IMPACTS OF INTRODUCED CRAYFISH ON ASH MEADOWS AQUATIC COMMUNITIES: ASH MEADOWS NATIONAL WILDLIFE REFUGE, NEVADA

BY

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THESIS

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Abstract

The single greatest concentration of endemic life in the United States, and second greatest in North America, can be found 90 miles northwest of Las Vegas, Nevada in Ash Meadows National Wildlife Refuge. This area is threatened by numerous non-native, introduced species, most notably the Red Swamp Crayfish (*Procambarus clarkii*). The interactions are of concern to the Refuge because of the presence of the federally protected endemic life, specifically pupfish (*Cyprinodon nevadensis mionectes* and *Cyprinodon nevadensis pectoralis*) and the federally threatened Ash Meadows naucorid (*Ambrysus amargosus*). Crayfishes may be potential predators and/or competitors for resources. I investigated the potential impacts *P. clarkii* poses to the sensitive aquatic systems and more specifically its food web. Within the Refuge, three springs lacking crayfish were compared to three springs which contained crayfish. I coupled habitat assessments, invertebrate community assessment and fish abundance and condition with stable isotope analysis and gut content analysis or crayfishes to better understand the interactions which the invasive *P. clarkii* has with the endemic system. Invertebrate communities, specifically snail populations, are less diverse in those springs with crayfish. Fish condition and abundance showed no significant trends between treatments. Stable isotope analysis indicated significant overlap at the same consumer level between crayfish, pupfish and even invertebrate grazers and predators in some seasons, but trophic position analysis showed no significant relationship to treatment. Gut contents revealed that crayfish shifted to a more animal based diet in the winter months, and animal material (specifically fish parts or snails) occurred in over a third of the stomachs examined. Results from this study highlighted the direct and indirect effects which this invasive crayfish poses to Ash Meadows aquatic communities and the need for future management techniques to include continued efforts to eradicate crayfish from the springs.

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Introduction

Non-native, invasive species are becoming one of the greatest threats to biodiversity around the world (Wilcove et al. 1998, Clavero and García-Berthou 2005). Recent estimates put a price tag upwards of $120 billion a year for damages and control costs associated with alien species (Pimentel et al. 2005). Alterations to an ecosystem's species composition, nutrient cycling, physical features and productivity are all consequences of invasive species and are being perpetuated by human activity (Mack et al. 2000, Crooks 2002). These consequences become even more troublesome when endangered species are involved. Competition or predation by non-native species is one of the primary threats to almost 50% of threatened and endangered species listed under the Endangered Species Act (Wilcove et al. 1998) and considering that we only have adequate information for 15% of all the known species in the United States, this number could be well below reality (Wilcove and Master 2005). Invasive species come in all forms – vertebrates, invertebrates and plants, and there is no discrimination when it comes to habitat – terrestrial, marine or freshwater environments are all facing challenges due to invasive species and few habitats have escaped the strain which introduced species create (Mack et al. 2000).

A particular taxon of freshwater invasive species that does not receive adequate attention is the crayfish. A common crustacean found natively on every continent except Antarctica and Africa, and inhabiting a variety of water bodies like streams, lakes, marshes and even caves, the crayfish is often overlooked for its importance in aquatic ecosystems, and as an aquatic invasive species. North America is home to over 75% of the world's 500+ crayfish species, with a majority of these species native to the southeastern part of the country (Taylor et al. 2007), but from this great biodiversity come some of the greatest threats to aquatic ecosystems. Over 20

years ago, 20 crayfish species had been documented as introduced into new river drainages, states or continents (Hobbs et al. 1989) and it is easy to assume that this number has increased. Crayfish have been introduced to Europe, Africa and Australia and it comes as no surprise that they are widely introduced across the Unites States. New records of non-native crayfish species are being recorded all over the United States, particularly west of the Mississippi River (Mueller 2001, Carpenter 2005, Rogowski and Stockwell 2006, Olden et al. 2009 and Larson and Olden 2011). These introductions become increasingly important when one considers the "keystone" role which crayfish play.

Because they often dominate invertebrate biomass and are omnivores, crayfish can have substantial effects on all parts of a food web, interacting with everything from detritus to fish (Momot 1995, Stenroth and Nyström 2003). It is this interactive role which puts them in the "keystone" species category, where crayfish have a disproportionate effect on lower organisms relative to their abundance (Paine 1969, Momot et al. 1978). Through omnivorous activities, crayfish have the ability to manipulate community composition and do so quite regularly. Thus, as an alien species, an introduced crayfish's keystone species role could become destructive to the entire ecosystem and food web. Crayfish can, in effect, cause changes to every trophic level of a system. There is no shortage of literature which illustrates the negative impact of these activities (Lodge et al. 2000, Hobbs and Lodge 2010).

Introduced crayfish have been linked to changes or reductions in periphyton, macrophytes and macroinvertebrates and can compete with native crayfish and fish species for habitat and food resources. Several species from the south and Midwest have become widely introduced across the United States and worldwide. There are a multitude of studies involving the Rusty Crayfish, *Orconectes rusticus*, a species native to the Ohio River drainage, which is

now widely introduced in the Great Lakes region in Wisconsin and Minnesota, New England, and isolated locations in Colorado and Wyoming (http://nas.er.usgs.gov). Using a combination of enclosure-exclosure studies and macroinvertebrate surveys along natural crayfish density gradient, researchers found direct and indirect effects of the invasive Rusty Crayfish. Reductions in total macroinvertebrate densities as high as 58% and herbivore densities up to 72% were seen in the enclosure-exclosure experiment with the longitudinal gradient study mirroring these results (Charlebois and Lamberti 1996). Lodge et al. (1994) conducted a similar enclosure-exclosure experiment and found similar results with significant declines in macrophytes and snails declining to 1% of original abundance. An intense invasion by the Rusty Crayfish in a Wisconsin lake resulted in a decline in snail abundance from more than 10,000/m^2 to just 5/m^2, a decrease in the mean abundance of multiple aquatic insect orders, and submerged macrophyte species richness declined by as much as 80% (Wilson et al. 2004). The Signal Crayfish, *Pacifasticus leniusculus*, which has been introduced in Europe, caused decreased total invertebrate biomass and taxon richness while increasing biomass of epilithic algae in in-stream enclosure experiments (Stenroth and Nyström 2003). The Virile Crayfish, *Orconectes virilis*, is yet another example of a widely introduced species and can be found throughout the United States, notably in Washington, Montana, Idaho, Arizona and New Mexico (http://nas.er.usgs.gov), with Arizona being an area where a native crayfish species has never existed (Carpenter 2005, Martinez 2012). This species also causes many negative impacts on the ecosystems it invades. Chambers et al. (1990) found Virile Crayfish to significantly affect biomass of particular macrophytes, either through indirect stimulatory means or direct reductions by grazing, in a series of mesocosm experiments. In those cases where *O. virilis* has been introduced into a system in which there was previously no native crayfish, as it now exists in the

Colorado River Basin, the presence of this crayfish decreased growth of certain native fish species (Carpenter 2005). Virile Crayfish were found to have equal biomass to all other macroinvertebrates and fish combined in a recent survey of the middle Yampa River in Colorado (Martinez 2012) which raises concerns for the sensitive ecosystem which exists here. The Virile Crayfish has negatively affected local aquatic communities in the Pacific Northwest and has been called the most widely invasive crayfish in that area (Larson and Olden 2011).

Not only do invasive crayfish interact at many levels within the food web, but also at many points with stream physical characters. The term "ecosystem engineers" is commonly used to describe the substantial effects that crayfish can have on stream ecosystems and how they interact with the stream in terms of substrate processing and sediment accumulation. A New Zealand study found that native crayfish filled a key leaf decomposition role (Usio 2000) and another study found that leaf decomposition was significantly increased in the presence of crayfish (Creed and Reed 2004). One particular study even termed crayfish as "geomorphic agents" and found that 10 cm Signal Crayfish, *Pacifastacus leniusculus*, were capable of displacing an average of 1.7 kg m^{-2} of sediment per day (Johnson et al. 2010).

Another crayfish species eclipses all the rest and is hailed as the most invasive crayfish species in the world. The Red Swamp Crayfish, *Procambarus clarkii*, is now established on every continent except Australia and Antarctica and has been spread through various means, most notable aquaculture and possibly the biological supply trade (Hobbs et al. 1989, Larson and Olden 2008). In North America this species has been documented in at least 13 states well outside its native home range of the Southeastern United States (http://nas.er.usgs.gov).

