travel between littoral and pelagic habitats to eat benthic macroinvertebrates, although prior mark-recapture studies of another polymorphic pumpkinseed sunfish population found considerable habitat fidelity (McCairns and Fox 2004). If the prior diet and morphological analyses of this and other populations, and mark-recapture studies are accurate, then my current results suggest that the $\delta^{13}C$ values from these sunfish cannot be used as evidence of a link between the pelagic sunfish ecomorph and zooplankton, even though a similar link to littoral resources is supported.

One possible explanation for the difference between $\delta^{13}C$ signals in zooplankton and the pelagic sunfish ecomorph is that the reference zooplankton used here were not those consumed by pelagic pumpkinseeds in the wild. I collected zooplankton from deep pelagic habitats surrounding the rocky shoals where pumpkinseed sunfish feed. My vertical plankton tows also started at 15m, which may be substantially below the depth where sunfish normally feed. Vander Zanden and Rasmussen (1999) found variation in the stable isotope signatures of zooplankton collected from depths of 0-2m and 12-15m in lakes similar to where this study took place. This difference between the depths may explain my results as pelagic sunfish feed near the surface, but the zooplankton collected for reference samples was gathered throughout the water column starting at 15m in depth.

As well, there may be spatial variation among zooplankton collected from different pelagic sites that affect their $\delta^{13}C$ values (Hobson 1999, Vander Zanden and Rasmussen 2001, Post 2002). This highlights how important it is to gather appropriate resource references samples when studying stable isotopes of natural populations.

$\delta^{15}N$ of consumers is expected to reflect the trophic level at which they feed and also may reflect base differences between alternate food chains. My results indicated that both littoral and pelagic pumpkinseed sunfish exhibit an increase in $\delta^{15}N$ over that of their prey resources consistent with the increase of 3-5‰ found elsewhere (Vander Zanden et al. 1997, Bearhop et al. 2002). Figure 1.4 suggests that $\delta^{15}N$ was less enriched in the pelagic ecomorph than the littoral ecomorph in relation to their reference prey $\delta^{15}N$ values, if they were actually consuming their respective diet sources. There are three mechanisms that may cause the observed differences in the amount of enrichment observed between sunfish ecomorphs. First, the enrichment rate may vary between the habitats for reasons currently unknown causing more enrichment to occur in $\delta^{15}N$ of the littoral sunfish. Secondly, the littoral food chain may have more trophic steps between invertebrate prey and sunfish than the pelagic food chain allowing the greater enrichment of $\delta^{15}N$ (Schluter 1995, Schindler and Scheuerell 2002). Finally, it is possible that my reference prey samples are not representative of the actual diets of sunfish in Ashby Lake. This may be especially true of the pelagic zooplankton resources used here because littoral ecomorphs actually appear to be more similar to my reference zooplankton than the pelagic sunfish!

Variation in Stable Isotope Values between Sunfish Tissues

I also tested if stable isotope signatures varied between tissue types, between sites and habitats within a single lake, and finally with the size of an organism. If any of these factors have a significant effect on stable isotope signatures, then they would need to be accounted for in order to make useful inferences about resource use. I also further tested sources of variation in stable isotopes of wild populations with a manipulative experiment of pumpkinseed sunfish diets using wild prey species (see Appendix).

In order to test the effect of tissue type on $\delta^{13}C$ and $\delta^{15}N$, I compared stable isotope values in a tissue with high metabolic activity (liver) to a less active tissue (muscle) which will reflect diets at different time scales. There were no significant differences between the tissues in $\delta^{13}C$, but the $\delta^{15}N$ of muscle tissue was significantly higher than that of liver tissue in both sunfish ecomorphs. Many studies of wild populations examine only a single tissue type (eg. Hobson and Clark 1993, Overman and Parrish 2001, Harrod et al. 2005, Hoeinghaus and Davis 2007) and do not explore variation in stable isotopes between tissues. In addition, studies of smaller organisms often blend the entire organism together (eg. Pinnegar and Polunin 1999, Adams and Sterner 2000, Focken 2001, Haubert et al. 2005, Paterson et al. 2006), making it difficult to evaluate the actual stable isotope value of the organism if the relative contribution of tissues with different signatures are unknown. Studies have shown different rates of integration between muscle and liver tissues (eg. Pinnegar and Polunin 1999, Perga and Gerdeaux 2005) that ignored, may lead to inaccurate inferences about the diet of an organism sampled from its natural habitat. My results indicated that $\delta^{13}C$ is uninfluenced by tissue type, but that $\delta^{15}N$ is 18.7% higher in muscle compared to liver tissues in the

same fish, and so potentially affects any interpretation of enrichment between resources and fish.

Variation in Stable Isotope Values in Response to Fish Body Size

The diet of fish is strongly influenced by their gape-width because this determines the size of prey that they are able to consume (Osenberg and Mittelbach 1989, Svanbäck and Eklöv 2002, Hjelm et al. 2003). The standard model of pumpkinseed sunfish life history starts with sunfish feeding exogenously on zooplankton from the water column until they are large enough to consume benthic macroinvertebrates in the littoral habitat (Gacria-Berthou and Moreno-Amich 2000). Size variation among adult littoral pumpkinseed sunfish may also cause smaller sunfish to feed on smaller insect larvae and soft-bodied macroinvertebrates while larger sunfish consume larger hard-bodied gastropods (Sadzikowski and Wallace 1976, Keast 1978). Pelagic ecomorphs are expected to either not switch to macroinvertebrates and continue to consume zooplankton, or may switch first to macroinvertebrates at an intermediate size and then switch back to zooplankton at a larger size perhaps because predation limits small sunfish to littoral habitats and prey (Osenberg et al. 1988, McCairns and Fox 2004). An understanding of how ontogenetic niche-shifts affect stable isotope signals is required if we are to use them in studies of natural populations that sample individuals across multiple sizes and life stages.

The results of this study indicated that there was little difference in stable isotope signatures related to the size range of the pumpkinseed sunfish collected here. Stable isotope signatures of $\delta^{13}C$ did not vary between the tissues or in relation to the size of the

fish sampled and $\delta^{15}N$ was greater in muscle compared to liver tissues but this was not affected by sunfish body size over a size range of 18.7g to 75.5g. The results of this study indicated that the diets of both the littoral and pelagic sunfish ecomorphs are not influenced by body size over the size range examined here. It would be interesting to examine the relationship of stable isotopes to body size across a greater range of body sizes, particularly involving smaller young-of-year fish where ontogenetic effects on diet may be greater.

Spatial and Temporal Variation in Stable Isotope Values

Spatial variation in $\delta^{13}C$ and $\delta^{15}N$ values is one of the main reasons that baseline stable isotope signatures of reference prey organisms are required in order to link consumers to particular prey. However, spatial variation may also occur among samples within a continuous habitat. Multiple sampling sites were used in this study allowing me to examine spatial variation in sunfish $\delta^{13}C$ and $\delta^{15}N$. I did not predict there to be a difference in the stable isotopes of sunfish between sampling sites except between the littoral and pelagic lake habitats. For example, liver tissue values of $\delta^{15}N$ varied between littoral and pelagic habitats, but not within sites of each habitat type. However, I found evidence of variation in the $\delta^{13}C$ stable isotope values of sunfish tissues sampled from different sites within the littoral but not within the pelagic habitat.

My evidence of spatial variation in $\delta^{13}C$ values among pumpkinseed sunfish sampled from different littoral sites in Ashby Lake must be treated with caution, however, because samples were collected over a five week period and as a result variation in space is confounded with time. The absence of significant variation in stable

isotope values between samples of pelagic sunfish though suggests that they may live in a more homogenous stable isotope environment than littoral sunfish. For example, the diversity and abundance of benthic macroinvertebrate taxa may vary from site to site in the littoral habitat of a lake creating distinct micro-habitats increasing the variation in stable isotope signatures (Zah et al. 2001). The more uniform and well mixed conditions of the upper pelagic habitat may distribute zooplankton more evenly throughout the surface waters (although I cannot discount variation in stable isotopes among pelagic sites because I only sampled sunfish from two pelagic sites of similar depths). While I cannot distinguish whether variation in stable isotope values in sunfish from multiple littoral samples reflects variation in time or in space, my results nonetheless demonstrate variation within a single species of fish that reduces the utility of stable isotope values for making inferences about prey and habitat use without considerable supporting information on spatial-temporal patterns of prey stable isotope variation.

Comparing Trophic Width of Sunfish Using Stable Isotopes

Assessing the trophic width of a population allows us to address if a population is composed of generalists or specialists. Bearhop et al. (2004) proposed that variation in the trophic width of a population should affect variation in stable isotope signatures, so that a population of generalists should have greater variation among individuals in stable isotope signatures than a specialist population.

I found little difference in the amount of $\delta^{15}N$ variation between the sunfish ecomorphs, which is interesting as Bearhop et al. (2004) expected $\delta^{15}N$ to be the measurement most likely to indicate trophic width because generalists are more likely o

consume prey from multiple trophic levels, which $\delta^{15}N$ is used as an indicator of. However, I did find that the littoral ecomorph was more variable in $\delta^{13}C$ than the pelagic ecomorph. This is consistent with what would be predicted if the littoral sunfish ecomorph consumes many different benthic macroinvertebrate taxa and a pelagic ecomorph consumed a more specialized diet of zooplankton (Robinson et al. 1996, Jastrebski and Robinson 2004, Parsons and Robinson 2006).

A second test of variation in trophic width between sunfish ecomorphs involved analyzing variation between tissue types that reflect different dietary time scales within each individual. I predicted that by comparing the amount of variation between a long term indicator of diet (white muscle) to a short term indicator (liver) I could make inferences about the trophic width of a population and classify them as resource generalists or specialists. The results again supported the hypothesis that the pelagic ecomorph of sunfish are more specialized than the littoral ecomorph (i.e. little difference in the amount of variation stable isotope signatures between the tissues, for both $\delta^{13}C$ and $\delta^{15}N$). However, the littoral ecomorph of sunfish also showed little difference in the variation of either $\delta^{13}C$ or $\delta^{15}N$ between liver and muscle tissues. This suggests that the littoral ecomorph are specialists as well, possibly for gastropods and bivalves based on the comparisons to reference prey samples.

One possible source of error to be considered in this comparison of two metabolically active tissues is that they integrate diet over more similar time scales than expected. A better long-term signal of diet may be metabolically inactive tissues such as scales or otoliths (Bearhop et al. 2004). While I can conclude there is evidence of

specialization in both the littoral and pelagic sunfish ecomorphs this is supported only over the time-scale it takes for stable isotope signatures of white muscle to change.

Conclusions

Differences in the resource use by sunfish ecomorphs is one of the key elements thought to maintain sunfish polymorphism. It is thought that the littoral ecomorph consumes a diet of benthic macroinvertebrates while the pelagic ecomorph consumes zooplankton suspended in the water column (Robinson et al. 1993, 2000). The stable isotope results of this study support that there are differences in the diets of littoral and pelagic sunfish based on the $\delta^{13}C$ values. An interesting feature of this study is that zooplankton collected from the deeper pelagic habitat surrounding the rocky shoals, where the sunfish reside, was significantly depleted in $\delta^{13}C$ compared to the pelagic sunfish. These $\delta^{13}C$ results alone seem to indicate that pelagic sunfish are either not eating zooplankton or that the reference zooplankton collected here were different from those consumed by sunfish. However, when looked at in conjunction with stomach contents collected by Jastrebski and Robinson (2004), who found that zooplankton constituted 98% of the pelagic ecomorph diet, I propose that there may be significant variation in stable isotope signatures of zooplankton between shallow and deeper areas (or between rocky shoals and open waters).

Resource specialization is also an important component of the sunfish polymorphism that can be examined using stable isotopes. The littoral ecomorph is thought to represent an ancestral state that feeds on a variety of benthic macroinvertebrates (Keast 1978, Werner and Hall 1979) and this was supported by the

stable isotope results here. There was also evidence that pelagic sunfish are more specialized because of less variation in stable isotopes between individuals compared to littoral sunfish. This is consistent with previous studies that have found variation in morphological traits between ecomorphs (Jastrebski and Robinson 2004) and selection on the pelagic ecomorph strongly favours individuals highly specialized for zooplankton (Robinson et al. 1996, Parsons and Robinson 2006).

Interestingly, there was also evidence that the littoral sunfish ecomorph show specialization when comparing white muscle to liver tissue in these fish. However, when comparing the two ecomorphs it was found that there was more variation in stable isotope signatures in the littoral ecomorph compared to the pelagic ecomorph. This indicates that pelagic sunfish are more specialized for their zooplankton resources and have less variation between individuals in their diets. While there appears to be some evidence of specialization in the littoral sunfish there is also evidence that they are the more generalist group of the two pumpkinseed sunfish ecomorphs.

Overall, stable isotopes of $\delta^{13}C$ and $\delta^{15}N$ provided some information consistent with previous analyses indicating the pumpkinseed sunfish ecomorphs consume different resources. There was strong evidence linking the littoral ecomorph to benthic macroinvertebrate prey groups. While the pelagic sunfish could not be linked directly to a prey resource group there was evidence that they are consuming resources different from the littoral sunfish. There is also evidence from this study that indicated stable isotopes are susceptible to variation caused by non-diet related factors that must be considered before making strong inferences about trophic ecology in these sunfish.

Table 1.1: ANCOVA model testing for a relationship between stable isotopes ($\delta^{13}C$ or $\delta^{15}N$) and sunfish ecomorph (littoral or pelagic) and tissue type (white muscle or liver), with body weight as covariate, and their interactions. * indicates statistical significance.

	$\delta^{13}C$			$\delta^{15}N$		
	F	df	P	F	df	P
Model Factors						
Body size (g)	0.35	1,92	0.56	1.27	1,92	0.26
Sunfish Ecomorph	12.40	1,92	<0.001*	0.58	1,92	0.45
Tissue Type	0.51	1,92	0.48	217.68	1,92	<0.001*
Interactions						
Body Size - Ecomorph	1.39	1,92	0.24	1.63	1,92	0.20
Body Size – Tissue	0.19	1,92	0.67	0.26	1,92	0.61
Ecomorph – Tissue	1.39	1,92	0.24	0.13	1,92	0.72
Body Size - Ecomorph – Tissue	0.002	1,92	0.96	0.23	1,92	0.63

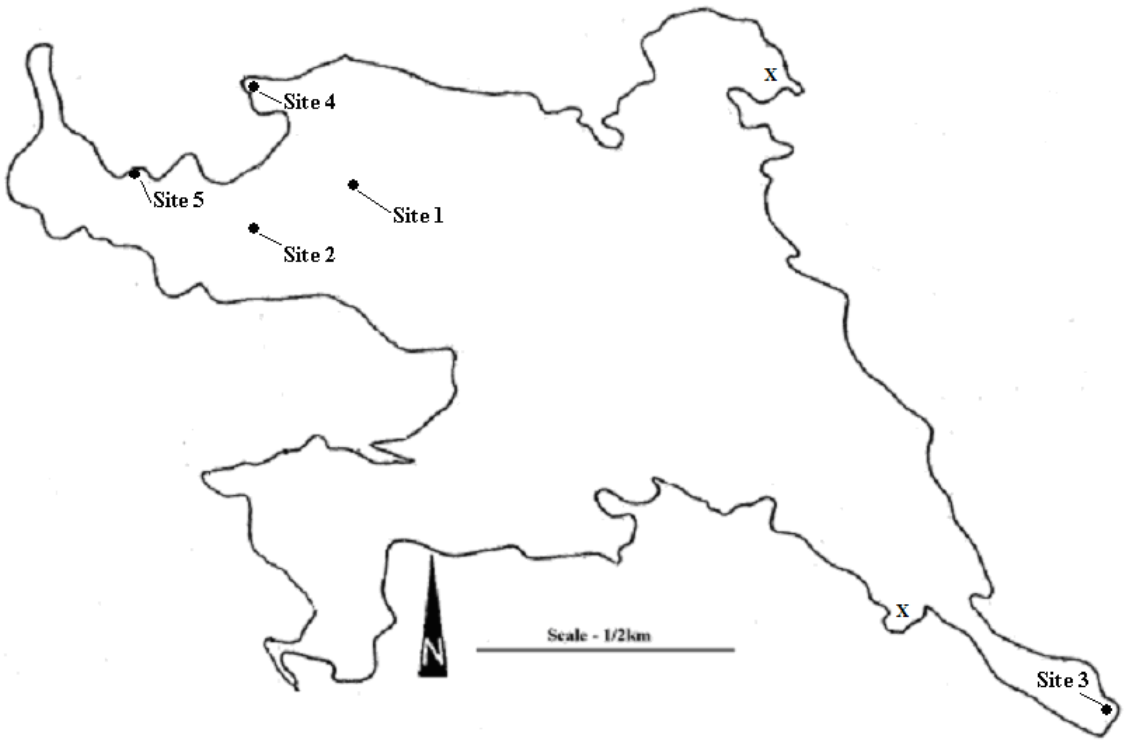


Figure 1.1: Location of the pumpkinseed sunfish sampling sites within Ashby Lake. Pelagic sunfish were sampled from Sites 1 and 2 located at rocky shoals in the pelagic habitat. Littoral sunfish were sampled from Sites 3-5 located near shore within areas of emergent and floating macrophytes. Littoral prey reference samples were collected from the sunfish sampling sites plus two additional sites (X). Pelagic reference samples were collected from depths >15m surrounding Sites 1 and 2.

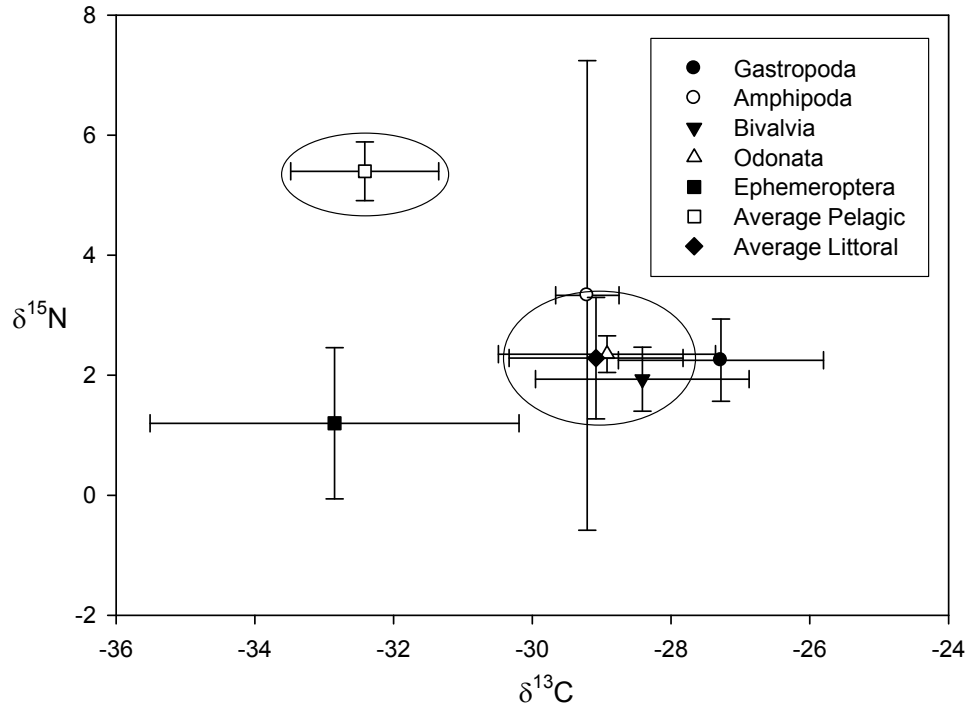


Figure 1.2: Mean $\delta^{13}C$ and $\delta^{15}N$ values (±1SE) of five benthic macroinvertebrate prey orders (gastropoda, bivalvia, amphipoda, odonata, ephemeroptera) sampled from littoral sites and mixed samples of zooplankton (copedpoda and cladocera) collected from pelagic sites. The mean signal of all littoral prey (averaged over the five groups), and pelagic zooplankton prey are centered in each ellipse with a radius of 1SE.