The Red Swamp Crayfish, similar to other introduced crayfish, has been shown to impact and cause some change to every level of the food web into which it is introduced. At the macrophyte level, *P. clarkii* has proven it can have dramatic negative effects - decreasing pondweed cover from 70% to 0% in a California wetland, showing selective feeding choice for particular macrophyte species, significantly decreasing biomass of macrophyte species in lake enclosure experiments, and increasing turbidity through consumption of macrophyte beds and decreasing abundance and distribution of submerged aquatic plants in African lakes (Feminella and Resh 1989, Cronin et al. 2002, Gherardi and Acquistapace 2007, Foster and Harper 2007, Matsuzaki et al. 2009). In Portugal, where *P. clarkii* often occurs in rice fields, it is known to disrupt the establishment of seedlings (Correia 2003). *Procambarus clarkii* has been introduced into streams within a national park in southern Spain and the area has seen rapid decreases in diversity of aquatic communities, specifically the extinction of various species of gastropods, as well as a general decline of macrophyte abundance and even extinction of certain species (Gutiérrez-Yurrita et al. 1998, Alcorlo et al. 2004). In other areas it directly preys upon and reduces native snail abundances or decreases the invertebrate biomass and density of invertebrates as a whole (Gherardi and Acquistapace 2007, Klose and Cooper 2012). Elsewhere, negative shifts in macroinvertebrate diversity were observed in experimental mesocosms (Correia and Anastácio 2008)

The Red Swamp Crayfish also exerts itself on upper trophic levels of an invaded system. In rearing ponds, this crayfish negatively affected survival of the native Razorback Sucker (*Xyrauchen texanus)* of the Colorado River (Lenon et al. 2002). In some areas *P. clarkii* has replaced native crayfish and affected amphibian populations through direct predation (Gamradt and Katz 1996, Gherardi 2006) and a study of isolated pools showed increased rates of fish

predation by Red Swamp Crayfish in shallow, densely populated areas, areas which contain valuable indigenous fish (Ilhéu et al. 2007). In some instances, the Red Swamp Crayfish may initially deplete all food resources available (Correia 2002).

The impacts of *P. clarkii* have not yet been fully assessed in most areas of the western United States where it is quickly becoming established and spreading (Larson and Olden 2011). The ability of *Procambarus clarkii* to exert such effects on multiple trophic levels gives it status as a "keystone" species, and its widespread introduction may be putting many biologically important ecosystems in jeopardy. This species is tied to a multitude of systems with endemic species as already noted. One such area of concentrated endemism which this species has been introduced is Ash Meadows National Wildlife Refuge, located in southern Nevada, USA. This particular area is home to at least 11 plants, 5 fish and 15 invertebrates found nowhere else on earth (Stevens and Bailowits 2008) while still more unique organisms are being assessed. The current study focused on this particularly important ecosystem and attempted to gain a better understanding of effects of an invasion of *Procambarus clarkii*.

Ash Meadows National Wildlife Refuge (Ash Meadows NWR) is located 90 miles northwest of Las Vegas, Nevada in the Mojave Desert. Its aggregation of unique plants and animals gives the Refuge the distinction of having the greatest concentration of endemic life in the United States, and the second greatest in North America (USFWS 2009, Crews and Stevens 2009). Ash Meadows NWR is a unique desert oasis comprised of more than 50 different warm spring seeps with temperatures ranging from 18°C to upwards of 33°C (Garside and Schilling 1979, Weissenfluh 2010) and cumulative discharge estimates of just under $0.7 m^3/s$ (Scoppettone et al. 1995). These springs pop up in a linear North-South fashion across 22,000 acres of wetland and upland habitat (Figure 1). The spring complexes are home to 17 endemic aquatic

taxa: five fish, nine snails and three aquatic insects. Of these, four fish are listed as endangered and one aquatic insect is considered threatened. Ash meadows NWR exhibits an "island" quality being isolated with no connections to any other aquatic systems. This characteristic makes it especially susceptible to alien species (Wilcove et al. 1998).

Previous use of the limited water source and land was for agriculture and even a potential housing development (Pister 1974, Riggs and Deacon 2002). Since the establishment of Ash Meadows NWR in 1984, the previous use of the land for agricultural purposes has ceased. The Refuge has continuously worked to restore and preserve the existing land by securing habitat and water sources, while re-establishing and monitoring new and existing populations of plants and animals. However, the aquatic native species within the Refuge still face numerous challenges, most notably, continued groundwater withdrawal from nearby municipalities and the increasing threat of non-native introduced and invasive species. For example, Bullfrog, *Lithobates catesbeianus,* Mosquitofish, *Gambusia affinis,* Largemouth Bass, *Micropterus salmoides*, Red-Rimmed Melania snails, *Melanoides tuberculata,* and the Red Swamp Crayfish, *Procambarus clarkii*, create poorly understood problems of predation and competition for resources within the unique desert system. Such non-native species have been known to the Refuge for decades (Miller 1948), and the Red Swamp Crayfish has been blamed for the extinction of the endemic Ash Meadows poolfish, *Empetrichthys merriami* (Soltz and Naiman 1978) as well as extirpation of a pupfish population from South Indian Spring (Martin 2010). With the ever present issue of non-native invasive species within Ash Meadows NWR comes a need to better understand the dynamics of such organisms and the effects they have on a system.

Though considered a native of the southeastern United States from the Mississippi and down through the Gulf Coast, the Red Swamp Crayfish has occurred in California since the

1920's (Holmes 1924). This species could have been introduced into the Ash Meadows area through numerous pathways including bait bucket, pet trade or aquaculture (Lodge et al. 2000).

Of particular interest in Ash Meadows NWR is the occurrence of *P. clarkii* within certain systems and its co-occurrence with other native species in those springs, but also the lack of the crayfish in other springs on the Refuge. Recent work by Kennedy et al. (2005) in Jackrabbit Spring, located within Ash Meadows NWR, found that crayfish and native pupfish are primary consumers and probably compete for the same resources through most of the year. However, this study only covered the Jackrabbit Spring system. Inclusion of food web assessments within other complexes on the Refuge could provide valuable information on how resources are being used elsewhere by both the native fish and aquatic invertebrates in conjunction with *P. clarkii*. In addition, this would clarify the position of the Red Swamp Crayfish in the food web as it occurs in this unique system. Because this species has been established on the Refuge for such an extended time period, complete removal is not always a viable option. Indeed, once invasive species become established, it can be a near impossible task to eliminate them (Molnar et al. 2008), though the Refuge has had success in some cases.

Multiple springs throughout the Warm Springs complex provide parallel environments where endemic Warm Springs pupfish (*Cyprinodon nevadensis pectoralis*) and the endemic Warm Springs Naucorid (*Ambrysus relictus*) are both found, yet through removal and restoration methods, the presence of *P. clarkii* is limited to one spring. Elsewhere on the Refuge, Ash Meadows Amargosa Pupfish (*Cyprinodon nevadensis mionectes*) inhabit the springs. Both subspecies of pupfish forage on algae and sometimes invertebrates (Naiman 1975, 1976) which creates the obvious problem of possible overlap for resources with the introduced omnivorous crayfish, yet little data exist that illustrate this overlap. Here I used multiple methods - habitat

surveys, invertebrate community analysis, fish abundance and condition assessment, stable isotopes analysis and crayfish gut content analysis - to gauge the extent to which crayfish are interacting with the endemic aquatic community and within the food web.

By combining an array of analyses with taxon specific sampling of spring inhabiting organisms, I addressed the following hypotheses in an effort to better interpret ecosystem structure and function in cases of the presence and absence of an introduced species within warm springs of Ash Meadows NWR.

H_0 1: Pupfish that occur in springs with crayfish have the same level of condition, trophic position and abundance as fish that occur in springs without crayfish.

H_0 2: Abundance and diversity of invertebrates and macrophytes will be similar at springs with and without crayfish.