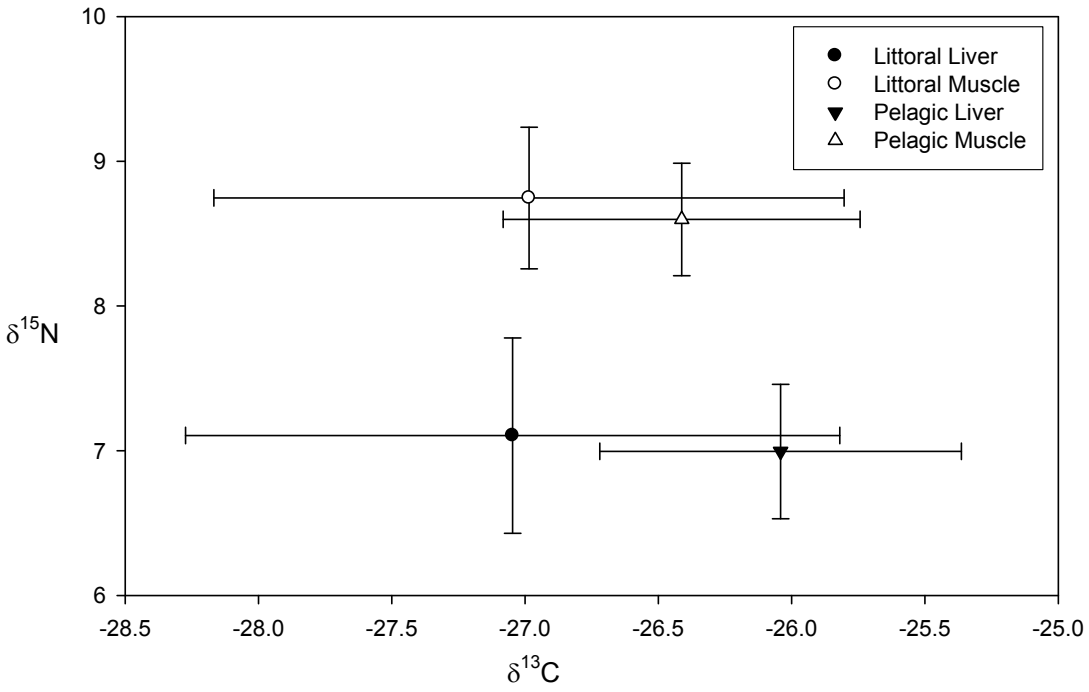


Figure 1.3: Mean $\delta^{13}C$ and $\delta^{15}N$ values (±1SE) of pumpkinseed sunfish ecomorphs sampled from the littoral (circles) and pelagic habitats (triangles) broken down by tissue type (white = white muscle and black = liver).

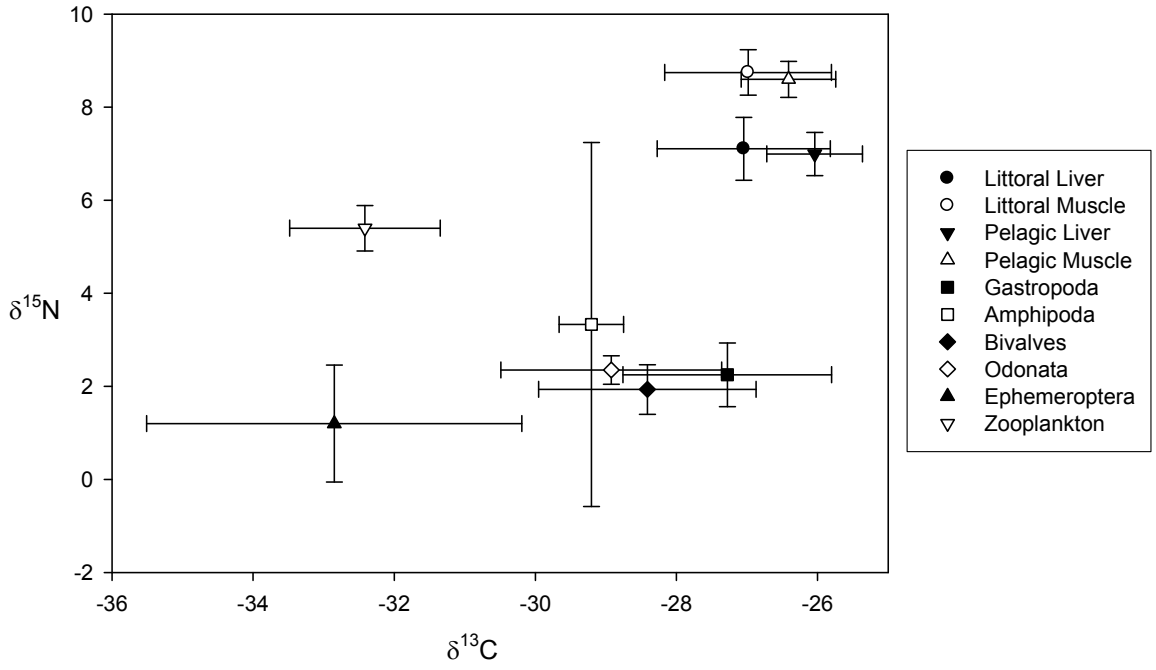
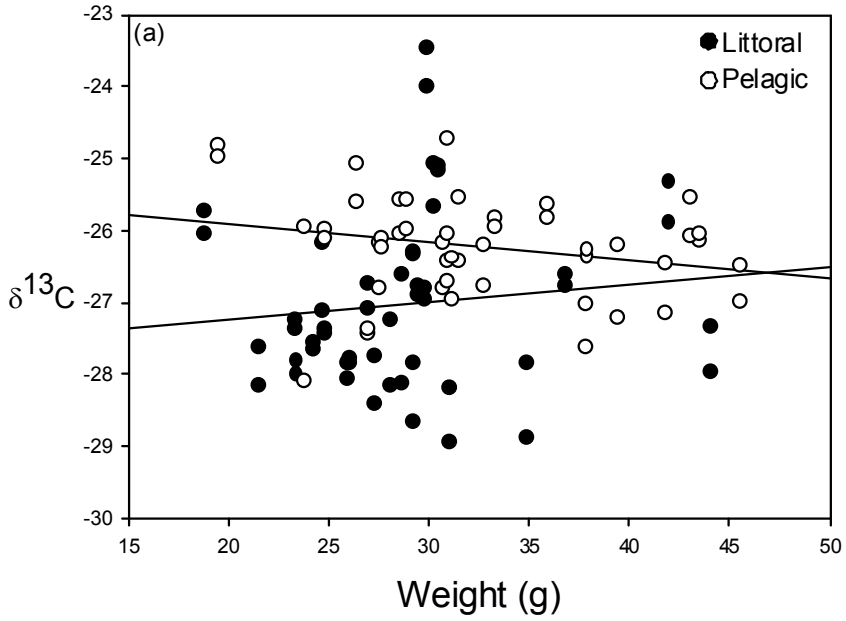
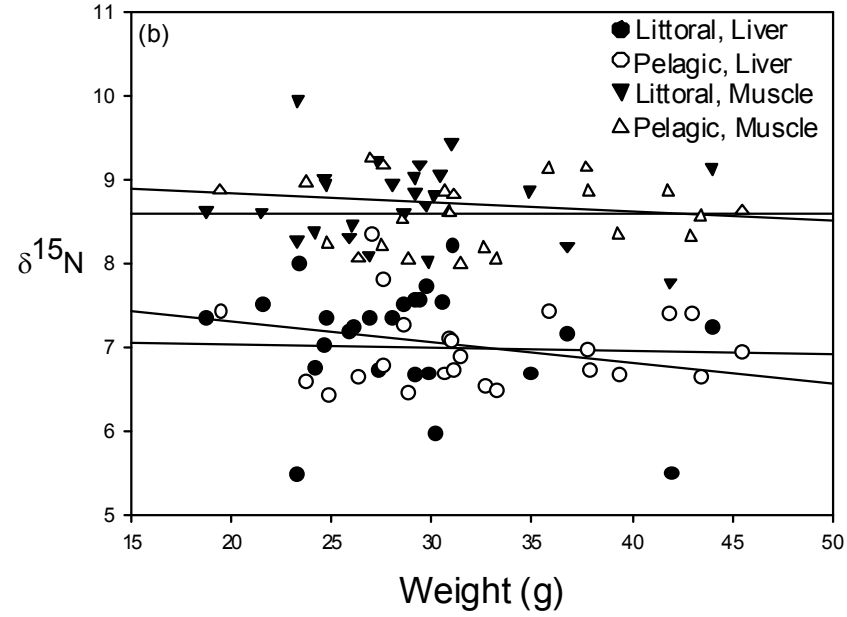


Figure 1.4: Variation in mean $\delta^{13}C$ and $\delta^{15}N$ (±1SE) values of pumpkinseed sunfish (by tissue type) relative to that of their possible prey reference mean values.


(a)
Littoral
Pelagic
δ13C
-23
-24
-25
-26
-27
-28
-29
-30
15
20
25
30
35
40
45
50
Weight (g)


(b)
Littoral, Liver
Pelagic, Liver
Littoral, Muscle
Pelagic, Muscle
δ15N
11
10
9
8
7
6
5
15
20
25
30
35
40
45
50
Weight (g)

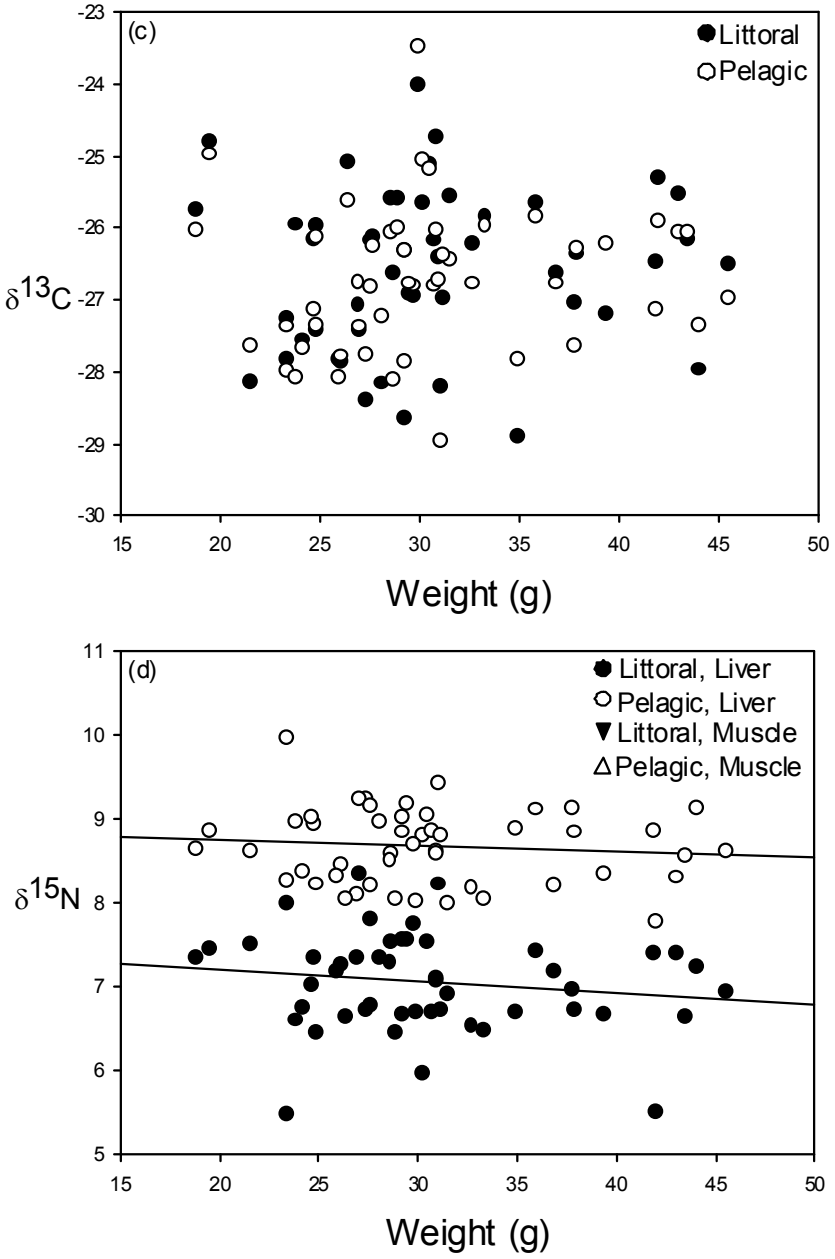


Figure 1.5: Relationships between stable isotope values and pumpkinseed sunfish body size (weight in g). (a) $\delta^{13}C$ values related to weight for each ecomorph (littoral or pelagic; tissue type combined), (b) $\delta^{15}N$ related to weight for each ecomorph and tissue type (white muscle or liver), and (c-d) respectively $\delta^{13}C$ and $\delta^{15}N$ related to body size for each tissue type (sunfish ecomorphs combined).

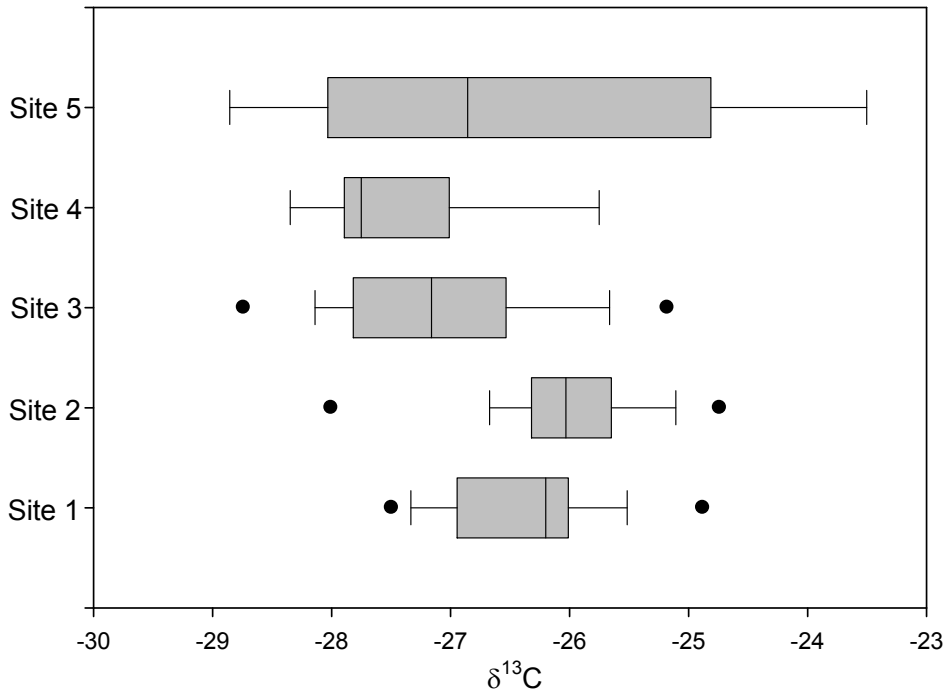
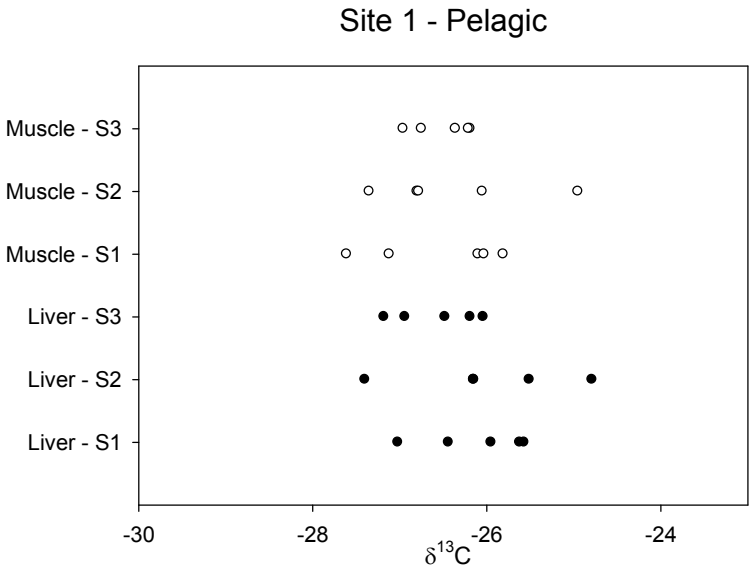
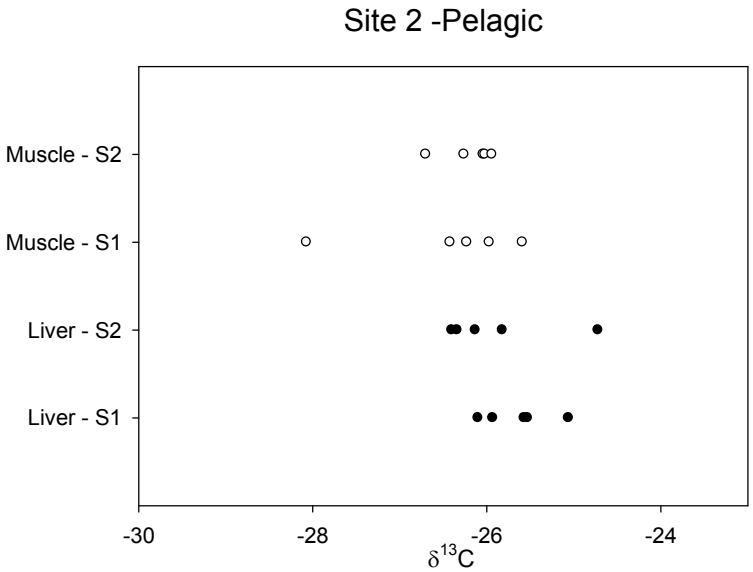
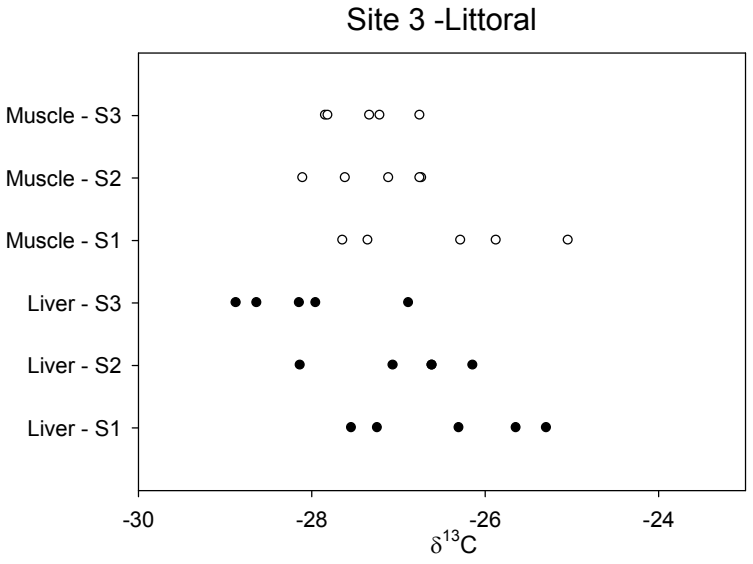


Figure 1.6: Boxplots of pumpkinseed sunfish $\delta^{13}C$ values (±1SE) from five sampling sites in Ashby Lake (Sites 1-2 are in the pelagic habitat and Sites 3-5 are in the littoral habitat). Stable isotope values were estimated from white muscle and liver tissue collected from each fish. Boxes represent the interquartile range (inner 50% of observations) separated by the median, whiskers represent the 90th and 10th percentile limits, and dots represent the 5th and 95th percentiles.


Site 1 - Pelagic
Muscle - S3
Muscle - S2
Muscle - S1
Liver - S3
Liver - S2
Liver - S1
-30
-28
-26
-24
δ13C


Site 2 -Pelagic
Muscle - S2
Muscle - S1
Liver - S2
Liver - S1
-30
-28
-26
-24
δ13C


Site 3 -Littoral
Muscle - S3
Muscle - S2
Muscle - S1
Liver - S3
Liver - S2
Liver - S1
-30
-28
-26
-24
δ13C

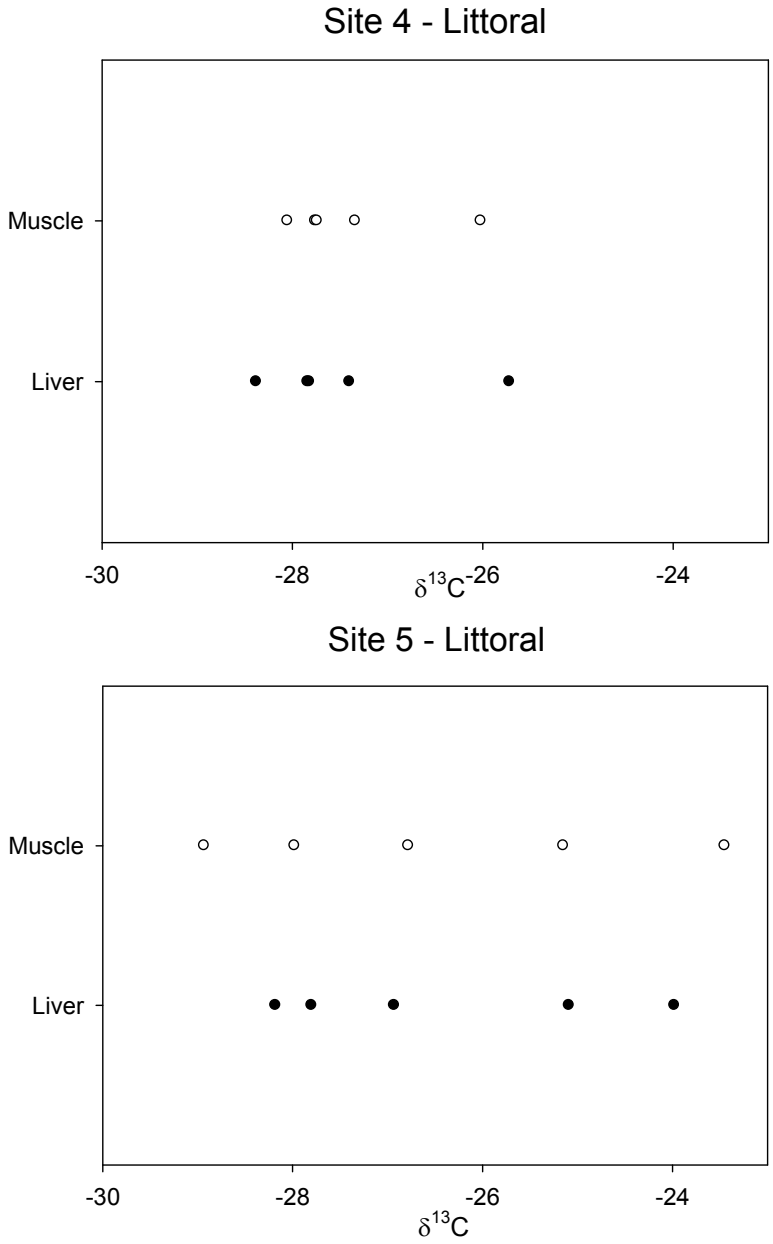
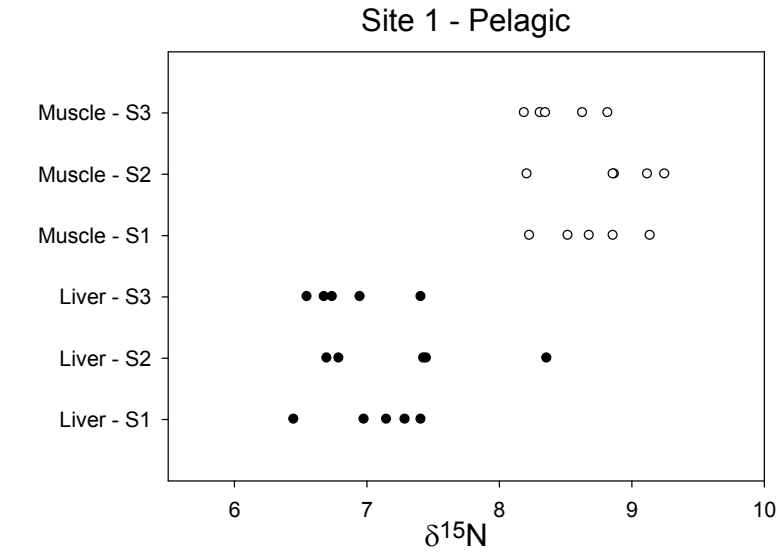
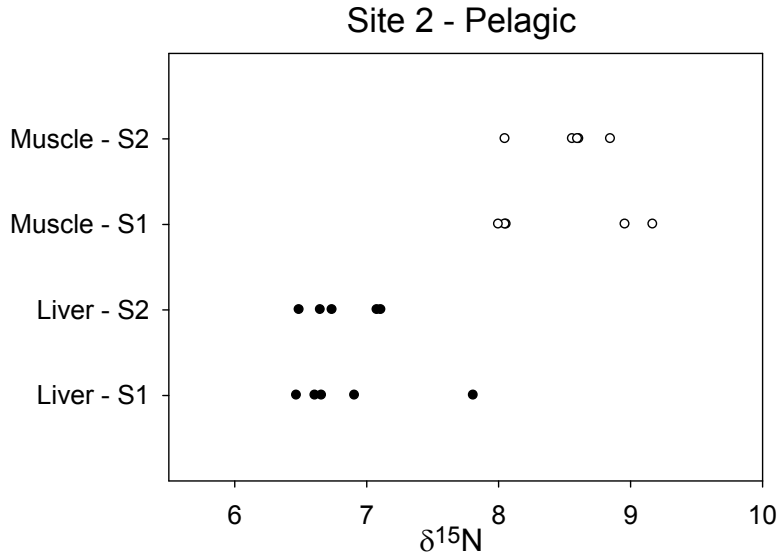
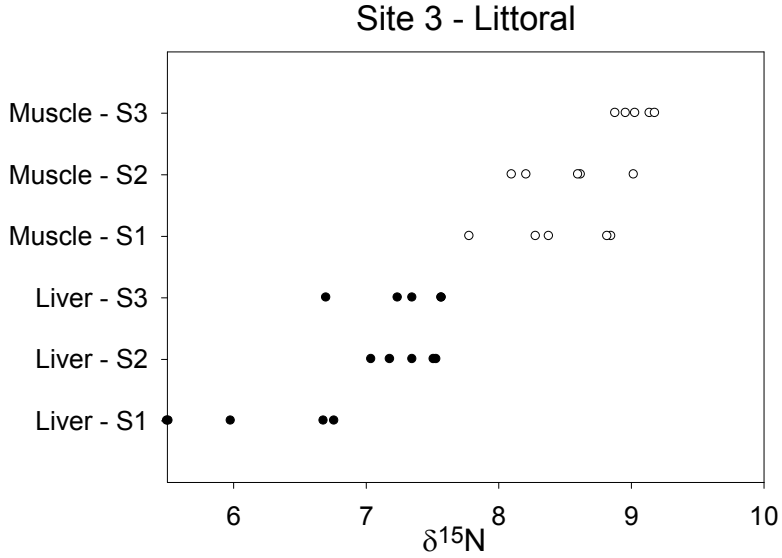


Figure 1.7: Variation in $\delta^{13}C$ values of individual pumpkinseed sunfish within and among up to five sites in Ashby Lake (two pelagic sites and three littoral sites), two tissue types (white muscle or liver), and up to three sampling periods.


Site 1 - Pelagic
Muscle - S3
Muscle - S2
Muscle - S1
Liver - S3
Liver - S2
Liver - S1
6
7
8
9
10
δ15N


Site 2 - Pelagic
Muscle - S2
Muscle - S1
Liver - S2
Liver - S1
6
7
8
9
10
δ15N


Site 3 - Littoral
Muscle - S3
Muscle - S2
Muscle - S1
Liver - S3
Liver - S2
Liver - S1
6
7
8
9
10
δ15N

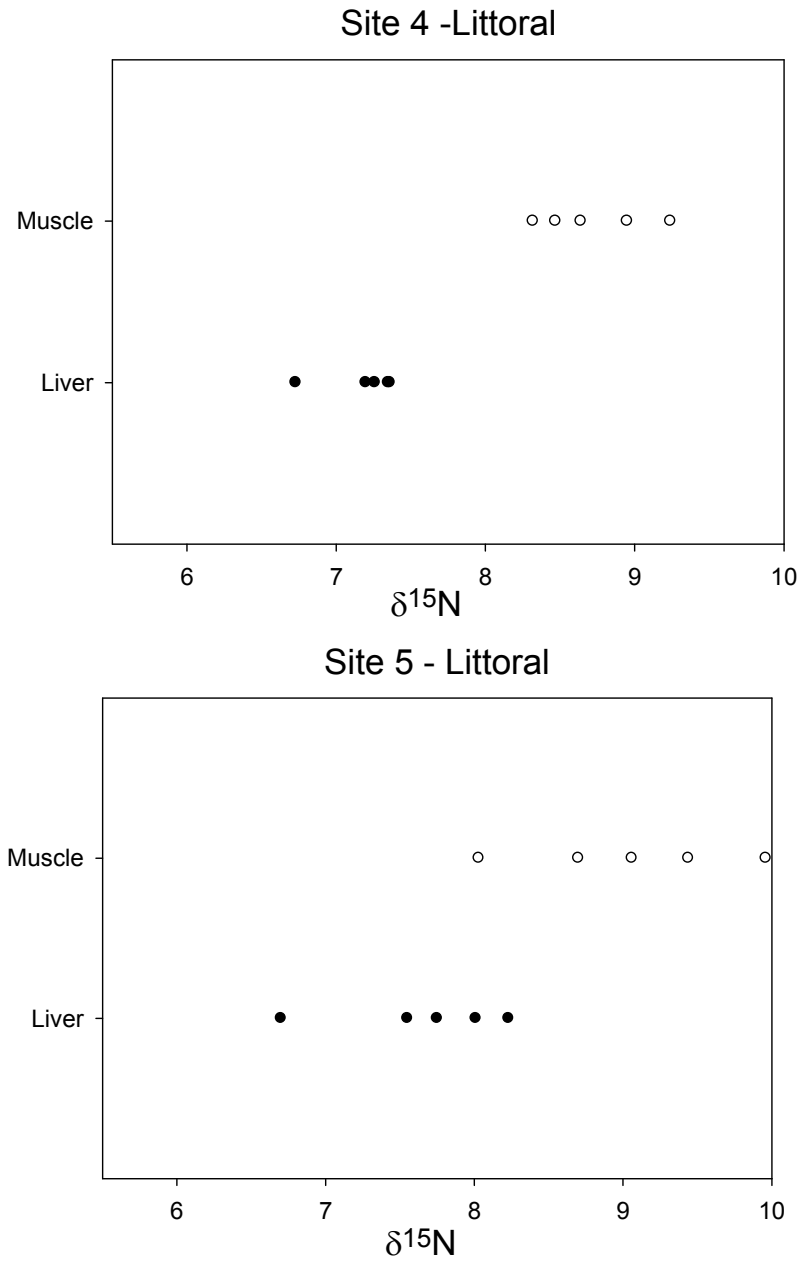


Figure 1.8: Variation in $\delta^{15}N$ values of pumpkinseed sunfish among five sites in Ashby Lake (two pelagic sites and three littoral sites), two tissue types (white muscle and liver) and up to 3 sampling periods.

Chapter 2

Experimental test of intraspecific competition and resource availability in the evolution of polymorphic pumpkinseed sunfish (*Lepomis gibbosus*)

Abstract

I examined the role of resource-mediated intraspecific competition in the diversification of pumpkinseed sunfish (*Lepomis gibbosus*). These sunfish usually inhabit the littoral habitat where they feed on benthic macroinvertebrate prey (ancestral form), but in some lakes they are also found in the pelagic habitat feeding on zooplankton (derived form). This polymorphism may have evolved because intraspecific competition in the littoral habitat favoured individuals who used resources in the pelagic habitat. Thus, two critical factors related to adaptive diversification are, 1) density-dependent competition differentially affecting the relative fitness of individuals feeding on different resources, and 2) availability of pelagic zooplankton as an alternate resource. I tested each of these factors in a manipulative field experiment involving experimental enclosures established in the littoral habitat of a lake. Sunfish density and the availability of zooplankton resources were manipulated in a fully crossed design and relative fitness was assessed as mortality and body condition for both ecomorphs stocked together in all treatments. Fitness did not vary with density treatments, presumably because variation in density did not result in variation in competition, which appeared to be high in all treatments. The addition of zooplankton increased mortality of both sunfish ecomorphs, although pelagic ecomorphs that survived were in better condition than surviving littoral sunfish. Zooplankton prey appear to be a more costly resource than

previously appreciated, perhaps requiring more specialization for effective use. The costs of zooplankton as an alternate resource represent a previously unexplored factor limiting adaptive diversification of pumpkinseed sunfish and possibly other fishes of postglacial lakes.

Introduction

The origin and maintenance of adaptive species-level diversity is widely thought to be influenced by many ecological factors including habitat type, available resources, competition (Bolnick 2004, Jastrebski and Robinson 2004, Pfennig et al. 2007), predation (Dewitt et al. 2000, Meyer and Kassen 2007), parasitism (Wiegmann et al. 1993, Nunn et al. 2004) and mutualism (Lutzoni and Pagel 1997, Kato et al. 2003). Adaptive diversity can develop between species (Darwin 1859, Hanson and Leggett 1985), among populations within species (Ehrlich and Raven 1969, Robinson 2000, Hegrenes 2001) and among individuals coexisting in a single population (Skulason and Smith 1995, Bolnick et al. 2003) when diversifying selection favours alternate phenotypes under different ecological conditions. Thus, it is through the study of mechanisms causing specialization that we can explore how polymorphic populations arise and are maintained.

Competition is a widely studied species interaction that encompasses a very complex and diverse group of interactions that can result in diversification. Competition is often associated with interactions between two or more species (interspecific competition; Werner and Hall 1976, Mittelbach 1984, Garvey et al. 2002, Gray et al. 2005, Pfennig et al. 2007). Although it is difficult to empirically test, interspecific

competition has been commonly studied because it is thought to contribute strongly to the adaptive diversification of populations and species by ecological character displacement (Schluter 1996a, Pfennig et al. 2007).

Resource competition occurs when organisms share a common resource that is required for survival and is of limited availability (Birch 1957, McIntosh 1992, Rubenstein 1981). One outcome of intense competition is population decline or even extinction. However, another possible outcome is an evolutionary response by a population away from the competitively limited resources and towards alternate resources if they are available. Variation in fitness due to competitive interactions may generate selection that favours phenotypes that do not use shared resources over phenotypes that do when availability of the shared resource declines (Pfennig et al. 2007). This results in resource-related characters evolving in the population. Three conditions are required for adaptive diversification, (1) resource competition, (2) the availability of alternate resources, and (3) the presence of genetic variation in the population (Parsons and Robinson 2006). In highly competitive communities, unfilled niches are expected to be rapidly filled because individuals can increase their fitness by occupying an alternate niche, resulting in endemism in isolated communities such as on islands (Schluter 2000, 2001, Kleindorfer et al. 2006).

The role of interactions between species in diversification is relatively well developed, but interactions within species can also play an important role. Competition among individuals within a single species (intraspecific competition) is expected to have significant ecological effects, such as limiting population growth, but it may also drive evolutionary change (Skulason and Smith 1995, Schluter 1996a, Smith and Skulason

1996). The role of intraspecific competition in adaptive divergence has been studied in several groups including insects (Bolnick 2001), amphibians (Pfennig et al. 2007) and fishes (Greenberg et al. 1997, Gross 1991). Selection can cause the adaptive divergence of phenotypes within a population when (a) the environment is heterogenous with respect to biotic or abiotic factors (eg. resource availability, competitors, predation risk, physiologically important abiotic factors) that influence fitness, (b) individual fitness is increased by using alternate resources as a way of avoiding density-dependent competition for increasingly rare resources, and (c) functional trade-offs in feeding performance on alternate resources favours specialization. If this last condition is not met then a single generalist phenotype may be favoured and little diversity would be expected across a wide range of resources being utilized.

The intensity of competition is determined by the number of individuals interacting and is therefore most often determined by the density of consumers and resources. At low consumer densities and high resource availability selection has a minimal role in changing the growth of a population or changes in phenotypes because there is little competition. As consumer density increases, resource density decreases and competition has an increased role in the fitness of individuals and possible effects on the evolution of new phenotypes. With this increase in competition there is now selection favouring those who can best use the resource and for phenotypic changes to use alternate resources (if available).

Freshwater communities provide the opportunity to study populations that have undergone resource-related divergence because there is some understanding of the structure of their heterogenous environments. Freshwater lakes have two general habitat

types (littoral and pelagic) that are very distinct from each other in structure, species assemblages, and available resources (Schluter 1995, Schindler and Scheuerell 2002). There are differences in which prey resources are most abundant in each habitat. Littoral habitats consist of dense vegetation supporting a complex community of often cryptic benthic macroinvertebrate taxa, while pelagic habitats are characterized by the open water column with abundant zooplankton dispersed throughout. These habitat differences appear to favour the evolution of fish phenotypes suited to each lake habitat and can therefore promote divergence in the phenotypes of life-history forms, ecomorphs and potentially species (Schluter 1996b, Robinson and Schluter 2000).

Postglacial lakes provide us with an opportunity to study divergence in young populations that have been present for ~10,000 years, establishing after the recession of glaciers (Robinson 2000, Gray et al. 2005). Postglacial lakes also present us with the opportunity to study communities that have simpler species assemblages then other fresh water communities, such as those found in relatively warmer lakes (Parsons and Robinson 2006). Furthermore, these lakes also represent environments that are more likely to have resources that are not being utilized or are being underutilized because many of the traditional consumers found in similar habitats may not have colonized them after the glaciers receded. Adaptive intraspecific diversity may be more likely in postglacial lakes because there are fewer interspecific interactions (predators, parasites, or interspecific competitors) allowing more ecological opportunities to exist for the species that are present.

Pumpkinseed sunfish (*Lepomis gibbosus*) are one of the most abundant fish species found in postglacial lakes of North America. They are found at the northern

limits of most sunfish species (Scott and Crossman 1998). These sunfish commonly inhabit rivers and the littoral zone of lakes, co-existing with yellow perch (*Perca flavescens*) and other sunfish, such as the bluegill (*Lepomis macrochirus*) and rock bass (*Ambloplites rupestris*; Jastrebski and Robinson 2004). Pumpkinseed sunfish feed on benthic macroinvertebrates, such as snails and bivalves (Keast 1978, Werner and Hall 1979). This form of pumpkinseed sunfish seems to be a result of interspecific competition, especially with bluegill sunfish that outcompete pumpkinseed sunfish for zooplankton resources (Werner and Hall 1976, Keast 1977). There are differences in the distributions of pumpkinseed and bluegill sunfish (Scott and Crossman 1998), meaning that in some areas there are populations of pumpkinseed sunfish without one of their strongest competitors.

Some pumpkinseed populations appear to be diverging along the classic littoral-pelagic resource axis, with an ancestral littoral ecomorph having a diet of benthic macroinvertebrates and a pelagic ecomorph feeding on zooplankton in addition to other benthic invertebrate prey when available (Robinson et al. 1993, Robinson et al. 2000, Gillespie and Fox 2003, Jastrebski and Robinson 2004). To date, this divergence is found only in the absence of bluegill sunfish (Robinson et al. 1993, Gillespie and Fox 2003), suggesting that interspecific competition shapes most pumpkinseed sunfish populations. However, these divergent populations also provide us with an opportunity to test the role of intraspecific competition in adaptive diversification.

Empirical testing of intraspecific adaptive diversification is still rare (Schluter 1995, Bolnick 2001, 2004) and generally focuses on testing if competition affects the relative fitness of individuals. I used prey additions to manipulate the prey spectrum

available and also altered density to manipulate the intensity of competition. These manipulations allowed me to study how competition, resource diversity, and functional trade-offs in feeding performance affect the relative fitness of divergent pumpkinseed sunfish ecomorphs.

The adaptive evolutionary model of polymorphism in pumpkinseed sunfish is best laid out in a series of steps. This model starts with ecological conditions with fitness consequences. These fitness differences then resulted in selection favouring new phenotypes and the formation of littoral and pelagic sunfish ecomorphs. The model proposes that ancestral population of pumpkinseed sunfish were found in the littoral habitat with abundant benthic macroinvertebrate species forming their resource spectrum. Over time the density of the ancestral littoral pumpkinseed sunfish increased, reducing the benthic macroinvertebrate availability which then resulted in greater intraspecific competition and a decrease in individual fitness. Increased competition in the littoral habitat then favoured any individuals that used alternate resources, such as pelagic zooplankton.

The first steps in this evolutionary model proposed that density-dependent competition affects the fitness of individuals. Specifically, that fitness at high sunfish density would be less than at low sunfish density in the littoral habitat. To test this, the fitness of pumpkinseed sunfish must be compared when benthic resources are abundant and when they are limited, accomplished by manipulating fish density.

A necessary condition of the evolutionary model is the availability of an alternate resource. In a population that is experiencing intense intraspecific competition, a replaceable resource will allow individuals that to use those resources to have higher

fitness than those that do not. In the pumpkinseed sunfish system, the alternate resource appears to be pelagic zooplankton. When littoral benthic macroinvertebrate resources are low in availability the availability of significant zooplankton should the fitness of pumpkinseed sunfish that are able to eat them. To test this component of the model, I compared the fitness of pumpkinseed sunfish in the presence and absence of zooplankton when benthic resources were rare.