H_0 3: Crayfish are not selectively feeding on endemic invertebrates within springs

Methods

Study Sites

Within the extensive spring system of Ash Meadows National Wildlife Refuge, three springs containing crayfish (Jackrabbit, Big and South Scruggs) and three springs without crayfish (Indian, School and North Scruggs) were studied (Figure 1). Four of these springs (North and South Scruggs, School and Indian) are located within the management unit of the Warm Springs complex. This complex is made up of small (width <1m, depth <15cm) low flow (<1cfs) springs. The complex is inhabited by the Warm Springs Pupfish (*Cyprinodon nevadensis pectoralis*) and the endemic Warm Springs Naucorid (*Ambrysus relictus*), among a variety of other endemic and non-endemic taxa. Jackrabbit and Big springs are both located south of the Warm Springs complex, are slightly larger springs (1-2m widths, average depth 35cm, flow 1-2cfs) and are inhabited by the Ash Meadows Amargosa Pupfish (*Cyprinodon nevadensis mionectes*) and the Ash Meadows Speckled Dace (*Rhinichthys osculus nevadensis*) but no other endemic taxa. Initial habitat surveys were conducted to assess the area within each spring that was accessible for efficient sampling and specific sampling sites at the springs were selected based on this accessibility. Each spring acted as its own sampling unit and within each spring 20 m of stream outflow length was designated as a site. If two separate sites were needed to reach 20 m (e.g. North and South Scruggs and School Spring), the lower site was denoted as (1) and upper as (2) and sample data from both reaches were combined for analysis. Sampling occurred in fall 2011, winter 2012 and spring 2012.

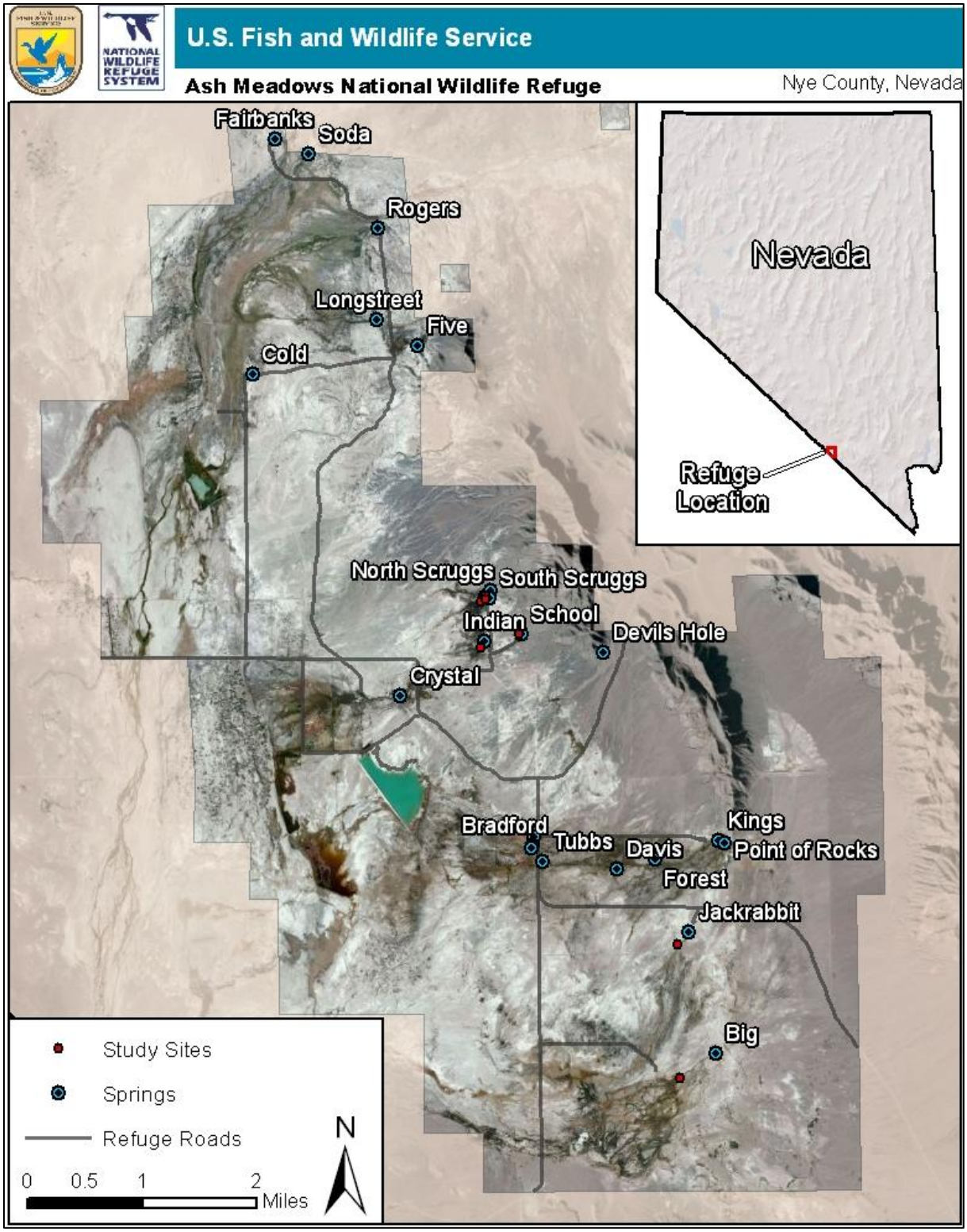

Figure 1: Map representing study sites locations within Ash Meadows National Wildlife Refuge, Nevada, USA.

In-Stream Vegetation and Habitat

At each sampling site, measuring tapes were stretched along each side of the reach, closely following bank profile (Figure 2), and length was measured from the left tape (facing downstream). Vegetation and in-stream habitat were sampled following modified methods from USGS National Water-Quality Assessment Program (Mouton et al. 2002), EPA Rapid Bioassessement Protocols (Barbour et al. 1999), and Hauer and Lamberti (2006). Bank vegetation was characterized at 0.5m intervals, by recording the dominate type or types (genus if possible) present at the specific point, and measuring the height of each type. Bank characterizations began with the left downstream side and working upstream, then crossing to the right side and going downstream. In-stream vegetation was assessed by cross section transects. Data were collected at 2m intervals, starting upstream and proceeding downstream. A 10cmx10cm quadrat was placed at the bank, and then at 0.5m intervals across the stream. If the channel was less than 0.5m wide, then only each bank was sampled and smaller intervals were noted if applicable. Within the quadrat, percent cover (NEAREST 5%) by either algae (filamentous) or macrophyte (genus if possible) was recorded, as well as type and size of substrate (modified pebble count method, Wolman 1954, Leopold 1970). Substrate size was then assigned a score (1 to 4, modified from Bain et al. 1985 and Litvan et al. 2010). Widths were recorded at 3 (6 total for springs with two reach lengths) of the cross section points. Water quality measurements [flow (Marsh-McBirney Flow Mate Meter), pH, temperature (Hanna Combo Meter), dissolved oxygen (YSI 85 Meter)] were taken on the same day at all springs and were measured at the top, middle and bottom of the reach. Habitat variables (temperature, DO, flow, substrate score, average % algae (filamentous) or vegetation cover) were analyzed using

principle components analysis in Primer v6 (PRIMER-E Ltd, Plymouth, UK) and compared sites with and without crayfish.

Figure 2: Sample site example illustrating bank vegetation sampling. Site pictured is Indian Spring, winter 2012.

Macroinvertebrate Sampling

Macroinvertebrates were sampled following modified methods from USGS National Water-Quality Assessment Program (Moulton et al. 2002), EPA Rapid Bioassessement Protocols (Barbour et al. 1999), and Hauer and Lamberti (2006). Macroinvertebrate sampling was conducted using small dip nets (17.78cm across the base). Specific points of collection were selected randomly but number of points sampled was proportionally split between habitat types (e.g. 5 riffle, 5 pool - if evenly distributed between the two) and always totaled 10 samples. Specimens were collected by placing the dip net downstream of area to be sampled then disturbing an area about18cm x 18cm front of the net (324cm² total). Contents of the 10 samples were then combined into a 5 gallon bucket. For fall 2011, samples were poured over a 300µm sieve, placed in a sample bottle and entire contents preserved in ethanol. The subsequent winter and spring 2012 samples were quartered (per USFWS request). This was accomplished through utilization of a modified splitting apparatus (Figure 3). The entire contents were poured evenly over the splitter two separate times. After the first pour, a coin was tossed to choose which bucket of contents was returned to the stream, then the process was repeated once more until only a quarter of the original sample remained. Next, the contents were spread between multiple sorting trays and endemic springsnails (Gastopoda: *Pyrgulopsis* and *Tryonia*), naucorids (Hemiptera: *Ambrysus relictus*) and occasionally Elmidae (Coleoptera) adults or larvae were removed from the sample, counted and placed back into the spring. Once these organisms were returned to the stream, the remainder of the dip nets contents were placed in containers, preserved in 70% ethanol and sorted in the laboratory at a later time.

Figure 3: Macroinvertebrate sample splitter used to quarter invertebrate samples taken during the winter and spring sampling periods. (Photo: Hal Fairfield)

Laboratory processing

Macroinvertebrate samples were returned to the lab where they were rinsed over a 295 µm mesh sieve and then placed in sorting trays. Samples were sorted in small increments and all macroinvertebrates were separated from plant matter and sediment. Specimens were sorted into separate taxa and then identified to lowest taxonomic level possible (Merrit and Cummins, 1996 Thorp and Covich 2010) and preserved in 95% ethanol. Macroinvertebrate abundance, richness, evenness, relative proportion and Shannon (H', Shannon and Weaver 1949) and Simpson's (Δ, Simpson 1949) indices were calculated for each spring, each season.

Taxa abundance was normalized by log transformation (log(x+1)), a Bray-Curtis similarity applied and then analyzed using non-metric multi-dimensional scaling (MDS) in Primer software (PRIMER v6: PRIMER-E, Plymouth, Clarke 1993). MDS was used because it ordinates samples based purely on community similarity and plots samples according to that similarity of the community composition.

Fish Abundance and Condition

Fish (and crayfish if applicable) sampling was conducted through a depletion estimate sampling method. The top and bottom of the reach was blocked with seine nets, which were well anchored across the bottom with rocks found from the surrounding area to ensure that a closed system was created. Then, a short seine net was placed at the lowest downstream point (beginning in front of the lowest block net) and, starting a few meters up from the net, one or two people splashed/agitated water and vegetation up to the net, attempting to scare fish into the net. This method was repeated two more times, with the seine net being placed at the starting point of the last section, splitting the first pass into three separate portions. This entire method was then repeated two more times for a total of three separate passes, with the goal of decreasing numbers of fish/crayfish collected each pass. Any pupfish collected during a pass were placed in a bucket with aerators and stress coat while crayfish or non-native fish were placed in a separate bucket. After each pass, the fish/crayfish were identified then pupfish and crayfish were measured (total length [TL], crayfish carapace length [CL]) and weighed (nearest 0.1g). Any pupfish or native fish were returned to the stream (upstream or downstream of block nets) and crayfish and non-native fish were placed on ice and later preserved and processed for stable isotope analysis. Fish condition was assessed using a simple length to body mass index. A modified version of Fulton's fish condition factor (Fulton 1911, Williams 2000) was used:

$$K = 100{,}000 * \frac{M}{L^3}$$

Where K = condition, M = mass and L= length).

Condition was assessed across sites and treatment using a mixed model ANOVA. Mixed model ANOVA's allow assignment of fixed (those set by the experiment) and random (sampled from a larger set of potential samples) effects. This type of analysis was used for its ability to incorporate the multilevel nature of the study (sites nested within season) and the treatment type (crayfish absence or presence). Here, treatment type (crayfish presence or absence) and season were fixed effects while stream (nested within season and within treatment) was a random effect. This method allowed for a broad application of results to other streams within the Refuge rather than thinking of each stream as an isolated example. All analyses were conducted using JMP version 9 (SAS Institute, Cary, NC, USA). Alpha level for all tests was set at 0.05.

Stable Isotopes

To assess food web interactions, materials from all potential trophic levels, including distinct invertebrate functional groups and higher level consumers were collected. This included detritus (fine particulate organic matter, FPOM), leaf litter, algae, aquatic and terrestrial plant sources, a macroinvertebrate grazer and a predator, crayfish (if applicable) and fish. Fine particulate organic matter, leaf litter and algae were collected by hand, placed in Whirl-pak bags and placed on ice. Invertebrate grazers and predators were collected from the split dip net collections. For most springs, the invasive snail *Melanoides tuberculata* was collected as the grazer species due to its abundance and to avoid taking large numbers of the endemic springsnails. However, in North Scruggs Spring this species was not available within the sample reach and thus endemic *Pyrgulopsis* and *Tryonia* spp. had to be collected to fill the grazer roll.

The invertebrate predator was usually an Odonate species. The fall sampling season was an exception. Here, both Odonate and *Ambrysus relictus* were collected to provide at least one signature example for the endemic naucorid species. Crayfish were mainly collected during seine net sampling, however in the case of South Scruggs Spring; specimens had to be collected by hand during night samples or by baited traps set overnight.

Fin clips were used to obtain stable isotope data for pupfish because of their endangered status. A non-lethal alternative to the traditional muscle plug (Jardine et al. 2005, Kelly et al. 2006), fin clip sampling was conducted on separate days from the previous sampling methods with a state biologist (K. Guadalupe, Nevada Department of Wildlife). In most cases, un-baited minnow traps (3-4) were placed within each sampling reach and allowed to set for 2- 3 hours. After this time, any pupfish collected were measured (total length); a fin clip was cut from the upper half of the caudal fin, and then were returned to the stream. Fin clips were placed into individually numbered vials and placed on ice. On one sampling occasion, and at two sampling sites (South Scruggs and Big Spring), baited traps had to be utilized in order to capture crayfish and pupfish.

Laboratory processing

Samples of FPOM, leaf litter, algae, macroinvertebrates (grazer, predator, other and crayfish) and fish fin clips were processed according to methods of Stenroth et al. (2006) and Taylor and Soucek (2010) and analyzed to determine the isotopic signatures of an individual or group. FPOM samples were vacuum filtered through 1.5µm glass microfiber filters (Whatman 934-AH) and then placed in individual tin weigh boats for drying. Crayfish muscle tissue was removed from the abdomen, snails bodies were removed from their shells and for Odonates or

other invertebrate consumers whole bodies were used. These were similarly placed in weigh boats and dried in a 55°C oven for 48 hours. Fish fin clips were individually placed in weigh boats dried for only 24 hours due to the small material amounts and sensitive nature. FPOM, leaf litter and algae were then placed in a desiccator with a beaker containing 100ml HCl for six hours to remove inorganic carbonates. Subsequently, all samples (except fin clips) were ground with mortar and pestle and packed into individual tin capsules (Stenroth et al. 2006, Taylor and Soucek 2010). FPOM samples required approximately 4mg of material to obtain a signature, leaf and algae approximately 3mg and all animal tissue ranged between 0.25mg and 1mg depending on the total amount that was available from field collections. Fin clips were left whole and material weights ranged from 0.25mg to 1mg (more if possible) due to the small size of some fin clips. Some fin clip samples required combination of multiple clips in order to reach the minimum weight of 0.25mg required to obtain a reliable isotopic signature. Fall 2011 samples were processed at University of California - Davis while winter and spring 2012 samples were processed by Southern Illinois University – Carbondale isotope facilities. UC Davis processed samples using a continuous flow Isotope Ratio Mass Spectrometer (IRMS) PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). SIUC facilities use a Costech 4010 ECS (Elemental Combustion System = EA) connected to a Delta V Plus Isotope Ratio Mass Spectrometer [CF-EA-IRMS (Continuous Flow-Elemental Analyzer-Isotope Ration Mass Spectrometry)] with analytical precision +/- 0.06 per mil for both $\delta^{15}N$ and $\delta^{13}C$.

Stable isotope ratios are expressed as δ in parts per thousand (‰) and calculated by the following:

$$\delta X = \frac{R_{sample}}{R_{standard}} * 1000$$

Where X = ^{15}N or ^{13}C and R = ratio of the respective isotopes in the sample and standard. Both facilities utilized the international standards V-PDB (Vienna PeeDee Belemnite) and air for carbon and nitrogen, respectively.

Trophic position (*TP*) was then calculated by the following equation:

$$TP_{consumer} = TP_{base} + (\delta^{15}N_{consumer} - \delta^{15}N_{base})/\Delta_N$$

Algae was used as the base and $TP_{base} = 1$. The $\Delta_N$was set at 3.4 based on an established average fractionation rate (Petersen and Fry 1987, Vander Zanden and Rasmussen 2001, Post 2002, Nilsson et al. 2012). Calculated trophic positions were then analyzed using the same mixed model ANOVA as stated above for fish condition, and conducted with the same fixed and random effects.