The evolutionary model also proposes that selection in the pelagic habitat favours specialization to consume zooplankton rather than a generalist phenotype. Ecological specialization implies trade-offs associated with feeding on zooplankton versus benthic macroinvertebrate species (Robinson et al. 1996, Parsons and Robinson 2007). If trade-offs occur as a result of consuming different prey then when benthic macroinvertebrates are rare and zooplankton is available the specialized pelagic ecomorph of pumpkinseed sunfish should have higher fitness than the littoral ecomorph. To test this prediction, I compared the fitness of pumpkinseed sunfish ecomorphs when benthic macroinvertebrates were rare, but zooplankton was available.

A field experiment was used to manipulate pumpkinseed sunfish density, resource availability, and the phenotype of sunfish (in this case ecomorph) by setting up enclosures in the littoral habitat of a lake. This experiment represents an opportunity to test multiple factors involved in the adaptive diversification of pumpkinseed sunfish in a single experiment. Testing the effects of competition and resource availability on fitness and for functional trade-offs associated with feeding should increase our understanding of the development of polymorphic populations.

Materials and Methods

Experimental Design

I used enclosures constructed in the littoral habitat of a natural lake to test if intraspecific competition and resource diversity affected resource use and short term fitness of pumpkinseed sunfish originating from littoral and pelagic habitats. My strategy was to manipulate intraspecific competition by changing the total fish density in each enclosure (high vs. low) while maintaining equal proportions of each type of sunfish, and to also manipulate the available prey resource spectrum by either supplementing the ambient benthic macroinvertebrate prey with pelagic zooplankton or not in a fully crossed design. The respective treatments were: (T1) 1 littoral + l pelagic sunfish, benthic macroinvertebrate prey; (T2) 2 littoral + 2 pelagic sunfish, benthic macroinvertebrate prey; (T3) 1 littoral + l pelagic sunfish, benthic macroinvertebrate prey supplemented with added pelagic zooplankton; (T4) 2 littoral + 2 pelagic sunfish, benthic macroinvertebrate prey supplemented with added pelagic zooplankton.

Study Lake

Ashby Lake is located in the Mazinaw region of Ontario (45˚05'N, 77˚21'W) where multiple lake populations of polymorphic pumpkinseed sunfish have been identified and studied (Gillespie and Fox 2003, Jastrebski and Robinson 2004). This lake occurs in the Canadian Shield and is characterized by extensive rocky pelagic shoals rising to just below the water surface where pumpkinseed sunfish congregate and feed on zooplankton. The littoral habitat is relatively small, representing less than 10% of the total lake area, due to a shoreline that rapidly drops off into pelagic habitat. However,

several shallow bays contain vegetation and provide ideal littoral habitat for pumpkinseed sunfish.

Enclosure Design and Deployment

Pumpkinseed sunfish were stocked into enclosures constructed out of semi-rigid polyethylene plastic fencing (3.2 mm mesh size) that enclosed an area of ~2m^2 of littoral substrate. Enclosures had a minimum water depth of 0.75m and a maximum depth of 1.25m, although this varied somewhat over the course of the experiment due to natural fluctuations in lake level. Posts anchored the enclosures against wave and wind action and stakes sealed the bottom of the enclosures to the sediment. A polyethylene fiber sheet (<1mm mesh size) was wrapped around enclosures receiving zooplankton additions in order to reduce the loss of zooplankton while still permitting water exchange. All native fish present in enclosures were removed by trapping prior to stocking enclosures with experimental fish.

Enclosure Placement and Monitoring

Ten replicates of each treatment were created for a total of 40 enclosures that were arrayed in a block design across a variety of littoral sites. These sites were selected on the basis of three primary characteristics: littoral zone depth (0.75 – 1.25m); quantity of floating or emergent vegetation (in amounts similar to sites were sunfish occurred naturally); and total littoral patch size (large enough to hold at least one complete experimental block without obvious variation in conditions among enclosures).

Five sites were identified in natural bays that met these criteria (1 inlet, 1 outlet and 3 without surface influent flows) and two experimental blocks were installed in each bay. Enclosure security and water temperature were checked daily. Three enclosures developed holes and were excluded from analyses.

Fish Capture and Initial Processing

Pumpkinseed sunfish were collected from multiple littoral and pelagic habitats by either angling or the use of large funnel traps. All fish stocked into the four treatments of a block were collected at the same time and systematically assigned to treatment groups. Prior to stocking, fish were anaesthetized for 5 minutes in benzocaine (stock solution created by adding ethyl-*p*-aminobenzoate to ethanol for a 2.5g/L solution, then diluted in 10L of water to a final concentration of 0.03mL/mL water), photographed (lateral digital image), weighed and dorsal spine clipped for unique identification. After handling they were placed in a 20g/L sea salt bath for 20 minutes for recovery and to reduce risk of infection.

Study Period

Fish lived in enclosures over 42 days from June - August 2007. Some mortality was expected and so all enclosures were monitored daily for dead fish, which were identified and replaced to maintain the treatment density of fish in each enclosure. Fish replaced during the first 28 days of the experiment were included in the analysis, while fish restocked after this point were not included in analyses.

Prey

Resources consisted of ambient macroinvertebrate prey present when an enclosure was installed in the littoral habitat. Pelagic zooplankton was collected daily from near shoals where the pelagic sunfish were collected and added live to treatments 3 and 4. Zooplankton was collected using vertical tows of a volume of water approximately equal to that of the average enclosure from a maximum depth of 15m. Live zooplankton were added to enclosures immediately after collection, dispersal was facilitated by gently mixing the upper water layers with a paddle.

Fish Retrieval

Fish were retrieved from enclosures on their 42nd day in the experiment by any of three methods applied in order: angling; bubbling CO_2 into an enclosure after wrapping its circumference in plastic in order to force fish to the surface for dip-netting; dip-netting of fish after removing all vegetation from the enclosure. Fish were euthanized in benzocaine (concentration as above) for approximately 10 minutes, identified, photographed, and blotted wet-weight was recorded.

Benthic Macroinvertebrate Sampling

Benthic invertebrates were collected inside and outside of enclosures by taking three Ekman grabs of the sediment and vegetation representing a bottom surface area of 74.25cm^2. Macroinvertebrates were then hand sorted through a series of nested sieves (mesh sizes of 12.7, 4, 2, 1 and 0.5 mm) and preserved in 70% alcohol. Samples were taken adjacent to the experimental enclosures in three randomly selected bays each week.

Benthic samples from inside of enclosures were collected only at the end of the study period so as not to remove prey available for feeding during the study. I compared the abundance and species richness of benthic invertebrates among treatment enclosures at the end of the experiment and between enclosures and natural conditions outside of enclosures.

Statistical Analyses

Differences in benthic macroinvertebrate prey species richness were compared between the different treatment groups using a blocked two-factor analysis of variance (ANOVA) with the factors: final sunfish density (no. of surviving fish) and diet treatment (zooplankton addition or not), their interaction, and type of bay as a blocking factor (inflow, outflow, or non-influent). Macroinvertebrate species abundance was also analyzed by ANOVA but without bay type as a factor as it was non-significant. Macroinvertebrate prey availability (abundance and species richness) inside and outside of enclosures were compared using a blocked one-factor ANOVA with location (inside vs. outside) as factor and bay type as a blocking factor.

I analyzed patterns of mortality among experimental pumpkinseed sunfish as a measure of fitness to test if it was related to ecomorph type, experimental treatment or their interactions. Relative mortality was analyzed with a 3-factor nested ANOVA consisting of 2 among-block factors (diet and density treatments) and 1 within-block factor (ecomorph) with diet and density being nested inside experimental enclosures. Relative mortality was calculated for each ecomorph in each enclosure as: the number of sunfish deceased divided by the total number of sunfish stocked into the enclosure. I

transformed this proportion using the arcsine transformation: 1/2 X [arcsin(√ Deceased / Total Input + 1) + arcsin(√ Dead + 1 / Input + 1], as described by Zar (1999).

I also analyzed patterns of change in body condition among surviving experimental sunfish as a second measure of (short term) fitness in the same way. Body condition was estimated following Williams (2000) as, $K= (100{,}000\ W) / L^3$, where K is body condition, W is the weight of the fish in grams and L is the standard length of the fish in mm. An initial estimate of body condition was made prior to placement in an enclosure and again at the end of the experiment (final condition measurement), and the loss in body condition was determined as the difference between the two measures (Initial body condition – Final body condition). In high density treatment enclosures, the mean body condition loss was determined for each ecomorph.

Loss of condition values were transformed using $\log_{10}$ (mean condition loss +1; Zar 1999). Transformed condition estimates were then compared using a three-factor ANOVA , factors: total enclosure density, diet, ecomorph, their interactions, and bay type as a blocking factor. This model was subsequently simplified by dropping the density factor (and all of its interactions) when effects of density were judged to be non-significant ($P > 0.05$).

All analyses were performed using the statistical software JMP IN (v. 5.1). P-values ≤ 0.05 were considered statistically significant although values from 0.05 - 0.10 were interpreted as weak evidence against the null hypothesis because of reduced control of additional unknown factors in field experiments.

Results

Effects of Sunfish Density on Littoral Prey Resources

The density of consumers should be related to the intensity of competition because increasing consumer numbers is expected to cause a decline in prey availability. After 42 days, benthic macroinvertebrate prey abundance inside the enclosures was not significantly different between high and low sunfish density treatments (ANOVA, $F_{1,11} < 0.01$, $P = 0.98$). There was weak evidence that benthic prey abundance was on average 38% greater in the zooplankton addition treatments compared to non-zooplankton addition treatments by the end of the study (ANOVA, $F_{1,11} = 3.40$, $P = 0.09$, Fig. 2.1). Neither sunfish density (ANOVA, $F_{1,9} = 2.15$, $P > 0.10$) nor zooplankton additions ($F_{1,9} = 0.11$, $P > 0.70$) affected the richness of benthic macroinvertebrate species among treatment groups. These results indicated that competition was either uniform and high among treatments or nonexistent.

Experimental sunfish inside enclosures likely experienced high levels of resource competition because macroinvertebrate prey abundance (quantified as number of individuals) was 52% less inside compared to outside of enclosures (ANOVA, $F_{1,30} = 33.62$, $P < 0.0001$, Fig. 2.2). The overall species richness did not vary significantly inside compared to outside of enclosures (ANOVA, $F_{1,31} = 0.03$, $P = 0.87$).

There was weak evidence that benthic macroinvertebrate species richness inside of enclosures was influenced by the type of bay (ANOVA, $F_{2,9} = 3.46$, $P = 0.08$), with the inflow bay having the highest species richness, followed by bays without influent water sources, and richness being lowest in the outflow bay (Fig. 2.3). The type of bay also

influenced the benthic macroinvertebrate species richness outside of enclosures (ANOVA, $F_{2,31} = 3.27$, $P = 0.05$, Fig. 2.3).

Effect of Density on Pumpkinseed Sunfish Fitness

I have established that resource levels were very low (relative to outside of enclosures) inside all sunfish enclosures. This likely contributed to the unexpectedly high 65% mortality of experimental sunfish in this study, but also provided an opportunity to test if mortality varied with any experimental factors. Mortality varied little with fish density (ANOVA, $F_{1,32} = 0.788$, $P > 0.10$; Fig. 2.4a), between ecomorphs (ANOVA, $F_{1,32} = 0.70$, $P = 0.41$), and the effect of density on mortality did not vary between the two sunfish ecomorphs (ANOVA, ecomorph X density interaction: $F_{1,32} = 2.57$, $P = 0.12$, Fig. 2.5).

Mortality is an important component of fitness, but patterns of mortality here may have been too coarse grained to relate to my experimental factors. I also compared body condition among surviving pumpkinseed sunfish because condition may contribute more subtly to sunfish fitness. Thirty-five percent of the pumpkinseed sunfish stocked into enclosures survived (including those from the starting day and those added over the first 28 days), and all but one lost body condition over the course of the experiment. I analyzed variation in the loss of condition (so that low values equate with higher fitness). Body condition loss was not related to density (Fig. 2.6a) nor to interactions involving density (ANOVA, all main effects: $F_{1,28} \leq 0.75$, all $P \geq 0.40$). Loss of body condition was the same in the low and high sunfish density treatments (7.1% and 6.9% respectively).

Effect of Zooplankton Prey Addition on Pumpkinseed Sunfish Fitness

Contrary to predictions, sunfish mortality increased on average by 27% with zooplankton additions (ANOVA, $F_{1,32} = 5.73$, $P < 0.05$; Fig. 2.4b). I also expected that zooplankton additions would reduce competition (and so mortality), particularly at high fish densities, but this was not found (ANOVA, diet X density interaction, $F_{1,32} = 0.19$, $P > 0.10$, Fig. 2.7). Zooplankton had the unusual effect of generally increasing sunfish mortality regardless of treatment density. Body condition loss in surviving sunfish did not vary significantly between zooplankton addition treatments (ANOVA, $F_{1,32} = 0.07$, $P = 0.79$, Fig. 2.6b).

Zooplankton additions were predicted to enhance the fitness of pelagic sunfish relative to littoral sunfish under competition if phenotypic differences between ecomorphs influence resource use. The increase in sunfish mortality with the addition of zooplankton did not vary significantly between sunfish ecomorphs (Fig. 2.8, littoral = 22.7%, pelagic =29.5%; ANOVA, ecomorph X diet interaction: $F_{1,32} = 0.34$, $P = 0.57$).

Effect of Sunfish Ecomorph on Fitness

The results above indicate uniformly high levels of resource competition among treatments resulting in general in the loss of body condition, increased mortality, and also a strong negative effect of zooplankton addition on sunfish survival. The question I now address is whether sunfish ecomorphs responded to combinations of these strong negative effects in the same or different ways.

Sunfish ecomorphs had different mortality responses to the effects of density and zooplankton (ANOVA 3-way interaction among diet, density and ecomorph, $F_{1,32} = 4.61$, $P = 0.04$). Pairwise comparisons showed that much of the difference in mortality between ecomorphs was related to the zooplankton treatment (see above), but also that sunfish density differentially affected mortality between ecomorphs (Table 1.1). In treatment groups without zooplankton additions, littoral ecomorph mortality increased with fish density ($P < 0.05$), whereas there was no corresponding change in pelagic ecomorph mortality with density ($P > 0.10$). This suggests that littoral ecomorphs were actually slightly more sensitive to increased competition in extremely comeptitive littoral conditions than pelagic ecomorphs.

Loss of body condition among surviving sunfish also varied in complex ways between the two sunfish ecomorphs. Overall, the loss in body condition was similar between littoral and pelagic ecomorphs (ANOVA, ecomorph factor, $F_{1,32} = 0.56$ $P = 0.46$; Fig. 2.6c). However, there was weak evidence that the effect of zooplankton addition on body condition loss differed between ecomorphs. The loss of body condition in the littoral sunfish ecomorph increased by 62% with zooplankton additions, while the pelagic ecomorphs loss of condition decreased by 66% with zooplankton present (Fig. 2.9; ANOVA; diet X ecomorph interaction, $F_{1,32} = 3.20$, $P = 0.08$).

Discussion

My goal was to examine three major factors (consumer density, the role of replaceable resources, and phenotype) hypothesized to be involved with the formation and maintenance of the pumpkinseed sunfish polymorphism. I first examined the roles of

fish density and intraspecific competition in creating selection favouring the evolution of the pelagic phenotype. Then, I tested the role of the alternate resources (zooplankton) in this system. Finally, I examined how performance trade-offs may maintain phenotypic diversity between pumpkinseed ecomorphs.

Pumpkinseed Sunfish Density and Intraspecific Competition

If density dependent selection in littoral sunfish promoted adaptive diversification in this system, then it was predicted that sunfish fitness would decline with increased fish density. I found no evidence that either mortality or body condition changed with increased sunfish density within these experimental treatments. It seems likely that the fish density (1 or 2 fish/m^2) was too high for this 42-day experiment. Both fish density treatments dramatically reduced the number of benthic macroinvertebrate prey individuals by more than 50% compared to prey abundance outside of the enclosures where fish density was much lower. The number of prey taxa did not vary inside enclosures compared to richness in the natural littoral community outside of enclosures. Thus, it seems unlikely that reduced prey availability inside of enclosures resulted from a broad scale emigration of prey out of enclosures, and instead the reduced prey abundance is likely due to sunfish predation. It seems reasonable to conclude that both fish density treatments experienced very high levels of resource competition with obvious fitness consequences such as high mortality and significant loss of body condition. I provisionally conclude that fitness is reduced at high densities of fish relative to availability of resources.

I was surprised to find that the littoral sunfish ecomorph appeared more susceptible to increased fish density and reduced benthic macroinvertebrate prey abundance than the pelagic ecomorph. This result is interesting because it suggests that the littoral ecomorph may experience stronger density-dependent selection than the pelagic ecomorph. This is further evidence of sensitivity to density-dependent processes in littoral pumpkinseed sunfish that could lead to selection favouring adaptive diversification.

The differential response to density by pumpkinseed ecomorphs could represent another phenotypic difference between them that potentially affects fitness. One possible hypothesis is that the different response to density may be related to territorial behavior. The littoral habitat is structurally complex and has a variety of food resources. Snails and other macroinvertebrates are found on the submerged vegetation and on the surface of the benthos, while other prey are found among the upper layers of sediment. Habitat complexity and spatially predictable prey may favor territorial behaviour in littoral pumpkinseed sunfish when the benefits of territoriality outweigh its costs. Male nest defense also involves considerable territorial behaviour in pumpkinseed sunfish. Pelagic ecomorphs feed extensively in the water column where the dispersed distribution of zooplankton is unlikely to yield a significant energetic return to a fish engaging in territoriality. Instead, foraging success may be a function of rapidly finding and consuming prey in a competitive scramble. If ecomorphs vary in territorial behaviour, then littoral fish may have attempted to maintain a territory within the study enclosures, and would have paid the costs of territoriality without receiving sufficient resource

benefits due to limited availability of benthic resources. This may have resulted in increased mortality and greater loss of body condition with density.

These results provide some indirect support that density-dependent competition can contribute to the diversification of pumpkinseed sunfish ecomorphs, at least at the very high fish densities imposed here (up to 2 fish / m^2). At these densities, pumpkinseed sunfish rapidly lose body condition and face increased mortality. Any individuals that could exploit an alternate more abundant resource would likely be at a significant fitness advantage (Schluter 2003, Steele and Forrester 2005, Davey et al. 2006, Amundsen et al. 2007, Svanbäck et al. 2008). Future experimental studies should strive for a more realistic low fish density (low competition treatment) or a test of shorter duration in order to better test the density-dependence of competitive effects in these sunfish.

Role of Zooplankton in Phenotypic Diversification

The availability of alternate resources is a critical component of most models of adaptive resource diversification. Endemic fauna on oceanic islands are thought to have evolved because of the availability of alternate resources and minimal competition (Schluter 2000). The ubiquity of intraspecific diversity in postglacial fishes across the littoral-pelagic gradient is often interpreted in the same way (Robinson and Schluter 2000). As a consequence, I was surprised when my results indicated an unexpectedly high cost to consuming zooplankton for both ecomorphs. Zooplankton are a ubiquitous and often abundant resource in lakes, and in the absence of interspecific competitors (e.g., bluegill sunfish) it is thought to be available as a replaceable resource for pumpkinseed sunfish (Gillespie and Fox 2003, Jastrebski and Robinson 2004). However,

while my results indicated that the zooplankton addition treatments increased mortality in both sunfish ecomorphs, it resulted in less loss of body condition in pelagic compared to littoral ecomorphs. Therefore, the pelagic ecomorph seemed to have some benefit from the availability of zooplankton as a resource relative to littoral sunfish.

In order to understand the role of zooplankton in pumpkinseed sunfish diversification, it is important to better understand these patterns of effects. It is possible that not enough zooplankton were available to sunfish in this experiment because not enough were supplied, or they leaked out despite wrapping each enclosure with fine mesh. The availability of benthic macroinvertebrate prey was limited inside enclosures, and so it seems likely that competition for any added zooplankton resources would also have been strong. If zooplankton was too rare to supply significant amounts of nutrients for sunfish in the first place, then both sunfish ecomorphs would have been equally negatively affected, as reflected in the similarity in mortality between ecomorphs. However, if enough zooplankton were available to have some positive nutrient impact on sunfish, and pelagic sunfish possessed traits that allowed them to eat more than littoral sunfish, then the littoral ecomorph should have fared worse under competition than pelagic sunfish, as reflected in the greater loss of body condition in littoral compared to pelagic sunfish. Thus, my results cannot discriminate between these two alternatives. In the first case, the role of zooplankton would not have been well tested in this study, while in the second case, I would have evidence that zooplankton represent an alternate resource, although one with additional unknown costs.