Gut Contents

Crayfish foreguts (cardiac and pyloric stomachs) were removed from crayfish and preserved in ethanol until analysis. The contents of foreguts were emptied onto a gridded petri dish (25 squares, 1cm² each) and spread out uniformly with 0.25ml increments of water. Once the contents were evenly dispersed, the total number of squares covered was recorded and then each square was assessed at 40x magnification for specific contents. Each individual square was split into quarters at this magnification and within each quarter, the percent composition (to nearest 10%) of six different material categories (macrophyte, woody material, detritus, sediment, algae or animal) was recorded. Animal material was identified to taxon if possible (lowest taxonomic unit). Total percent coverage was then divided by total squares occupied and thus total percent composition of the six materials could also be estimated. A total of 52 guts were examined.

Results

Instream Habitat and Vegetation

Principle components analysis resulted in three main axes (Table 1) which explained 84% of the variation for between site differences. PC1 explained 47% of the variation and was related to a combination of depth and % vegetation cover, where high (+) scores of PC1 corresponded to greater depth and higher vegetation cover with the inverse being true for low (-) PC1 scores (Figure 4). PC2 represented variations in DO and explained 20.9% of spring variation. PC2 scores were high (+) if the spring had higher DO and low (-) if the inverse was true (Figure 4). PC3 (16.2%) was Temperature (°C) and DO dependent with positive scores relating to lower temperature and higher DO.

Table 1: Eigenvector values for the three main principle components of principle components analysis for temperature, dissolved oxygen, flow, substrate score, depth, percent algae cover, percent vegetation cover and the percent variation explained by each variable. Those variables in bold represent main components of that vector.

Eigenvectors			
Variable	PC1	PC2	PC3
Temp °C	-0.4	0.282	**-0.527**
DO	-0.04	**0.647**	**0.521**
Flow	**0.397**	-0.381	-0.298
Substrate Score	-0.338	-0.331	0.314
Depth	**0.459**	-0.199	0.383
% Algae cover	-0.348	-0.311	0.339
% Veg cover	**0.485**	0.334	-0.048
% Variation explained	47	21	16

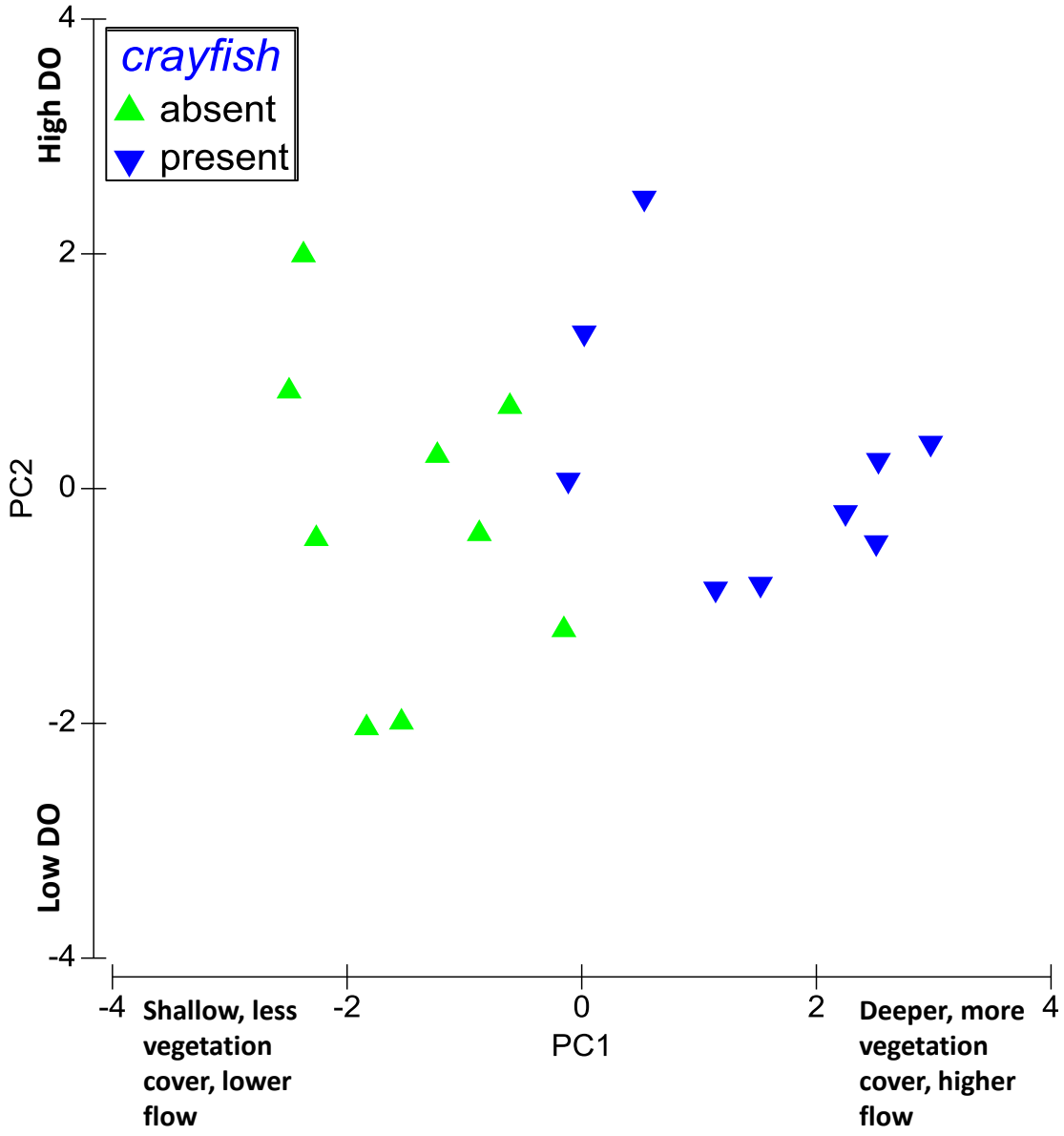

Figure 4: Principle components analysis plot representing principle component 1 (PC1) and principle components 2 (PC2) for all sites and fall, winter and spring sampling events. PC1 is represented by depth, percent vegetation cover and flow. PC2 is represented by dissolved oxygen.

Invertebrate Abundance and Diversity

Counts of invertebrate taxa present at all spring sites are presented in Table 2. Overall, 16 orders and 9 functional groups [grazer, scavenger, scraper/collector-gather, collector-gather, piercer-herbivore/shredder, collector-filterer, herbivore (algae), predator (engulf), predator (piercer)] (Merrit and Cummins, 1996) of invertebrates were represented between the six springs across three seasons of sampling. The Warm Springs Complex tended to be dominated by the gastropods *Tryonia* and *Pyrgulopsis* (exceptions: Indian and South Scruggs) while Jackrabbit and Big Spring were dominated by either Amphipoda or *Melanoides tuburculata* and contained fewer species. Most abundant groups across all springs were Gastropoda, Amphipoda and Chironomidae. Indian Spring had recently undergone restoration (completed March 2011) and exhibited successional patterns in its invertebrate communities. Both North and South Scruggs showed jumps in Chironomidae, Elmidae and *Hyalella* abundance (though relative % remained similar) from fall to spring sampling events. North Scruggs revealed a dramatic increase in springsnail abundance with successive seasons while South Scruggs actually showed a decreasing trend and *Pyrgulopsis* snails were actually not found in the spring sample.

Jackrabbit, Big and South Scruggs Springs had significantly lower richness (ttest, $p<0.001$) than sites without crayfish (Table 3) yet evenness, Shannon and Simpson's indices were similar between all sites and all seasons (ttest, $p>0.05$). The MDS plot (Figure 5) illustrates the relationship of invertebrate communities between sites. The plot overlay represents percent similarity between site communities with site similarity equaling proximity on plot. Big and Jackrabbit Springs become separate at only the 40% resemblance level, while other springs still group at that level. At 60% similarity a difference in crayfish presence and absence springs is apparent. South Scruggs becomes isolated, as well as the fall samples of Indian and North

Scruggs. The only samples that remain grouped after 80% similarity are the fall and winter samples from School Spring. Special note should be taken for Jackrabbit Spring. This spring has dried completely in the past as a result of pumping for irrigation (Pister 1974) and thus its invertebrate community composition may have been compromised, and no pre-drying data of invertebrate communities exists.

Table 2: Raw invertebrate counts for each spring in each season. Winter and spring samples were multiplied by four to correct for quartering of samples. F=fall, W= winter, S=spring.