Trade-offs Associated with Phenotypic Specialization

The final component of the pumpkinseed diversification model tested here predicted that strong trade-offs related to resource use occur in these sunfish. If pumpkinseeds are equally good at consuming macroinvertebrate prey and pelagic zooplankton (i.e., a weak trade off in feeding performance), then perhaps even weak density-dependent competition in the littoral population would drive some individuals to consume zooplankton prey. Evolutionary responses to selection in the pelagic habitat to improve feeding performance would not necessarily come at the expense of reduced performance on macroinvertebrate prey and so reflect generalist feeding ability. On the other hand, if sunfish feeding on macroinvertebrate prey were poor at consuming zooplankton prey and vice versa, as suggested in other studies (Robinson et al. 1996, Parsons and Robinson 2007), then density dependent competition among ancestral littoral ecomorphs would have to have been stronger before fish exploited pelagic zooplankton. Evolutionary responses to selection in the pelagic habitat for improved feeding on zooplankton would more likely come at the expense of reduced feeding performance on macroinvertebrate prey and so reflect some degree of phenotypic specialization on zooplankton prey. This second scenario is more likely to yield phenotypic diversification, such as resource polymorphism, than the first scenario.

I have some evidence that the pelagic ecomorph of pumpkinseed sunfish here exhibits some degree of specialization on zooplankton because pelagic fish lost less condition when zooplankton were available than did the littoral fish. The response to zooplankton addition suggests that pelagic sunfish were better able to use available zooplankton resources than the littoral ecomorph. Pelagic sunfish were not in better

condition than littoral sunfish when placed into the enclosures and so this does not explain the differential loss in condition. Morphology has been linked to feeding strategy in fishes through predictable co-variation between resource use, body shape and gill raker structure (e.g., Sanderson et al. 1991, Robinson et al. 1993, Robinson and Wilson 1994, Robinson et al. 2000, Gillespie and Fox 2003). From this I conclude that benthic macroinvertebrates and zooplankton are very different prey groups that can be considered alternate resources, and that this study provides further evidence of ecomorph specialization that would have occurred during adaptive diversification.

General Conclusions

My results suggest new features related to the evolution of sunfish polymorphism. First, zooplankton appear to be a costly alternative resource for littoral fishes, like pumpkinseed sunfish, and so may impose stronger selection for phenotypic specialization than initially anticipated. What is it about zooplankton that is so costly for these fish? Fish parasites (Marcogliese 1995) that use zooplankton as intermediate hosts may more easily infect littoral fish taxa because of little prior exposure, but it is unlikely this would have a major impact over the course of the experiment. Switching to a diet of zooplankton may also require extensive behavioural changes in order to optimize foraging returns against different risks of predation in the pelagic compared to littoral habitats. Finally, zooplankton are much smaller than many benthic macroinvertebrate prey groups and may have different nutritional quality. As a result, the energetic return from zooplanktivory and feeding on macroinvertebrates may be very different. There is evidence of adaptive diversity between these ecomorphs (Robinson et al. 1993, 2000,

Gillespie and Fox 2003, Jastrebski and Robinson 2004) which may improve capture and ingestion of zooplankton (Parsons and Robinson 2004).

Competition can promote diversifying selection when individuals vary in the intensity of resource competition, so that those in less competitive situations do better than those in more competitive situations. However, if the replacement of one resource with another is costly, then this will decrease the fitness advantage of the noncompeting phenotypes and so reduce the intensity of diversifying selection. The strength of diversifying selection is therefore a function of the variation in competitive intensity experienced by the population and the costs of replacing standard resources with novel ones. We are just beginning to understand and empirically study the costs of replacing resources in the context of competitively mediated divergence, and this study demonstrates the potentially high cost of switching to a new zooplankton resource. It is important for future work to consider the limiting role of resource replacement in adaptive diversification and to examine the possible trade-offs associated with resources as this is potentially a major factor that limits how species can diverge between resources.

Table 2.1: Comparison of the relative sunfish mortality (per enclosure) based on the density treatment, sunfish ecomorph and diet treatment. Relative mortality values indicate that mean (±1SE) for each category. * indicates that these values are significantly different (t-test, $P < 0.05$)

Ecomorph	Diet	Density	Relative Mortality
Littoral	Macroinvertebrate	Low	0.33 ± 0.33 *
		High	0.63 ± 0.29 *
Littoral	Macroinvertebrate + Zooplankton	Low	0.69 ± 0.38
		High	0.80 ± 0.24
Pelagic	Macroinvertebrate	Low	0.50 ± 0.33
		High	0.33 ± 0.38
Pelagic	Macroinvertebrate + Zooplankton	Low	0.66 ± 0.35
		High	0.83 ± 0.24

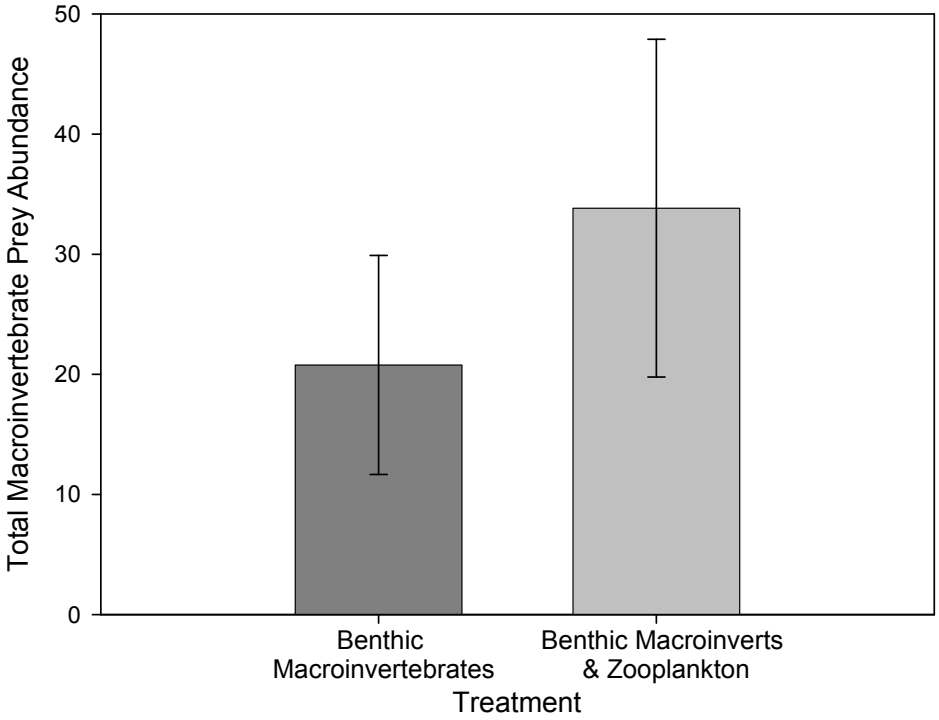


Figure 2.1: Total abundance per sample of all benthic macroinvertebrate species sampled from inside experimental enclosures for the two diet treatments. Bars represent the mean number of invertebrates per sample and enclosure (± 1SE). The macroinvertebrate diet represents ambient availability of the resources and no external additions, Macroinvertebrate and Zooplankton represents ambient with a daily addition of pelagic zooplankton to each enclosure. Total abundance represents the number macroinvertebrate organisms caught in a surface area of $0.74m^2$.

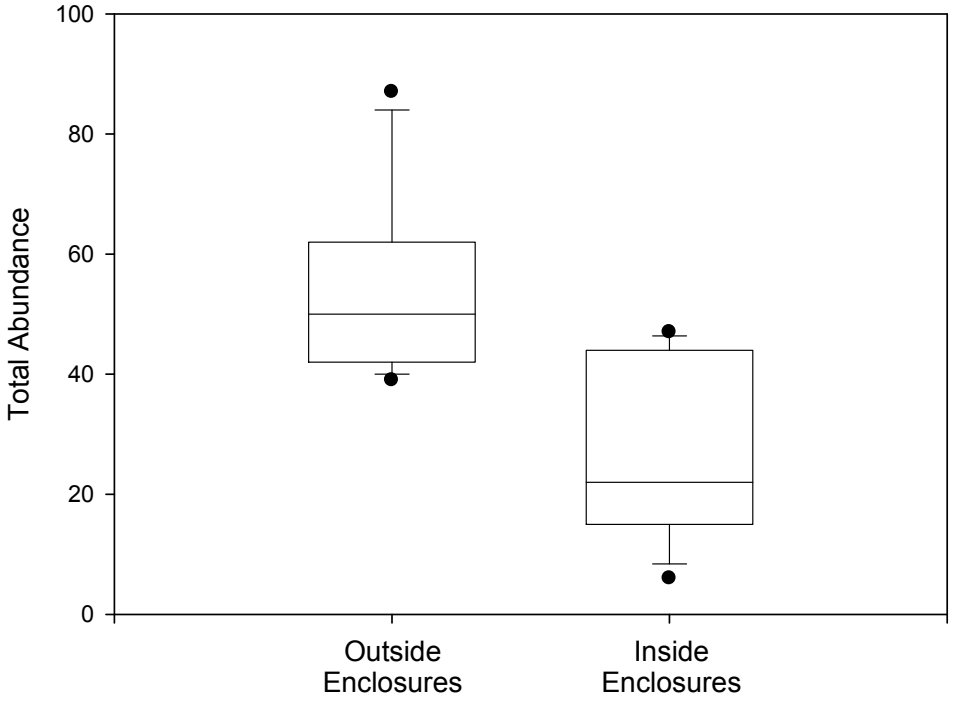


Figure 2.2: Boxplots comparing the mean abundance per sample of benthic macro-invertebrates outside to inside of experimental enclosures. Boxes represent the interquartile range (inner 50% of observations) separated by the median, whiskers represent the 90th and 10th percentile limits, and dots represent the 5th and 95th percentiles.

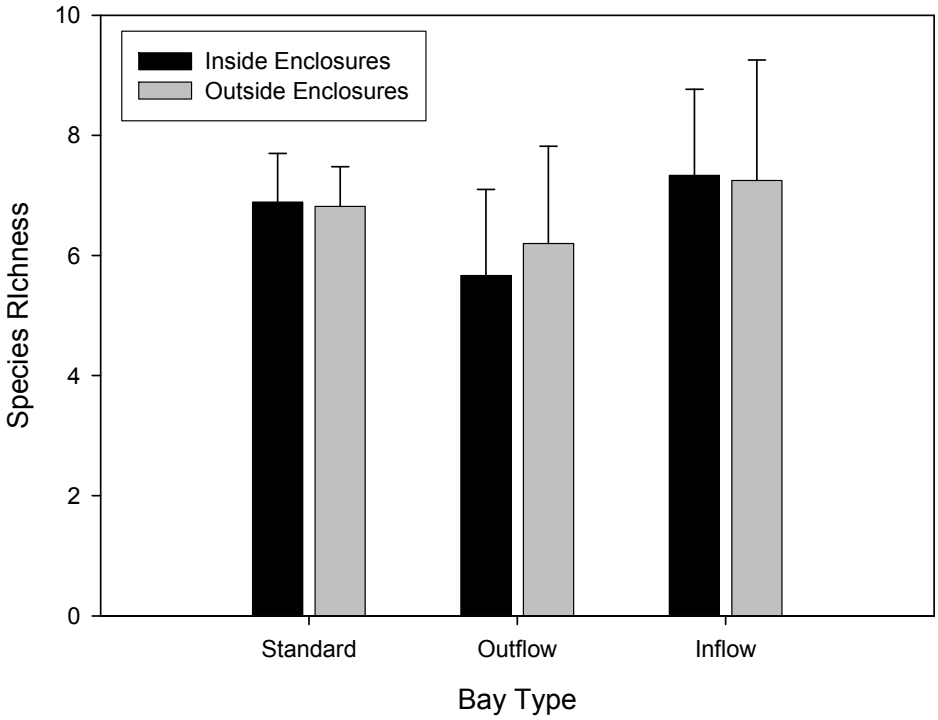


Figure 2.3: Variation in benthic macroinvertebrate species richness (no. of orders) inside and outside experimental enclosures based on bay type showing that richness does not significantly change from inside to outside enclosures but that variation occurs among types of bay. 'Standard' represents bays with no known surface water outflow or inflow in contrast to outflow and inflow bays. Inside represents samples taken from within experimental enclosures at the end of the experiment, while outside samples were taken adjacent to enclosures from the same bay at intervals throughout the 6-week study period. Bars represent the mean species richness of benthic invertebrates (± 1SE). Species richness is based on the macroinvertebrates captured over a surface area of $0.74m^2$.

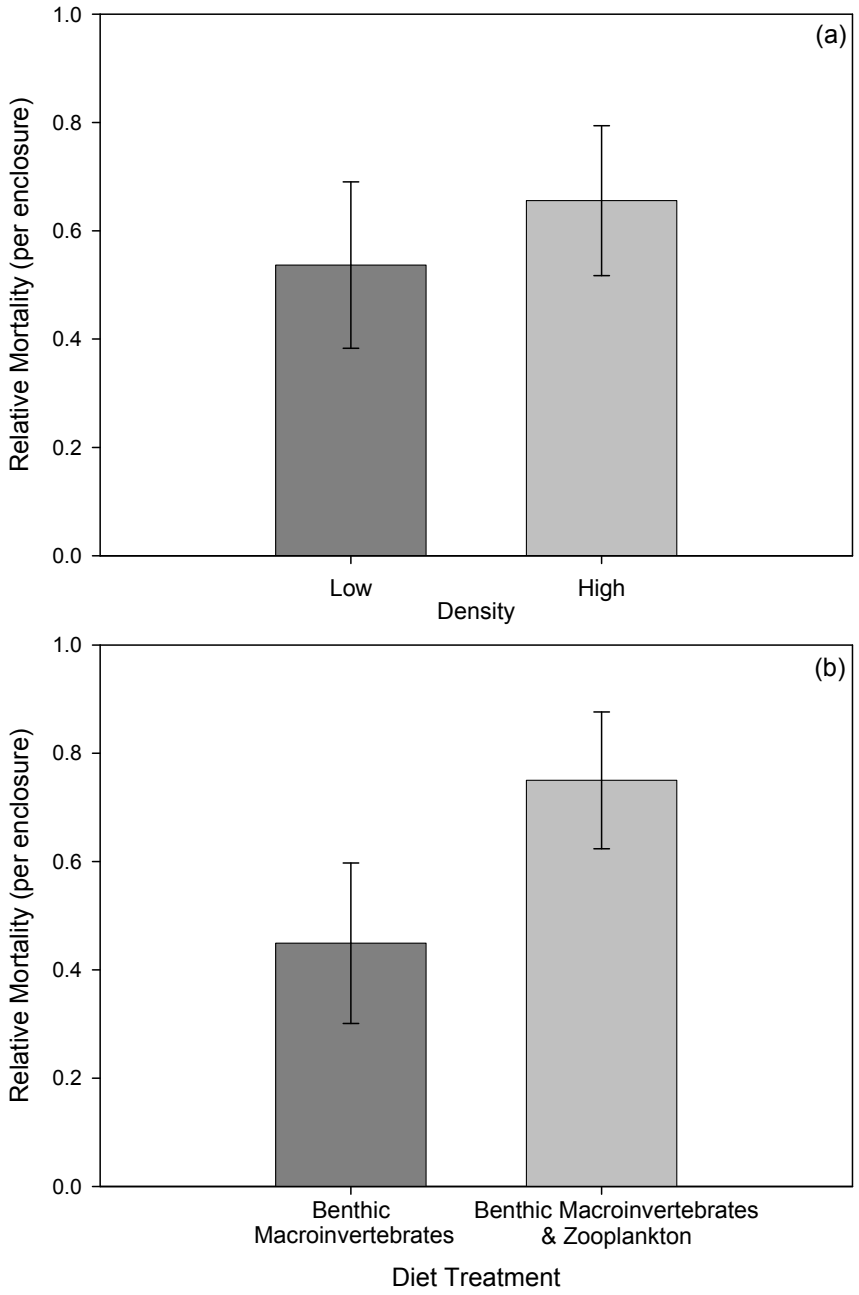


Figure 2.4: Effect of (a) fish density and (b) diet treatments on relative mortality of pumpkinseeds (number dying by experiment end in an enclosure / total no. of sunfish stocked in that enclosure). Density treatments were not significantly different but the addition of zooplankton significantly increased mortality. Bars represent the mean mortality by category (± 1SE).

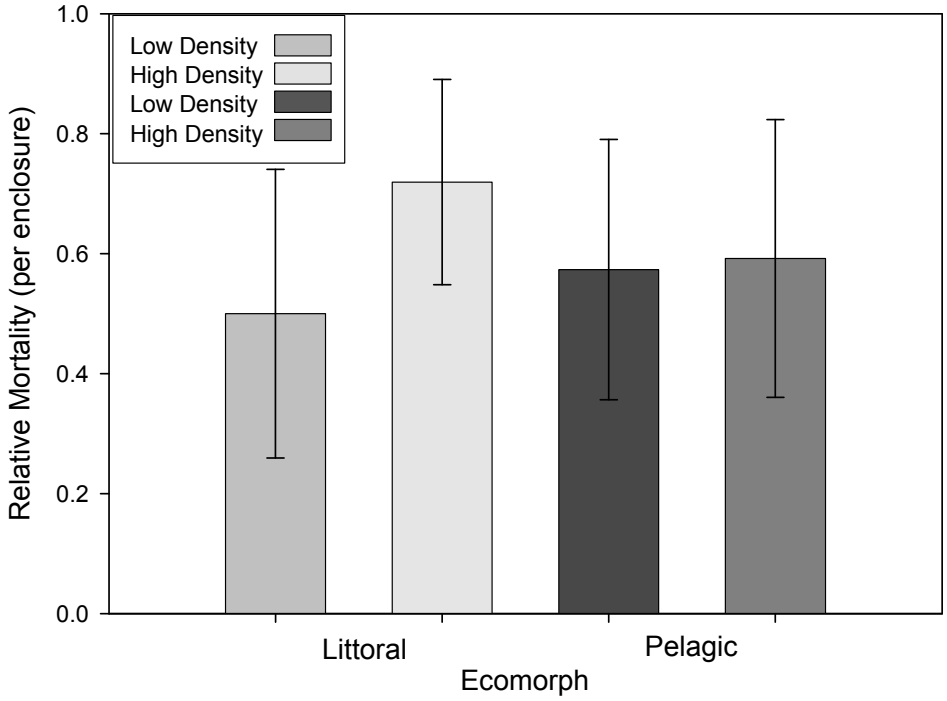


Figure 2.5: Relative mortality (refer to Fig 2.4 for calculation) based on experimental density treatment and ecomorph showing that the littoral ecomorph of sunfish had increased mortality at high density, while pelagic ecomorph mortality was almost unchanged. Bars represent the mean mortality per enclosure (±SE).

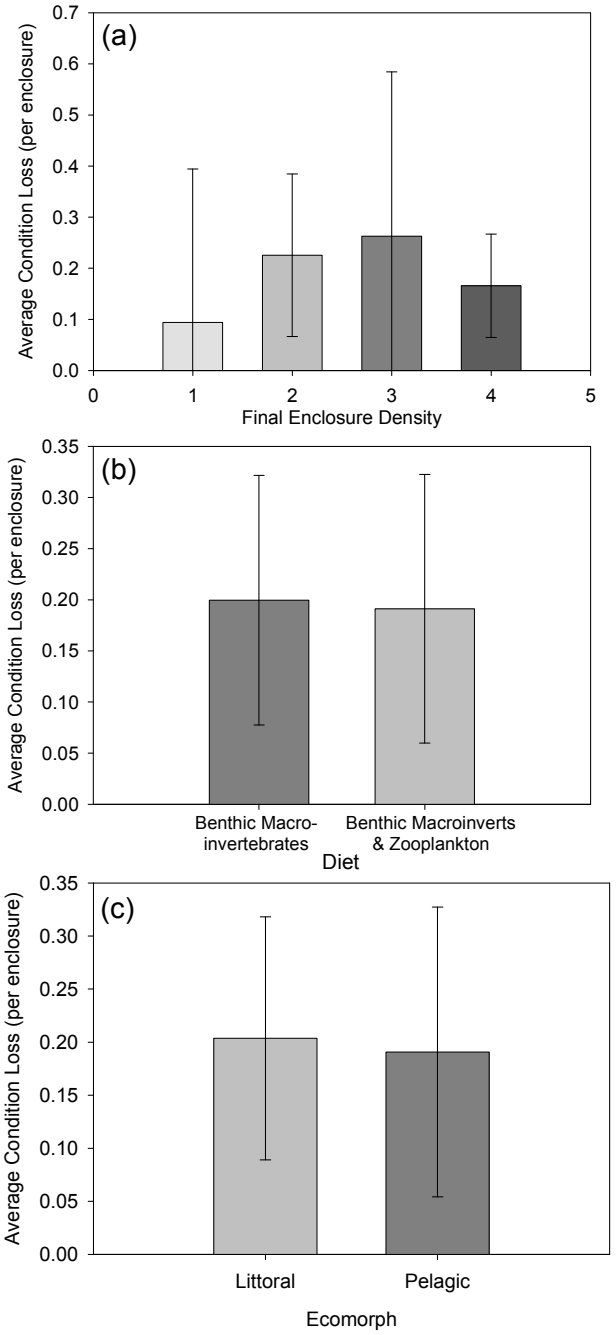


Figure 2.6: Mean average loss of body condition, calculated by taking the initial condition – final condition of each sunfish and finding the average condition loss for each enclosure, (± 1SE) for (a) the final density of fish per enclosure, (b) diet treatment of ambient benthic macroinvertebrates or ambient + zooplankton addition, and (c) ecomorph. There were no significant differences found between the groups for any of these factors.