Spring	North Scruggs			South Scruggs			School			Indian			Jackrabbit			Big		
Season	F	W	S	F	W	S	F	W	S	F	W	S	F	W	S	F	W	S
Ephemeroptera																		
Baetidae				1		4			16	56		16			4			
Anisoptera																		
Gomphidae	7	16	8	11	16	24	19	12	4	46	20	16	2	4	16			24
Libellulidae				5			12			3								
Zygoptera																		
Coenagrionidae	7	16	8	7	20	24	12	16	8	481	40	12	3	12	4	1	12	4
Calopterygidae										1							4	
Hemiptera																		
Gelastocoridae																		
Nertha	1					4												
Naucoridae																		
Ambrysus	21	16	24	23	16	12	4	20	16									
Veliidae	8	4				12	1			4	4					1		
Belostomatidae	1																	
Trichoptera																		
Hydroptilidae			192				29	20	20	7	32		7	24	256	1		40
Philopotamidae							6	12										
Lepidoptera																1		
Coleoptera																		
Elmidae																		
Stenelmis	30	84	288	6	4		50	44	156	23	20	340						
Microcylloepus	39	332	504	83	156	504	114	88	144	31	8	828			8			
Dytiscidae										3								
Hydrophilidae	2						3			46	8							

Table 2(cont.): Raw invertebrate counts for all springs and all seasons. Winter and spring samples were multiplied by four to correct for quartering of samples. F=fall, W= winter, S=spring.

Spring	North Scruggs			South Scruggs			School			Indian			Jackrabbit			Big		
Season	F	W	S	F	W	S	F	W	S	F	W	S	F	W	S	F	W	S
Diptera																		
Stratiomyidae	2	8	8	4			2	8	4	191								
Tanypodinae									16	53								
Ceratopogonidae			8			4	7		40	521	44	20						
Chironomidae	3	36	392	42	8	456	31	24	92	2068	144	816	106	372	1328	9	12	68
Tricladida																		
Planariidae																		
Dugesia			80			8		12	20			4						
Gastropoda																		
Hydrobiidae																		
Tryonia variegata	191	464	3704	14	24	36	965	1036	748	5	68	476						
Pyrgulopsis pisteri	817	1572	2504	11			363	448	512	3	4	56						
Tryonia?				34	8													4
Physidae	6			4														
Thiaridae																		
Melanoides tuburculata				246	32	88	152	44	40	1	148	196		236	164	21	164	700
Oligochaeta		40	232		16	32					36	12						32
Hydrachnidia		36	20				52	68	216	19	36	40	371	720	1108	24	16	96
Ostracoda	1	8	20	1			47		24	103	16		23	8	12			
Isopoda				2														
Copepoda														4				
Amphipoda																		
Hyalella	41	68	1660	242	148	308				5		484	237	868	2172			
Decapoda																		
Procambarus clarkii				7	7	2							23	12	28	5	11	14

Table 3: Table summarizing invertebrate indices comparison for crayfish present versus crayfish absent sites within season. R=richness, E=evenness, H'=Shannon Index, Δ=Simpson's index. P = crayfish present site, A = crayfish absent site. Values with asterisk indicate significant difference (t-test, $p<0.001$) between crayfish absent and present springs for that index.

Season	Crayfish	Site	R	H'	E	Δ
Fall	P	Big	7*	1.3	0.67	0.67
	P	Jackrabbit	7*	1.18	0.61	0.63
	P	South Scruggs	17*	1.8	0.64	0.76
	A	North Scruggs	16	1.12	0.4	0.49
	A	School	18	1.64	0.57	0.68
	A	Indian	19	1.42	0.48	0.62
Winter	P	Big	5*	0.79	0.49	0.37
	P	Jackrabbit	9*	1.39	0.63	0.71
	P	South Scruggs	11*	1.76	0.73	0.76
	A	North Scruggs	14	1.37	0.52	0.61
	A	School	14	1.43	0.54	0.62
	A	Indian	15	2.25	0.83	0.86
Spring	P	Big	8*	1.03	0.5	0.46
	P	Jackrabbit	10*	1.36	0.59	0.7
	P	South Scruggs	14*	1.67	0.63	0.75
	A	North Scruggs	16	1.68	0.61	0.75
	A	School	17	1.92	0.68	0.78
	A	Indian	14	1.91	0.72	0.82

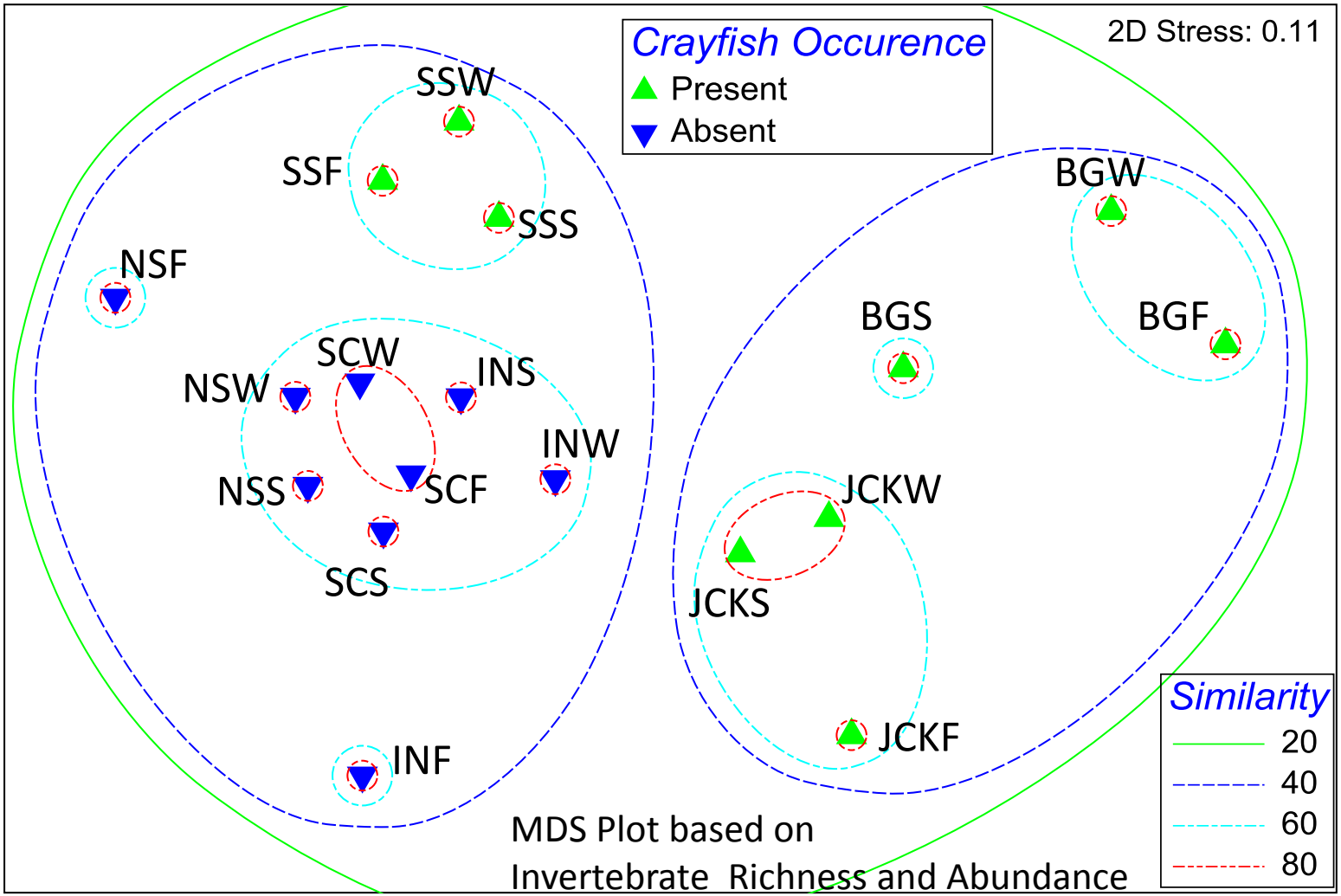

Figure 5: Non-metric multidimensional scaling (MDS) plot showing similarity of invertebrate richness and abundance values for study sites. Springs are plotted by season. BGF, BGW, BGS = Big Spring fall, winter and spring; JCKF, JCKW, JCKS = Jackrabbit Spring fall, winter and spring; SSF, SSW, SSS = South Scruggs fall, winter and spring; NSF, NSW, NSS = North Scruggs fall, winter and spring; INF, INW, INS = Indian Spring fall, winter and spring; and SCF, SCW, SCS = School Spring fall, winter and spring.