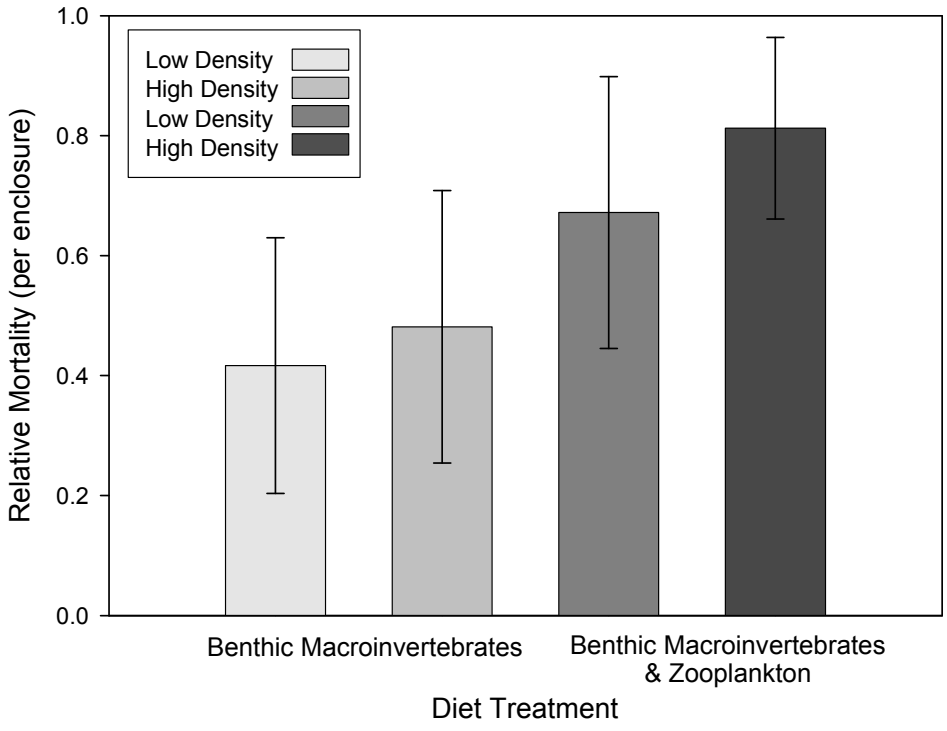


Figure 2.7: The relative mortality of sunfish (refer to Fig. 2.4 for calculation) based on the density treatments and diets provided for the fish. The addition of zooplankton had the strongest affect on increasing mortality. Bars represent the mean mortality per enclosure (± 1SE).

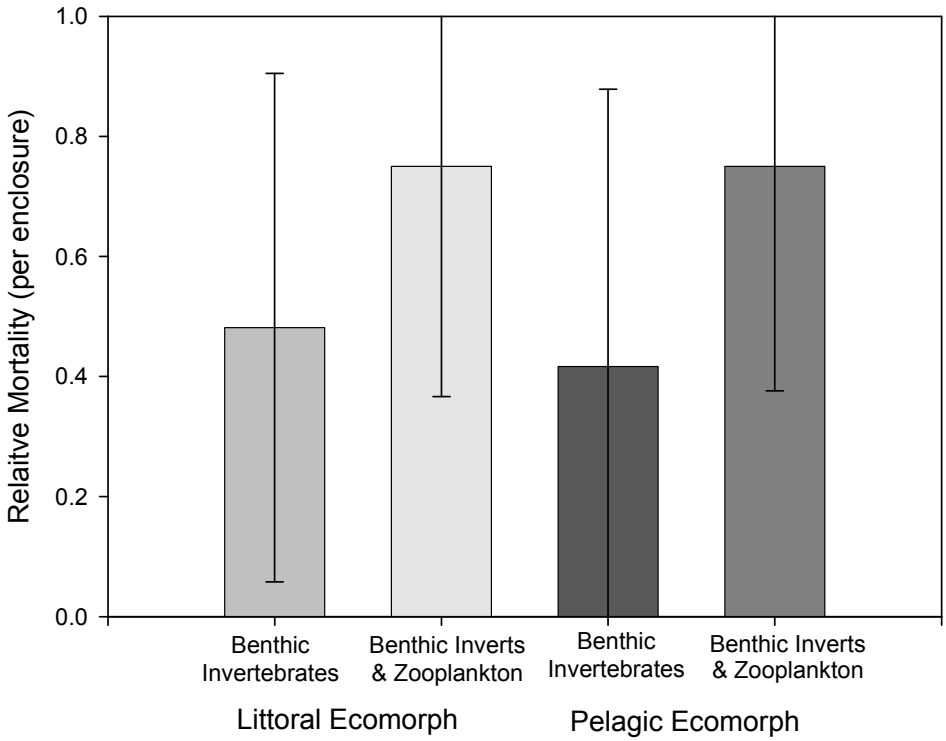


Figure 2.8: Mean relative mortality (refer to Fig. 2.4 for calculation) of pumpkinseed sunfish per enclosure (± 1SE), by diet treatment (ambient benthic macroinvertebrates alone or ambient plus zooplankton) and pumpkinseed ecomorph. There was similar mortality for both ecomorphs in both of the diet treatments, both show increased mortality with the addition of zooplankton.

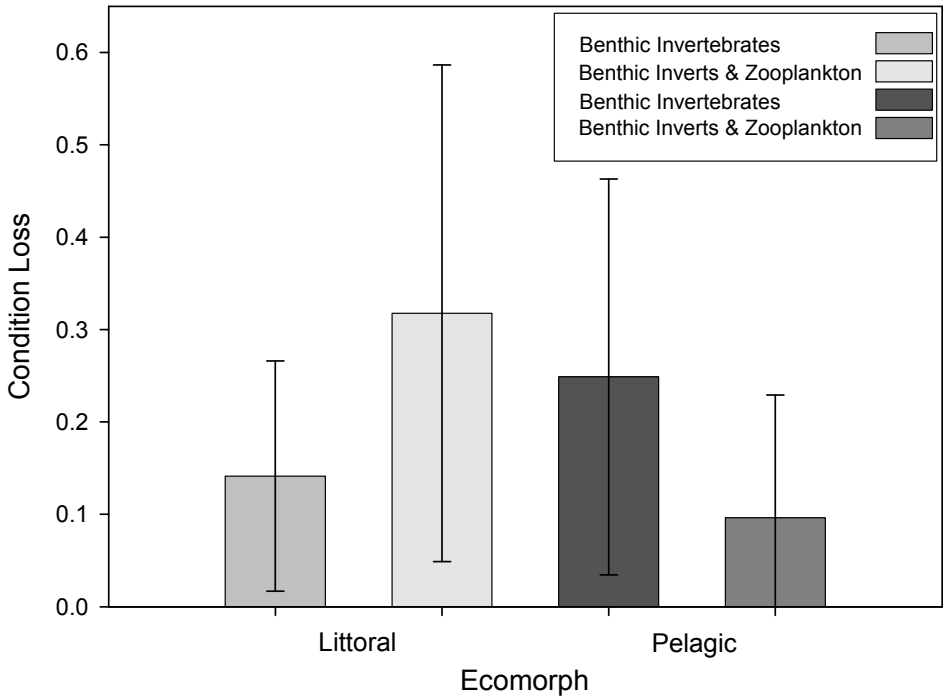


Figure 2.9: Mean loss of body condition per pumpkinseed sunfish and enclosure (± 1SE), estimated as discussed in Fig. 2.6. The addition of zooplankton increased the condition loss of the pumpkinseed sunfish littoral ecomorph, but decreased the loss of condition for the pelagic ecomorph.

General Discussion

In the first part of this thesis, I used stable isotope analysis to infer resource use by pumpkinseed sunfish ecomorphs. In principle, stable isotopes allow examination of resource use over longer time scales than studies of stomach contents. In this study of fish sampled directly from their natural habitat I found differences in the $\delta^{13}C$ signatures between the pelagic and littoral sunfish ecomorphs. This is consistent with the idea that there are general differences in stable isotope values between the littoral and pelagic habitats of lakes due to the way in which primary producers integrate organic nutrients (France 1995, Post 2002, Vander Zanden et al. 2003). However, I was not able to link the diets of pelagic sunfish directly to zooplankton resources using stable isotope values. In fact, stable isotope signatures indicated that the pelagic sunfish were eating something different from pelagic zooplankton. This highlights the importance of gathering reference prey directly from the area in which the fish are feeding to compare with predator stable isotope values. It is possible that my reference zooplankton samples did not represent the zooplankton being consumed by pelagic sunfish. Stomach content analysis on this population by Jastrebski and Robinson (2004) found that zooplankton made up 98% (by no. of prey items) of pelagic sunfish diets, therefore it is possible that there are differences in the stable isotopes of zooplankton between where I collected reference samples and where the sunfish actually feed.

This study also set out to examine factors unrelated to diet that may cause variation in stable isotope values. I found differences in $\delta^{15}N$ based on the type of tissue sampled from fishes, indicating that choosing a single tissue type for stable isotope analysis can add some complexity regarding the trophic level of individuals. On average,

values of $\delta^{15}N$ in fish tissues were the same for both sunfish ecomorphs and so would suggest that both ecomorphs eat at the same trophic level, although values of reference prey were substantially different. I also tested if sunfish body size affected stable isotope signatures. Body size often determines prey use by an organism, especially in predators that are gape-width limited (Osenberg and Mittelbach 1989, Svanbäck and Eklöv 2002, Hjelm et al. 2003). However, I found no evidence that stable isotope signatures varied with body size here eliminating this as a possible source of variation in stable isotope values.

The final important component of Chapter 1 was the use of stable isotopes to infer additional information about a population, specifically the trophic width. I expected that the pelagic ecomorph of sunfish faces strong selection to specialize on zooplankton prey while the littoral ecomorph is more of a generalist consuming a variety of benthic macroinvertebrates (Robinson et al. 1996, Parsons and Robinson 2006). By analyzing the amount of variation in signatures of both $\delta^{13}C$ and $\delta^{15}N$, I found that the pelagic ecomorph is made up of more specialized individuals that all share a similar diet. There was more variation in the isotope signatures among littoral individuals indicating somewhat less specialization. In an experimental manipulation detailed in the appendix, I found that starvation can both alter stable isotope values so that they do not accurately reflect the diets of individuals and that tissue types varied in how they responded. Overall, stable isotope signatures could be used to infer general information about the trophic width of a wild population and differences in resource use, but were much less useful in actually identifying particular prey taxa in the diet, especially for pelagic sunfish.

Overall, my stable isotope analyses used indicate that there are a variety of factors that contribute to variation in $\delta^{13}C$ and $\delta^{15}N$ signatures, and that these must be considered when making inferences about wild populations. However, stable isotopes are a useful tool in the study of both resource use and trophic width of a population, and so present a great opportunity to further our understanding of how species use resources and their environment.

In Chapter 2, I examined the origins of the pumpkinseed sunfish polymorphism using experimental manipulations of resources and density. Resource competition is predicted to increase with density and can result in selection favouring any phenotypes that consume alternate resources. I conclude from my enclosure experiment that both the 'low' and 'high' density treatments resulted in similar, large reductions in the abundance of benthic macroinvertebrate resources. As a result, levels of intraspecific competition were very high at both density levels reducing fish fitness.

The diversification of pumpkinseed sunfish into the pelagic habitat is thought to require an alternate resource (pelagic zooplankton). Despite the high level of competition occurring in all of the treatment groups, I was still able to address if supplying zooplankton prey influenced the fitness of sunfish. I found that zooplankton increased mortality in sunfish and so appear to constrain, rather than promote, resource diversification in sunfish as expected. This suggests that sunfish would likely require phenotypic specializations to use zooplankton as their primary food source. There was some evidence that sunfish competed for the zooplankton resources as well in this study, and the pelagic ecomorph appeared to be more effective at using it than the littoral ecomorph because pelagic fish had reduced rates of body condition loss.

The results of this experiment suggest that the density of sunfish in the littoral habitat will have to reach high levels before it becomes beneficial to diversify into a pelagic ecomorph because of unexpectedly high costs associated with consuming zooplankton prey. As well, competition with bluegill sunfish (*Lepomis macrochirus*) that forage on zooplankton (Keast 1977, Werner and Hall 1976) may not be the primary factor constraining pumpkinseed sunfish diversification and the formation of the pelagic ecomorph (Robinson et al. 2000). Instead it may be direct costs associated with feeding on zooplankton. If true, then competition with bluegill sunfish may not be the first constraint on pumpkinseed sunfish diversification, as studies on interspecific interaction between these species have generally concluded. The results of my study in the absence of bluegill sunfish suggested, instead, that bluegills may only represent an additional constraint on pumpkinseed sunfish diversification.

The results of my enclosure experiment from Chapter 2 indicated that pelagic ecomorphs have some degree of specialization allowing them to consume zooplankton prey more effectively than littoral ecomorphs. If individuals in the ancestral population of littoral sunfish varied in their ability to consume zooplankton, then selection may have favoured individuals that could feed on zooplankton. This result is consistent with the results of the stable isotope analysis in Chapter 1, which found that the pelagic sunfish ecomorph is more ecologically specialized, although the degree to which specialization is for zooplankton prey remains vague at this point. Thus, two different approaches provide independent evidence of specialization for zooplankton in the pelagic ecomorph of pumpkinseed sunfish.

In summary, my thesis investigated the role of intraspecific competition, resource availability and use on the divergence of polymorphic pumpkinseed sunfish. My results are not only applicable to pumpkinseed sunfish but also to the adaptive diversification of other fishes. I also evaluated the utility of stable isotopes of $\delta^{13}C$ and $\delta^{15}N$ to investigate the resource use of a wild population, and sources of variation unrelated to diet that may also affect stable isotope values in fish tissues.

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Appendix

Factors influencing stable isotope signatures of $\delta^{13}C$ and $\delta^{15}N$ in pumpkinseed sunfish (*Lepomis gibbosus*)

Abstract

Stable isotope analysis is increasingly used to assess long term resource use in wild populations. This is only possible if all sources of variation that can influence $\delta^{13}C$ and $\delta^{15}N$ values other than diet are well understood. Laboratory studies have found that differences in the rate at which tissues incorporate stable isotope signals and starvation can alter $\delta^{13}C$ and $\delta^{15}N$ values. Field studies have demonstrated spatial and temporal variation in diet signals. My goal here was to use wild fish and resources to experimentally explore the relative importance of three factors that may influence stable isotope values in sunfishes: 1) a change in diet (from pelagic zooplankton to littoral macroinvertebrate), 2) starvation, and 3) type of tissue (white muscle, liver, blood plasma, and blood cells). The pelagic ecomorph of pumpkinseed sunfish (*Lepomis gibbosus*) was used because they normally consume zooplankton but can also eat benthic macroinvertebrates. I found no change in $\delta^{13}C$ but some evidence of enrichment of $\delta^{15}N$ in fish reared for 42 days on the macroinvertebrate diet. Loss of body condition in all fish suggested that partial starvation occurred and masked the effects of the diet treatment. The effect of starvation on stable isotope values was greater in more metabolically active tissues (liver and blood plasma) than less active tissues (muscle and blood cells). Thus a trade-off may exist between sensitivity to diet effects and susceptibility to starvation effects that varies with the metabolic activity of tissues.

Stable isotope analysis of populations where some individuals face partial or chronic starvation could result in inaccurate estimates of resource use if this trade off is not recognized.

Introduction

Stable isotopes have the potential to inform us about the diets of individuals that are either hard to observe, or when sampling stomach contents does not accurately reflect all dietary components or is impossible. While stomach contents provide a precise measure of what an organism has recently eaten, it provides little information about what is consumed on a regular basis (Bearhop et al. 2004). Stable isotope ratios of $\delta^{13}C$ and $\delta^{15}N$ can provide an integrated picture of what an organism has eaten over longer intervals of time (Hesslein et al. 1993) as long as other factors that potentially influence variation in stable isotope signature are nonexistent or at least well understood.

Researchers sometimes use stable isotope signatures to establish both what resources consumers have used and also their trophic level in a local food chain. For example, a consumer's $\delta^{13}C$ usually reflects that of their resources with only weak enrichment of less than 1‰ per trophic level (Harrigan et al. 1989, Post 2002, Bearhop et al. 2004). A common pattern in aquatic ecosystems is for taxa inhabiting littoral and pelagic habitats to have distinct $\delta^{13}C$ values because of differences in the relative uptake of ^{13}C and ^{14}C by primary producers (France 1995, Post 2002, Vander Zanden et al. 2003). Macrophytes are the primary producers in the littoral habitat of most lakes and have significantly enriched $\delta^{13}C$ signatures compared to pelagic phytoplankton because phytoplankton discriminate between carbon isotopes and incorporate less ^{13}C during

carbon cycling (France 1995, Paterson et al. 2006). On the other hand, $\delta^{15}N$ indicates trophic position because it typically increases between 2.5‰ and 5‰ per trophic level (Vander Zanden et al. 1997, Bearhop et al. 2002).

Stable isotope signatures are thus a potentially valuable tool for the study of fish and their diets. Observing fish can be difficult in their natural environment and dietary information is usually obtained by destructive sampling of stomachs, that can only provide a snapshot of what has been consumed recently. Stable isotopes can be used to infer habitat use by what fish are eating (Cunjak et al. 2005, Hicks et al. 2005), what prey species are likely eaten (MacAvoy et al. 2001, Perga and Gerdeaux 2005, Paterson et al. 2006), and even the proportions of different prey in the diet of an average individual in the population (Beaudoin et al. 1999, Bolnick et al. 2002). Our ability to infer such information from stable isotopes though, depends on a clear understanding of any other major sources of variation that alter stable isotope signatures. It thus becomes important to identify what external and internal factors may impact $\delta^{13}C$ and $\delta^{15}N$ signatures in order to reliably use stable isotopes signatures to make inferences about wild populations.

A variety of potential external factors can influence $\delta^{13}C$ and $\delta^{15}N$ values including resource type and spatial location. Resource isotope signatures can differ greatly between locations, even within same habitat types (Hobson 1999, Vander Zanden and Rasmussen 2001). Ideally, an array of potential resources from a variety of sites should be sampled in order to establish reference $\delta^{13}C$ and $\delta^{15}N$ signatures and their variability in space and time for comparison with the values of a focal population. It is only by establishing clear reference signatures and their variability, that it is possible to

compare consumer samples to resource samples and between habitats in order to infer the diets of individuals in the wild.

Various factors internal to organisms may also affect variation in stable isotopes of $\delta^{13}C$ and $\delta^{15}N$. First, there is evidence that various tissues react to changes in diet at different rates due to variation in metabolic rate among tissues (Tiezen et al. 1983, Hobson and Clark 1992, Hobson et al. 1996, Pinnegar and Polunin 1999). The rate at which tissues are replaced affects how long it takes for stable isotopes to turnover (MacAvoy et al. 2001). White muscle tissue is widely used in isotope studies because it is less metabolically active than many others and so is expected to represent a longer term signature of diet (Fry and Parker 1979, Cabana and Rasmussen 1994). Metabolically active tissues, such as the liver (Hesslein et al. 1993, Perga and Gerdeaux 2005) and blood (Hobson and Clark 1992, Bearhop 2002, Pearson et al. 2003), are expected to change in response to dietary changes over a much shorter period of time. Variation in tissue integration rates may influence what can be inferred about diet, especially when consumers experience seasonal shifts in diet (Perga and Gerdeaux 2005). If this is the case, then more active tissues should reflect recent diet $\delta^{13}C$ and $\delta^{15}N$, while less active tissues should average recent with more historic diets. It is important, therefore, to identify which tissues are more vs. less active as this can influence choice of tissue and the required sampling period depending on the scale of dietary change experienced by the population.