Fish Abundance and Condition

Catch rates varied across sites, sampling method, seasons, and crayfish presence or absence. No clear biological pattern was evident and a broad scale analysis of crayfish present versus crayfish absent pupfish abundance gave no significant difference (t-test, $p>0.05$) for either minnow trap or seine method. At a finer scale however, a significant difference (ANOVA, $p=0.04$) was seen in only the winter season in crayfish absent sites Here, the winter abundance of pupfish captured in traps was significantly higher than either fall or spring. Due to the low numbers of fish captured and the small area assessed, population estimates were not calculated. Native and non-native fish capture numbers are represented in Table 4 and 5. Non-native fish were not captured within the crayfish absent reaches assessed in this study. The seining method was not used in School Spring because it had both shallow runs (1-3cm) and deep pools which would decrease capture rates of pupfish. No pupfish were captured by seine net in South Scruggs or by funnel traps in Indian Spring in the spring sampling season.

Pupfish condition also varied across sites and season, as well as with crayfish presence or absence. Average condition factor ranged from 1.1 to 2.33 with individual condition factors ranging from 0.3 to 4.78. The mixed model ANOVA showed no significance effect of season, treatment or the interaction of season and treatment, and means of treatment (crayfish absent or present) by season (Figure 6, Table 6).

Table 4: Pupfish capture rates from crayfish absent springs in each season, separated by method (minnow trap or depletion method with seine). N = number of pupfish caught.

Site	Season	Method	N
School	Fall	Seine	-
		Trap	5
	Winter	Seine	-
		Trap	46
	Spring	Seine	-
		Trap	11
Indian	Fall	Seine	38
		Trap	5
	Winter	Seine	42
		Trap	35
	Spring	Seine	47
		Trap	-
North Scruggs	Fall	Seine	13
		Trap	12
	Winter	Seine	42
		Trap	13
	Spring	Seine	28
		Trap	5

Table 5: Pupfish and non-native species capture rates for crayfish present springs in each season, separated by method (minnow trap or depletion method with seine). GAAF = *Gambusia affinis*, POLA = *Poecilia latipinna*, and PRCL = *Procambarus clarkii*.

Site	Season	Method	Pupfish	GAAF	POLA	PRCL
South Scruggs	Fall	Seine	5	1	-	-
		Trap	13	-	-	7
	Winter	Seine	3	-	-	7
		Trap	19	-	-	-
	Spring	Seine	-	-	-	1
		Trap	3	-	-	1
Jackrabbit	Fall	Seine	19	18	3	23
		Trap	19	-	-	-
	Winter	Seine	15	6	9	12
		Trap	28	-	-	-
	Spring	Seine	32	17	5	24
		Trap	7	-	-	4
Big	Fall	Seine	20	55	79	5
		Trap	9	-	-	-
	Winter	Seine	3	6	15	11
		Trap	7	-	-	-
	Spring	Seine	49	25	58	13
		Trap	11	-	-	1

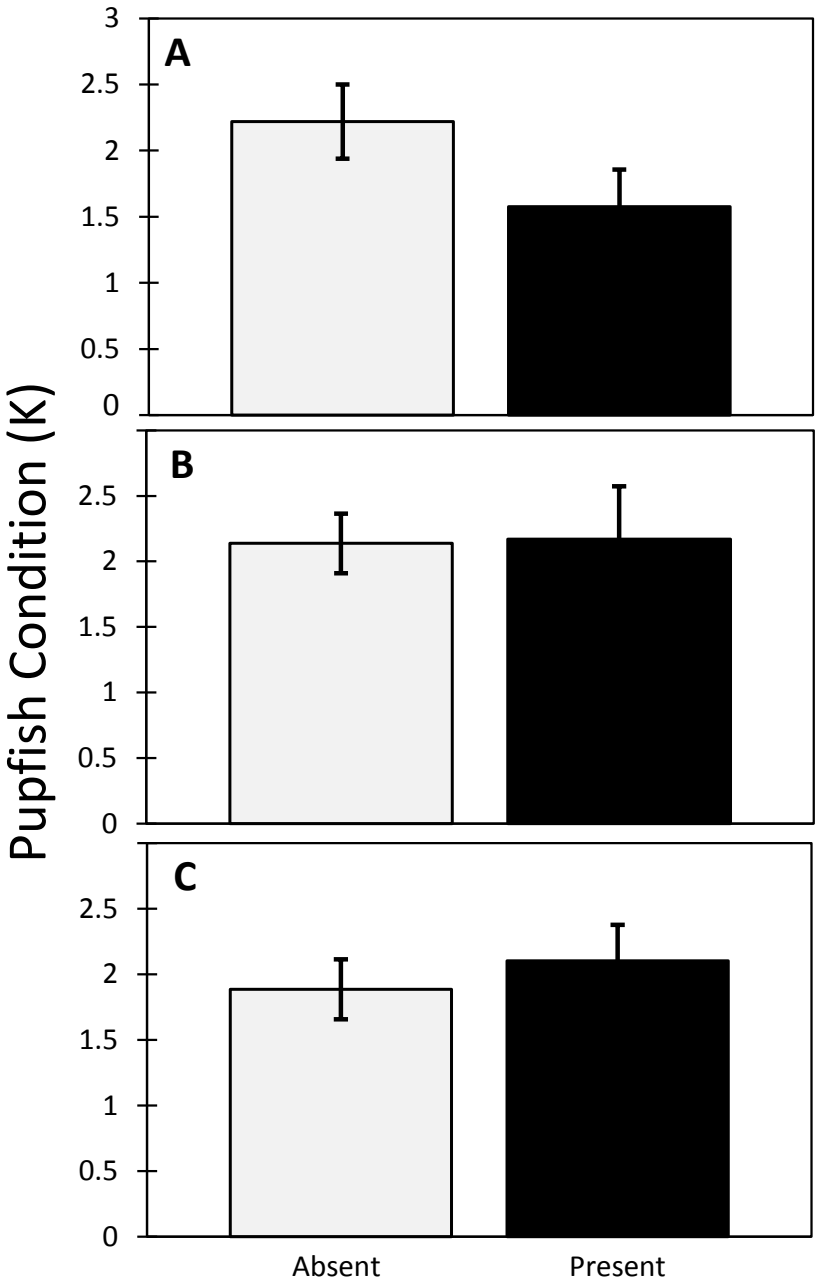

Figure 6: Pupfish condition (K) means for crayfish absent versus crayfish present springs for each season. Error bars show (± 1 SE) and A = fall, B = winter, C = spring.

Table 6: Parameter result of mixed model ANOVA for pupfish condition factor (K) fixed effects of season, crayfish treatment (present/absent) and the interaction of season and crayfish treatment.

Parameter	**p-value**	***α* level**
Season	0.711	0.05
Treatment	0.596	0.05
Interaction	0.317	0.05

Stable Isotopes

To illustrate a general pattern of energy flow in crayfish present and absent springs, $\delta^{13}C$:$\delta^{15}N$ plots were constructed for the three sampling seasons (Figures 7, 8, 9). The $\delta^{13}C$ and $\delta^{15}N$ of food web base items (FPOM, leaf litter and algae) tended to vary across season and even within a season. The $\delta^{13}C$ of the May 2012 algae samples from crayfish present springs ranged by 19‰ (Figure 9). Invertebrate grazers appeared to reflect similar variation; $\delta^{13}C$ differed by more than 7‰ in the crayfish absent springs in the fall. Also, considerable overlap occurs among all consumer species when crayfish were present.

Trophic position was also highly variable between seasons and between sites (Table 7). On average and across all seasons, pupfish trophic position in crayfish absent springs was 2.43 versus 2.20 in crayfish present springs (ranges: 1.51 to 3.04 compared to 0.92 to 2.91, respectively). Crayfish averaged a 1.96 (SD ±0.16) trophic position, while invertebrate grazers averaged 1.75 (SD ±0.42) and predatory invertebrates 1.77 (SD ±0.46). In some instances, the pupfish trophic position was just slightly higher than the algae baseline. Pupfish from Big Spring in May and South Scruggs Spring in January averaged 1.33 and 1.79 respectively.