A second internal factor worth considering during studies of wild populations is the effect of starvation on stable isotope signals. Starvation or poor resource conditions experienced by an organism cause physiological changes in the use of stored energy

reserves and noncritical tissues that may also affect $\delta^{13}C$ and $\delta^{15}N$ stable isotope signatures. There is evidence from invertebrates (Adams and Sterner 2000, Haubert et al. 2005), birds (Hobson and Clark 1992, Hobson et al. 1993) and mammals (Cormie and Schwarz 1996) that starvation can cause enrichment of $\delta^{15}N$ signatures which mimic an increase in trophic level by a consumer. There is also some evidence that $\delta^{13}C$ can be enriched during starvation (Oelbermann and Scheu 2002, Olive et al. 2003, Gaye-Siessegger et al. 2004), although this is not always the case (Kempster et al. 2007) and so may be taxon specific.

There is a lot of interest in the use of $\delta^{13}C$ and $\delta^{15}N$ stable isotopes in the study of fish and their diets. Prior tests of the factors that influence stable isotope signatures in fishes have either involved observational studies in the field or laboratory studies of fish given artificial diets. To date, no study has tested if wild fish exposed to wild diet treatments vary in their stable isotope signatures in predictable ways.

Previous studies with lake fishes have found that stable isotope signatures reflect prey species differences between the littoral and pelagic habitat (Schindler and Scheuerell 2002, Schluter 1995). By switching the diet of fish from a pelagic source to a littoral source, I expected to observe changes in stable isotope signatures indicative of these different categories of dietary prey. $\delta^{13}C$ values were expected to be similar to that of their prey while $\delta^{15}N$ was expected to reflect trophic enrichment of ~3‰ from the prey sources to consumers.

I also tested if various internal factors affected stable isotopes signatures of fish tissue. I predicted that metabolically active tissues would vary in their ability to reflect the dietary change, with those that are more metabolically active reflecting the changed

diet treatment compared to less active tissues. The order of increasing tissue metabolic activity was predicted to be: white muscle, liver, blood cells, and blood plasma. I analyzed $\delta^{13}C$ and $\delta^{15}N$ stable isotope signatures of these fish tissues over a 42 day interval in two treatments involving pelagic ecomorphs of pumpkinseed sunfish: one fed exclusively on a diet of pelagic zooplankton the other on a diet of benthic macroinvertebrates collected from littoral lake habitats. If sunfish were fed a sub-maintenance diet, then I also predicted that $\delta^{15}N$ would be enriched over the course of the experiment, with similar changes possible in $\delta^{13}C$ values.

Materials and Methods

Pumpkinseed Sunfish

Pumpkinseed sunfish (*Lepomis gibbosus*) are one of the most abundant fish species in North American postglacial lakes because they have the most northern distribution of all sunfish species (Scott and Crossman 1998). Pumpkinseed sunfish usually inhabit the littoral habitat of lakes where they specialize on a diet of benthic macroinvertebrates, such as snails and bivalves (Keast 1978, Werner and Hall 1979). Significant competition among juveniles can occur with a close relative, the bluegill sunfish (*Lepomis macrochirus*) in the littoral habitat, but adult bluegill usually leave the littoral habitat and generalize on pelagic zooplankton (Keast 1977, Werner and Hall 1976). The ranges of pumpkinseed sunfish and bluegill sunfish do not completely overlap (Scott and Crossman 1998), and in lakes without bluegill sunfish, some pumpkinseed sunfish are ecologically and phenotypically divergent between littoral ecomorphs that primarily eat benthic macroinvertebrates and pelagic ecomorphs that feed on zooplankton

in addition to some macroinvertebrates (Gillespie and Fox 2003, Robinson et al. 1993, Robinson et al. 2000, Jastrebski and Robinson 2004). I use the pelagic ecomorph of pumpkinseed sunfish here because even though they consume pelagic zooplankton resources (Gillespie and Fox 2003), they retain the ability to also consume littoral benthic macroinvertebrates (Robinson et al. 1993, Jastrebski and Robinson 2004). The ability to consume littoral and pelagic prey resources allows the dietary manipulation at the center of this experiment.

I used pelagic pumpkinseed sunfish sampled from Ashby lake, located in the Mazinaw region of Ontario (45˚05'N, 77˚21'W), because this population has been previously identified as polymorphic (Jastrebski and Robinson 2004) and also provided a shore site for the experimental infrastructure.

Pumpkinseed Sunfish Sampling and Experimental Procedure

Ninety pelagic sunfish were collected adjacent to pelagic shoals at the northern end of Ashby Lake and randomly assigned to one of two treatment groups, for a total of 45 fish per treatment. Treatment group 1 received a 'pelagic' diet of live zooplankton collected daily from the pelagic habitat. Treatment group 2 received a 'littoral' diet of live benthic macroinvertebrates collected daily from the littoral habitat.

Each treatment group was maintained in a circular fiberglass tank (1.2m dia. x 0.55m deep) placed in the shade along the shoreline. Approximately 25% of the water in each tank was exchanged with lake water each day and air pumps were used to maintain water circulation. Live zooplankton prey were collected using vertical tows of a plankton net (mesh size 0.5μm) from a depth of 15m at various points in water > 30m in depth.

Live benthic macroinvertebrates were collected by sweeping D-nets through the macrophyte vegetation and the upper layers of sediment in shallow littoral sites, and then separated from coarse material using a set of nested sieves (openings of 12.7, 4, 2, 1, 0.5mm). At the beginning and at regular intervals throughout the study, prey samples were preserved to provide reference measurements of $\delta^{13}C$ and $\delta^{15}N$.

A fraction of fish were randomly sampled from each treatment group at the start and then every seven days over six weeks. Starting (day 0) samples consisted of 10 fish from each treatment, while 4 fish were sampled every 7 days from each treatment over the next 42 days, with the final (day 42) sample consisting of 10 macroinvertebrate diet treatment fish and 8 zooplankton diet treatment fish (reflecting differences in mortality). Fish were anaesthetized in benzocaine (stock solution created by adding ethyl-*p*-aminobenzoate to ethanol for a 2.5g/L solution, then diluted in 10L of water to a concentration of 0.03mL/mL water) for 5 minutes, and a 50µL sample of blood was drawn from the posterior base of the anal fin and immediately separated into plasma and cellular components by spinning in a centrifuge at 500xG for 12 minutes. Fish were then euthanized, a sample of white muscle tissue was dissected from the base of the dorsal fin without scales and the liver was removed. All tissues were frozen individually at -20˚C until processing. Size measurements (blotted wet weight and standard length) and photographs were taken of all fish at this time. The body condition factor (K) of each fish was estimated following Williams (2000):

$$K = (100{,}000\ W) / L^3$$

where W is the weight of the fish in grams, and L is the standard length of the fish in mm.

All frozen tissue samples were freeze dried at -50˚C for 24 hours, ground into a fine powder and thoroughly mixed. Lipids were removed from tissue samples following Bligh and Dyer (1959) in which a 2:1 chloroform-methanol solution was mixed into the tissue, spun, and the lipids extracted three times per sample. Samples were then dried at 60˚C, re-ground and weighed to the nearest 0.001mg to obtain a final sample weight of 0.25 - 0.30mg.

Prey samples were also analyzed to provide base-line signatures of different taxa. Zooplankton and benthic macroinvertebrate samples were both prepared by freeze drying, grinding and weighing following the procedures above. However, carbonates were first removed from the benthic macroinvertebrates with a 10% HCl solution, except for snails and bivalves whose outer shells were manually removed.

Measures of stable isotope levels (N^{15}, N^{14}, C^{12}, and C^{13}) on all samples were made using the Isochrom spectrophotometer at the University of Waterloo Environmental Isotope Laboratory. Samples of an internal reference material were analyzed after every 5th specimen sample. The standard reference material for C is Pee Dee Belemnite (PDB) and atmospheric N_2 for N. Precision of the internal standards are ±0.3‰ for nitrogen and ±0.2‰ for carbon. The material of every 8th specimen sample was replicated and measured. Isotope ratios are expressed as parts per thousand (‰) differences from the standard reference material:

$$\delta X = (R_{sample}/R_{standard} - 1) \text{ X } 10^3$$

where X is N^{15} or C^{13}, R is the ratio of N^{14}:N^{15} or C^{13}:C^{12} and δ is the measure of heavy to light isotope in the sample.

Statistical Analysis

(i) Variation Due to Diet

The significance of variation in mean prey isotope signatures of $\delta^{13}C$ and $\delta^{15}N$ sampled from littoral and pelagic habitats, and variation in sunfish tissue $\delta^{13}C$ and $\delta^{15}N$ for each diet treatment (zooplankton or benthic macroinvertebrates) and each tissue type (muscle, liver, plasma, and blood cells) were evaluated using two-tailed t-tests. We expected dietary treatments to differ in $\delta^{13}C$ and $\delta^{15}N$ signatures, and so compared mean dietary stable isotope signals using a t-test. Prey taxa were combined in each diet treatment for this analysis (pelagic diet included cladocera and copepoda while the littoral diet was composed of gastropoda, amphipoda, bivalvia, odonata, and ephemeroptera). Prey taxa may also vary in their stable isotope signatures within each diet, and so we evaluated variation in prey order signatures within the littoral diet treatment using ANOVA, followed by student's t-test for multiple comparisons if a significant ANOVA main effect was detected.

The sunfish sampled on day 0 were tested for baseline differences in $\delta^{13}C$ and $\delta^{15}N$ between diet treatment groups and among different types of tissues. A two-factor ANOVA was analyzed for each stable isotope ($\delta^{13}C$ or $\delta^{15}N$) with the diet treatment group and tissue type as factors, along with their interaction. If a significant difference was found, then multiple pair-wise comparisons were performed using t-tests.

In order to infer the reference diet of sunfish at the start of the experiment, the $\delta^{13}C$ or $\delta^{15}N$ value of each type of fish tissue sampled on day 0 was compared to the mean stable isotope values of the two diet treatments using Dunnett's multiple comparisons tests. Pelagic zooplankton were selected as the comparison control because

they represent the presumed initial dietary signature that pelagic pumpkinseed sunfish should reflect.

The $\delta^{13}C$ and $\delta^{15}N$ values of the pumpkinseed sunfish were then examined for changes in each diet treatment group (pelagic zooplankton or littoral benthic macroinvertebrate) and each type of tissue sampled (white muscle, liver, blood cells, or blood plasma). Since I was looking for the $\delta^{13}C/\delta^{15}N$ change in each tissue and diet treatment separately this was analyzed using two-tailed t-tests.

(ii) Variation Due to Starvation

In order to test for any effect of starvation, fish body condition was first compared at four sampling intervals using a one-way ANOVA with Kruskal-Wallis rank sums for each diet treatment separately. An ANCOVA model was used to test if body condition changed over time in the same way for the two diet treatments (Factor: diet treatment, covariate: time and their interaction).

The relationship between body condition (dependent) and $\delta^{15}N$ (independent) was tested for each tissue type and diet treatment using linear regressions. For each tissue, ANCOVA was then used to test for differences in the relationship between body condition and $\delta^{15}N$ for the two diet treatments (Factor: diet treatment, covariate: $\delta^{15}N$ and their interaction). Interaction effects were tested first, and if non-signficant were removed and a simplified model of the main effects was reanalyzed.

(iii) Variation Due to Tissue Type

To test if different tissues have different rates of response to the stable isotope signal of diet treatments, I compared the $\delta^{15}N$ among the four tissue types and between

Day 0 and 42 using a two-factor ANOVA (Factors: tissue type and day, and their interaction).

All analysis were completed using JMP IN v. 5.1. with statistical significance assessed at an $\alpha < 0.05$, although values between 0.05 and 0.10 were also interpreted as weak evidence of effects.

Results

Variation Due to Diet

The first step to analyzing change in stable isotopes in consumers is to evaluate variation in the stable isotope values in their resources. Littoral benthic macroinvertebrates used in the diet treatment here were enriched in $\delta^{13}C$ compared to pelagic zooplankton (t-test, $t_{10.79} = 4.23$, $P = 0.002$). One littoral prey group, ephemeroptera though, had $\delta^{13}C$ values similar to that of pelagic zooplankton, but the four other common littoral prey types were all distinct from zooplankton (Fig. A.1). Littoral prey groups also had significantly lower $\delta^{15}N$ values than pelagic zooplankton ($t_{15.93} = -5.89$, $P < 0.0001$).

I next determined the stable isotope values of the sunfish tissue at the start of the experiment (day 0) and tested if they varied between diet treatments. $\delta^{15}N$ values of sunfish between diet treatments were similar (ANOVA, $F_{1,79} = 0.03$, $P = 0.86$) while $\delta^{13}C$ values were initially slightly higher in sunfish assigned to the benthic macroinvertebrate diet treatment (macroinvertebrate diet -27.83 ± 0.96, zooplankton diet -27.55 ± 0.95; ANOVA, $F_{1,79} = 5.94$, $P = 0.02$). There were also initial differences found in the $\delta^{13}C$ values of the different tissue types (white muscle -26.65 ± 0.13, liver -26.62 ± 0.13, blood

plasma -29.82 ± 0.14, and blood cells -27.64 ± 0.13; ANOVA, $F_{3,79} = 117.50$, $p < 0.001$). However, when comparing the individual tissues to each other in the diet treatments no significant differences were found (Table A.1), indicating that the initial values were equal for each type of tissue in both diet treatments at the start of the experiment.

The next step was to compare the $\delta^{13}C$ and $\delta^{15}N$ values of the sunfish collected at the start of the experiment (day 0) with the stable isotope values of the prey sampled from the lake. Pelagic pumpkinseed sunfish sampled prior to start of the experimental diet treatments have $\delta^{13}C$ values higher than what would be expected based on a specialized diet of only pelagic zooplankton (Dunnett's, all $P_{99} < 0.0001$; Fig. A.2). Similarly, all four tissue types examined were significantly higher in $\delta^{15}N$ from that of pelagic zooplankton (Dunnett's, all $P < 0.002$; Fig. A.2), consistent with the expected elevation of $\delta^{15}N$ in consumers due to moving up a trophic level in the food web.

After 42 days the $\delta^{15}N$ signatures of all pumpkinseed sunfish tissues significantly increased in the zooplankton diet treatment (t-test, all $t_{16} \leq -2.28$, all $P \leq 0.04$). Similarly, $\delta^{15}N$ increased in the muscle, liver and blood plasma tissues of the benthic macroinvertebrate diet (t-test, all $t_{18} \leq -2.79$, all $P \leq 0.01$), but did not change significantly in the blood cells ($t_{18} = -1.4$, $P = 0.17$; Fig. A.3). Conversely, $\delta^{13}C$ values did not change significantly in any of the tissues examined in both the zooplankton (t-test, all $t_{16} \geq 0.95$, all $P \geq 0.10$) and benthic macroinvertebrate (all $t_{18} \geq -1.40$, all $P \geq 0.20$) diet treatments (Fig. A.3).

Variation Due to Starvation

My second goal was to test the relative importance of additional possible factors, beyond dietary, that may have influenced $\delta^{13}C$ and $\delta^{15}N$ variation in pumpkinseed sunfish. For example, starvation resulting in use of stored tissues can enrich $\delta^{15}N$ in fishes. Starvation appears to have been a factor here because body condition decreased by 11% over the course of the study in the zooplankton diet treatment (ANOVA, $F_{3, 22} = 3.09$, $P = 0.06$) and by 8% in the benthic macroinvertebrate diet treatment (ANOVA, $F_{3, 24} = 2.21$, $P = 0.08$) (Fig. A.4). The decrease in body condition and the timing at which it occurred over the 42 day experimental period was not significantly different between diet treatments (ANCOVA, $F_{3,45} = 0.19$, $P = 0.90$; Fig. A.4) indicating that sunfish in both the zooplankton and benthic macroinvertebrate treatments groups experienced similar starvation conditions and rate of condition loss.

If starvation causes $\delta^{15}N$ to become enriched, then fish body condition should be negatively related to $\delta^{15}N$. This was found for muscle (linear regression, slope = -0.52, $t_{1, 36} = -1.87$ $P = 0.07$), liver (slope = -1.31, $t_{1, 36} = -2.97$, $P < 0.01$), and blood plasma (slope = -1.11, $t_{1, 36} = 0.04$, $P = 0.04$; data from both diet treatments combined). The negative relationship between body condition and $\delta^{15}N$ did not differ significantly between diet treatments for any tissue (ANCOVA, $\delta^{15}N$ X diet treatment interactions, all $F_{1, 34} \leq 1.50$, all $P > 0.23$). This indicates that in both diet treatments fish with lower body conditions had higher $\delta^{15}N$. Diet treatments also did not vary significantly in the intercepts of their negative relationships in any of the tissues (ANCOVA; Diet, all $F_{1, 35} \leq 1.10$, all $P > 0.30$).

Variation Due to Tissue Type

The four different tissue types (muscle, liver, blood plasma, and blood cells) permitted a test for different rates at which isotope signatures changed due to starvation. Because $\delta^{15}N$ changed over the experimental period while $\delta^{13}C$ did not, I only consider the effects of tissue type on $\delta^{15}N$ values. $\delta^{15}N$ was enriched in all of the tissues tested. However, there were differences between the tissues in how much they enriched, white muscle changed the least, followed by blood cells, while liver and blood plasma had the greatest $\delta^{15}N$ enrichment (ANOVA, $F_{3, 144} = 12.75$, $P < 0.0001$; Fig. A.5).

Discussion

External Factors Influencing Stable Isotope Signatures

The $\delta^{13}C$ and $\delta^{15}N$ baseline measurements of invertebrate prey species from littoral and pelagic habitats indicated that except for one littoral prey group there were differences in stable isotope signals between littoral and pelagic prey groups. The considerable variation among littoral prey taxa may reflect differences in their own trophic ecology. Some groups, such as amphipods, are opportunistic scavengers (Peckarsky et al. 1995) that eat prey from different trophic levels depending on availability. This can potentially increase the variation in $\delta^{15}N$ from one sample to the next depending on what amphipods have recently scavenged. Other groups are more specialized, such as bivalves, which feed by filtering microscopic prey and fine particulate organic matter from the water column (Strayer 1995) and so have a less variable diet and reduced variation in both $\delta^{13}C$ and $\delta^{15}N$ values. My baseline measurements reveal some overlap in stable isotope values among prey groups based on

habitat type, but for the most part show that prey groups can be distinguished by $\delta^{13}C$ and $\delta^{15}N$ values. This provides the opportunity to study how changing the diet of pumpkinseed sunfish affects variation in sunfish stable isotope signatures.

I expected that changing the diet of pumpkinseed sunfish from pelagic zooplankton to littoral macroinvertebrates would change $\delta^{13}C$ and $\delta^{15}N$ in sunfish tissues because the prey groups varied in stable isotope values. However, there is no evidence that a diet switch resulted in predicted changes to stable isotope signatures of sunfish in any of the tissues tested. The mean value of $\delta^{15}N$ in littoral macroinvertebrates was lower than in pelagic zooplankton, but I did not find a similar decrease in the $\delta^{15}N$ of tissues sampled from sunfish fed the macroinvertebrate diet treatment. In fact, the $\delta^{15}N$ values showed significant enrichment, the opposite of what was expected. As well, $\delta^{13}C$ values of pelagic pumpkinseed sunfish fed a littoral macroinvertebrate diet enriched in $\delta^{13}C$ did not change over the course of this experiment. Thus, there was no response in sunfish tissues to manipulation of dietary stable isotope values.

Pumpkinseed sunfish sampled from the pelagic habitat on day 0 had $\delta^{13}C$ values more similar to littoral macroinvertebrate values than to $\delta^{13}C$ values of pelagic zooplankton (Fig. A.2). It is possible then, that the littoral macroinvertebrate diet treatment imposed only minor changes on the baseline $\delta^{13}C$ signatures of sunfish tissues. However, if this was the case, then sunfish consuming the pelagic zooplankton diet treatment should have decreased their $\delta^{13}C$ values in response to the zooplankton diet treatment; but there was no such change. In either case, there is no evidence that sunfish tissue responded strongly to either dietary treatment.

Effect of Partial Starvation on Stable Isotope Signatures

There is evidence that experimental sunfish lost body condition despite daily feeding and so did not receive a diet sufficient to meet basal energetic demands. I predicted that starvation would cause enrichment of $\delta^{15}N$ in sunfish and this was detected in white muscle, liver, and blood plasma tissues of both diet treatments and blood cells of the pelagic zooplankton diet treatment. $\delta^{13}C$ values did not show changes during this period of starvation, consistent with some other studies of starvation (Kempster et al. 2007). It is also interesting that pumpkinseed sunfish switched to a diet of littoral macroinvertebrates did not have the expected elevation in $\delta^{13}C$. As mentioned above, if the $\delta^{13}C$ values of the pumpkinseed sunfish collected directly from the pelagic environment are similar to the $\delta^{13}C$ of the littoral resources then this change would not be expected. Instead the $\delta^{13}C$ should have decreased in the pelagic zooplankton treatment, but this was not found. Overall, these results indicate that starvation affected $\delta^{13}C$ values and masked any effect of diet manipulation.

Previous studies have experimentally imposed complete starvation (no food provided) to measure the response of both $\delta^{13}C$ and $\delta^{15}N$ stable isotopes (eg. Cormie and Schwarz 1996, Oelbermann and Scheu 2002, Olive et al. 2003, Haubert et al. 2005). It is conceivable that organisms will experience periodic food shortages that do not meet all of their energetic demands and so results in partial starvation. Perga and Gerdeaux (2005) found that $\delta^{15}N$ values in wild populations of Whitefish (*Coregonus lavaretus*) were highest at the end of the winter and decreased during the spring, and so came to the conclusion that the winter samples did not reflect the $\delta^{15}N$ signature of diet so much as starvation stress over winter. This seems reasonable, although neither prey availability

nor fish condition were assessed in that study. Individuals may experience periods of reduced feeding at any time that may affect stable isotope signatures.

The enrichment of $\delta^{15}N$ with starvation is likely caused by the preferential use of ^{14}N isotopes over that of ^{15}N which cause changes in the ratio used to calculate $\delta^{15}N$ (Adams and Sterner 2000) but this has been primarily studied during complete starvation when the body is able to draw only upon body stores. My results indicate that enrichment of $\delta^{15}N$ will even occur during periods of partial starvation. If populations are stressed by limitations in food availability, then this may affect the accuracy of studies evaluating stable isotope signatures in the wild. Partial starvation could result from seasonal variation in prey abundance or from suboptimal energetic returns by individuals that change resources as they grow (e.g., ontogenetic niche shifts) or use suboptimal niches as in 'sink' populations.

Tissue-specific Integration Rates of $\delta^{13}C$ and $\delta^{15}N$

Another possible source of variation in stable isotope signatures of consumers can be related to tissue-specific integration rates based on metabolic activity. Differences in tissues are predicted to occur when consumers consume new resources that differ in $\delta^{13}C$ and $\delta^{15}N$ values because metabolically active tissues (eg. liver and blood) will equilibrate to the new stable isotope values quicker than less metabolically active tissues (eg. white muscle) which instead reflect the historical diet (Hobson et al. 1996, Pinnegar and Polunin 1999, Bearhop 2002, Perga and Gerdeaux 2005). Similarly, based on these principles it could also be predicted that during periods of starvation the metabolic

activity of a tissue will affect the rate at which the stable isotopes change in response to the use of body stores to meet basal energy needs.

I found evidence of tissue specific differences in the rate at which isotope values change during starvation. Based on previous work, I expected to find that muscle has the longest integration time and so would change the least under the influence of diet (Perga and Gerdeaux 2005), blood plasma the shortest integration time (Hobson and Clark 1992, Bearhop 2002, Pearson et al. 2003), and the other two tissues somewhere in between. This pattern was confirmed here, blood plasma had the greatest amount of change, followed by liver and blood cells, and white muscle changed the least. The consistency of my results with previous work in other taxa supports the theory that stable isotope signatures of highly metabolically active tissues (i.e. liver and blood) change more quickly than in less metabolically active tissues such as muscle.

Thus, while using tissues with high metabolic rates allows one to evaluate hypotheses about recent dietary shifts, this may come at the expense of greater sensitivity to other internal and external factors. Tissues with quick integration of dietary isotope signatures will reflect dietary changes more quickly, but in cases where starvation or less than ideal conditions are possible they will also be the first ones to have isotope signatures affected by non-dietary factors. However, less metabolically active tissues present an opportunity to look at a longer term diet signature less affected by periods of starvation. As a result, tissues that rapidly integrate stable isotope signatures increases sensitivity to both external factors (diet) and internal factors (starvation), while tissues with a longer integration period are more likely to reflect dietary effects than other factors, but at the expense of limited ability to detect recent changes in resource use.

Conclusions

The $\delta^{13}C$ and $\delta^{15}N$ of pumpkinseed sunfish were affected more by moderate starvation and tissue type than by variation in prey stable isotope values in this experiment. I have shown that even partial starvation over a period of weeks alters the $\delta^{13}C$ and $\delta^{15}N$ signatures enough to mask large changes in diet that occur at the same time. Some degree of starvation is a potential factor affecting individuals in any wild population and so its affects may be underappreciated in studies that use stable isotopes to infer the diets of wild populations.

Differences in the metabolic rates of tissues provide researchers with an opportunity to select certain tissues to integrate dietary signals over shorter or longer time intervals. However, the metabolic rates of different tissues may also cause the stable isotope signatures of some tissues to respond to the effects of starvation or other factors more quickly than other tissues. Thus, inferences about diet over short versus long time intervals require tissues with short versus long integration rates respectively. However, the susceptibility of tissues with high metabolic rates to the effects of starvation makes them less reliable than tissues with low metabolic rates. By identifying the appropriate time scale of a stable isotope study it may be possible to select tissues that limit the likelihood of $\delta^{13}C$ and $\delta^{15}N$ values that reflect sources of variation other than diet.

Stable isotopes may be a valuable tool in ecological studies if the factors that drive variation in values are well understood. To date, more focus has been put on external factors that influence stable isotope signatures rather than on internal factors, such as starvation. The degree to which internal factors drive variation in stable isotope

signatures itself may vary among species and so requires more studies like this but involving different taxa.

Table A.1. Comparison of each tissue type (muscle, liver, blood plasma and blood cells) for differences between sunfish assigned to the diet treatments (littoral benthic macroinvertebrates or pelagic zooplankton) immediately before the start of the experiment using the student's t-test for multiple comparisons.

	Diet Treatment			
Tissue Type	Macroinvertebrates	Zooplankton	t_{79}	p
White Muscle	-26.90	-26.60	1.13	0.27
Liver	-26.78	-26.52	1.00	0.32
Blood Plasma	-30.05	-29.61	1.60	0.12
Blood Cells	-27.80	-27.49	1.20	0.25

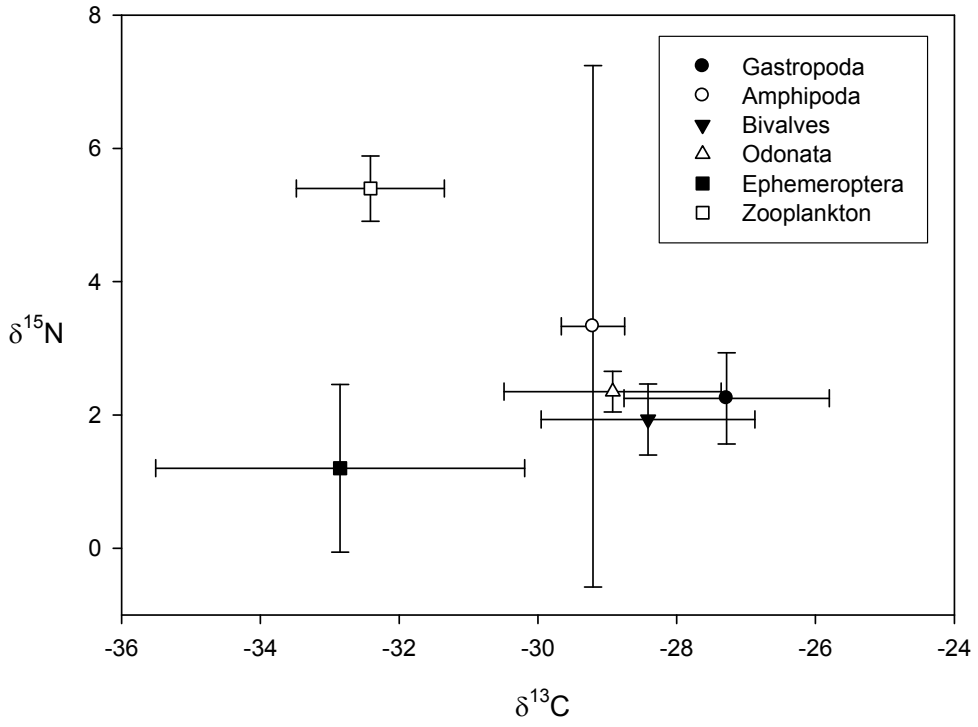

Figure A.1: Mean stable isotope values of $\delta^{13}C$ and $\delta^{15}N$ (±SE) for the 6 major prey groups used in this experiment. The stable isotope signature of zooplankton combines cladocerans and copepods and represents the pelagic zooplankton diet treatment. All other prey groups were present in the littoral macroinvertebrate diet.

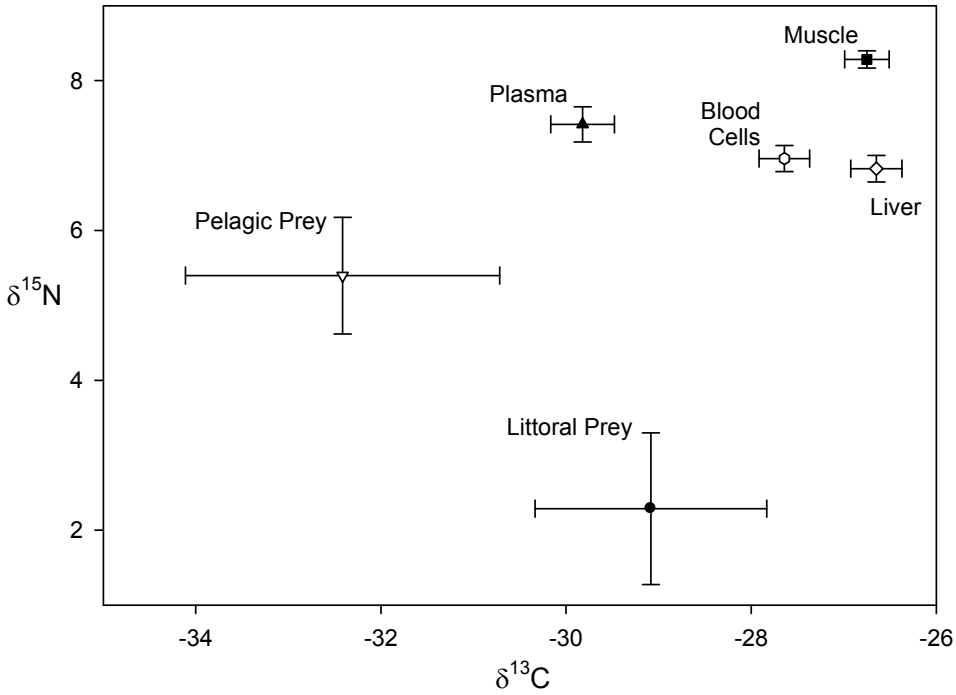

Figure A.2: Mean pre-experiment stable isotope values of $\delta^{13}C$ and $\delta^{15}N$ (± 1SE) of four types of tissue from pelagic pumpkinseed sunfish relative to that of the mean of littoral prey (gastropoda, amphipoda, bivalvia, odonata, and ephemeroptera) and pelagic prey (cladocera and copepods).

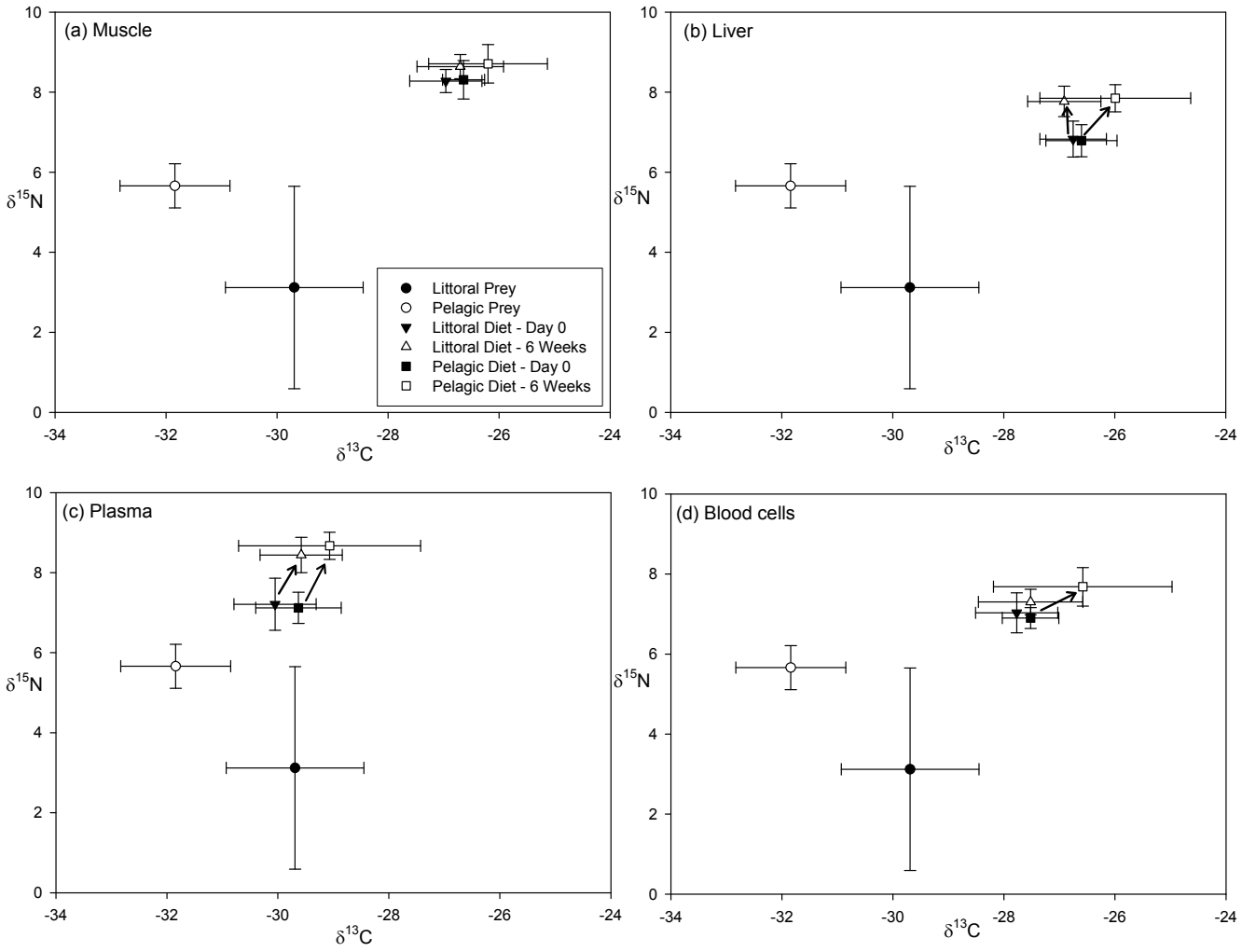

Figure A.3: Changes in stable isotope values of $\delta^{13}C$ and $\delta^{15}N$ (± 1SE) from day 0 to 42 days for four tissue types (muscle, liver, blood plasma, and blood cells) of pelagic pumpkinseed sunfish fed either an exclusive diet of pelagic zooplankton or a diet of littoral macroinvertebrate prey along with the mean values for prey composing each diet treatment.

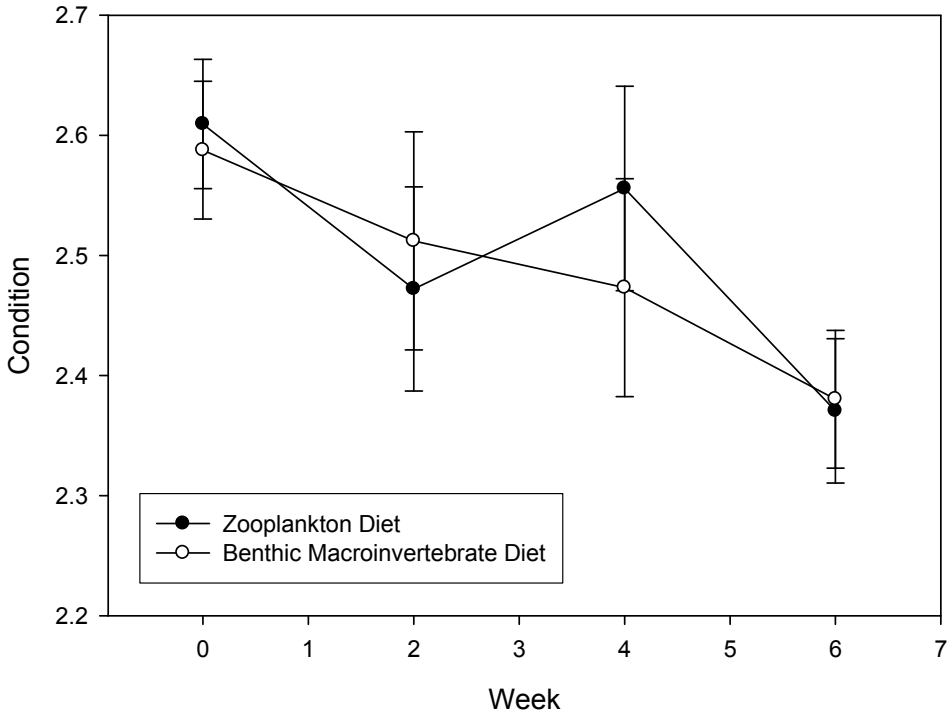

Figure A.4: Change in mean pumpkinseed sunfish body condition (± 1SE) for each diet treatment (zooplankton or benthic macroinvertebrate) from Day 0 to 42 days.

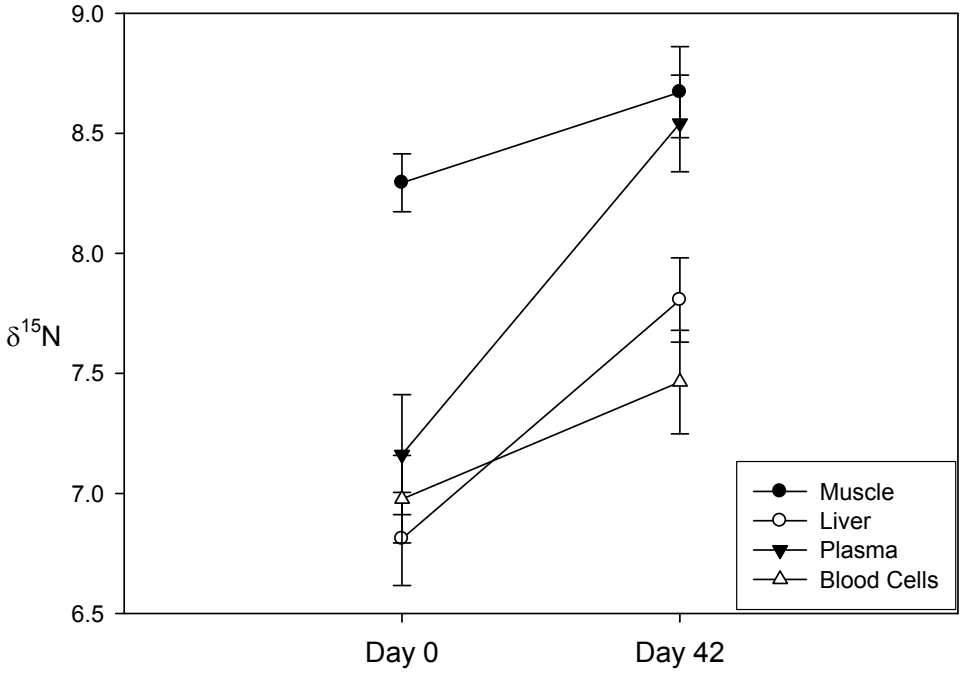

Figure A.5: Change in mean $\delta^{15}N$ values from Day 0 to 42 Days in four different tissues (pelagic zooplankton and littoral macroinvertebrate diet treatments combined).