Pupfish trophic position did not significantly differ by treatment or season with the mixed model ANOVA ($p > 0.05$) (Figure 10, Table 8) but did result in a significant difference ($p=0.005$) when a broad scale t-test was applied. The mixed model only assessed samples which contained more than one signature, so School, Indian and Big Spring in fall and Big Spring winter samples did not contribute to the analysis. Also, spring samples for both School and South Scruggs Springs were lost during isotope analysis and thus only the remaining four springs could contribute trophic position signatures.

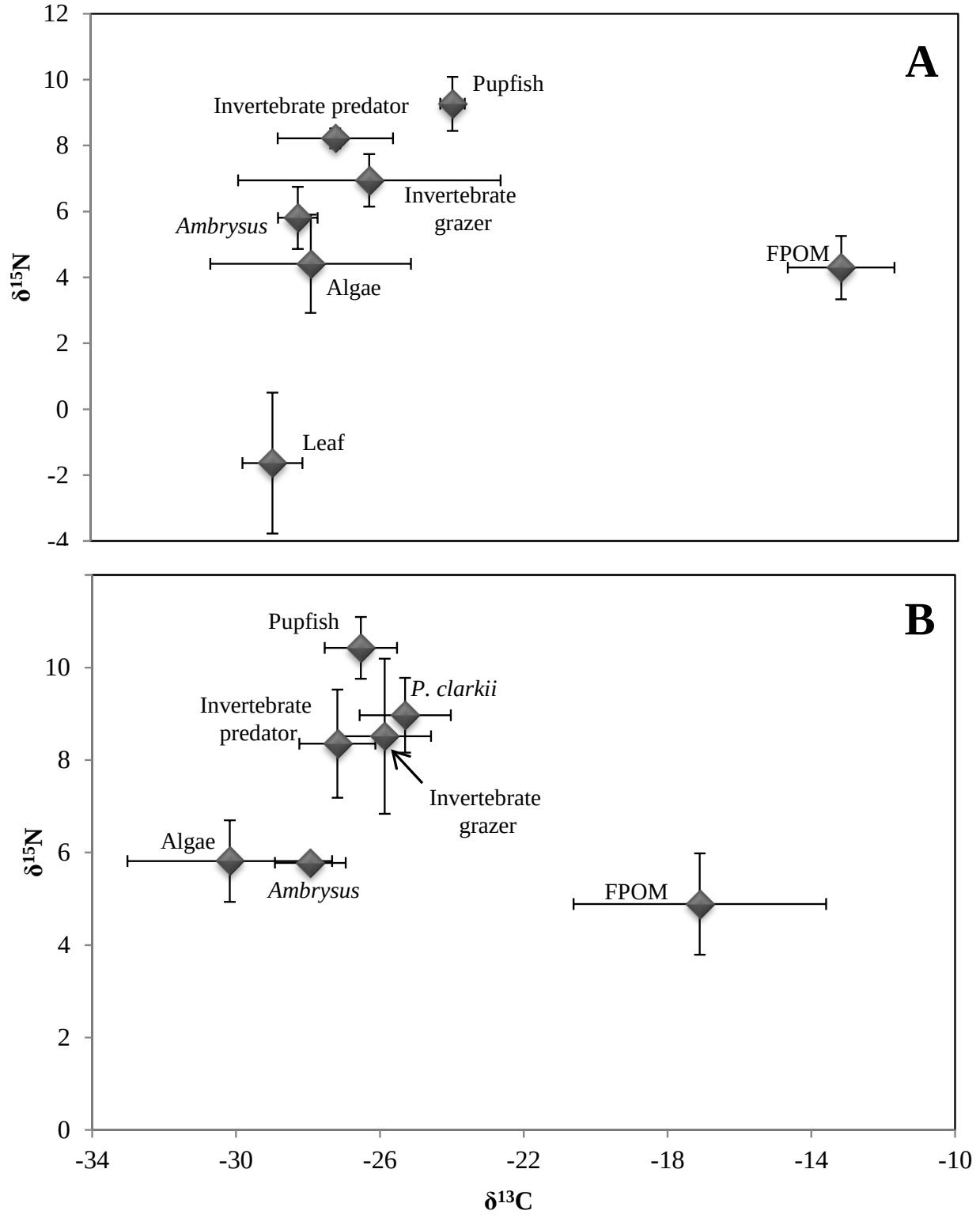

Figure 7: C:N plot for all materials collected in the fall sampling season for crayfish absent (A) and crayfish present (B) springs. Error bars represent ± 1SD from the mean.

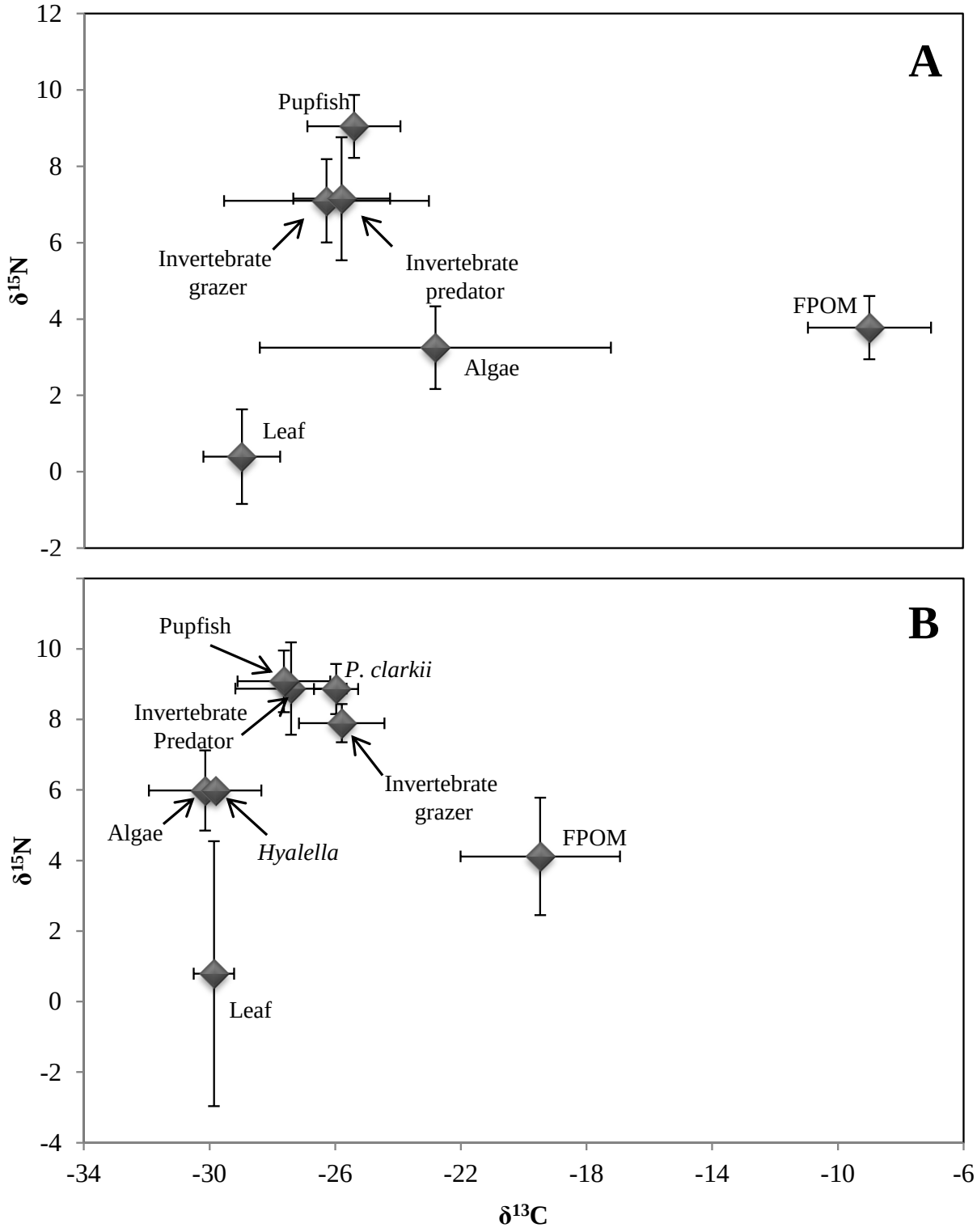

Figure 8: C:N plot for all materials collected in the winter sampling season for crayfish absent (A) and crayfish present (B) springs. Error bars represent ± 1SD from the mean.

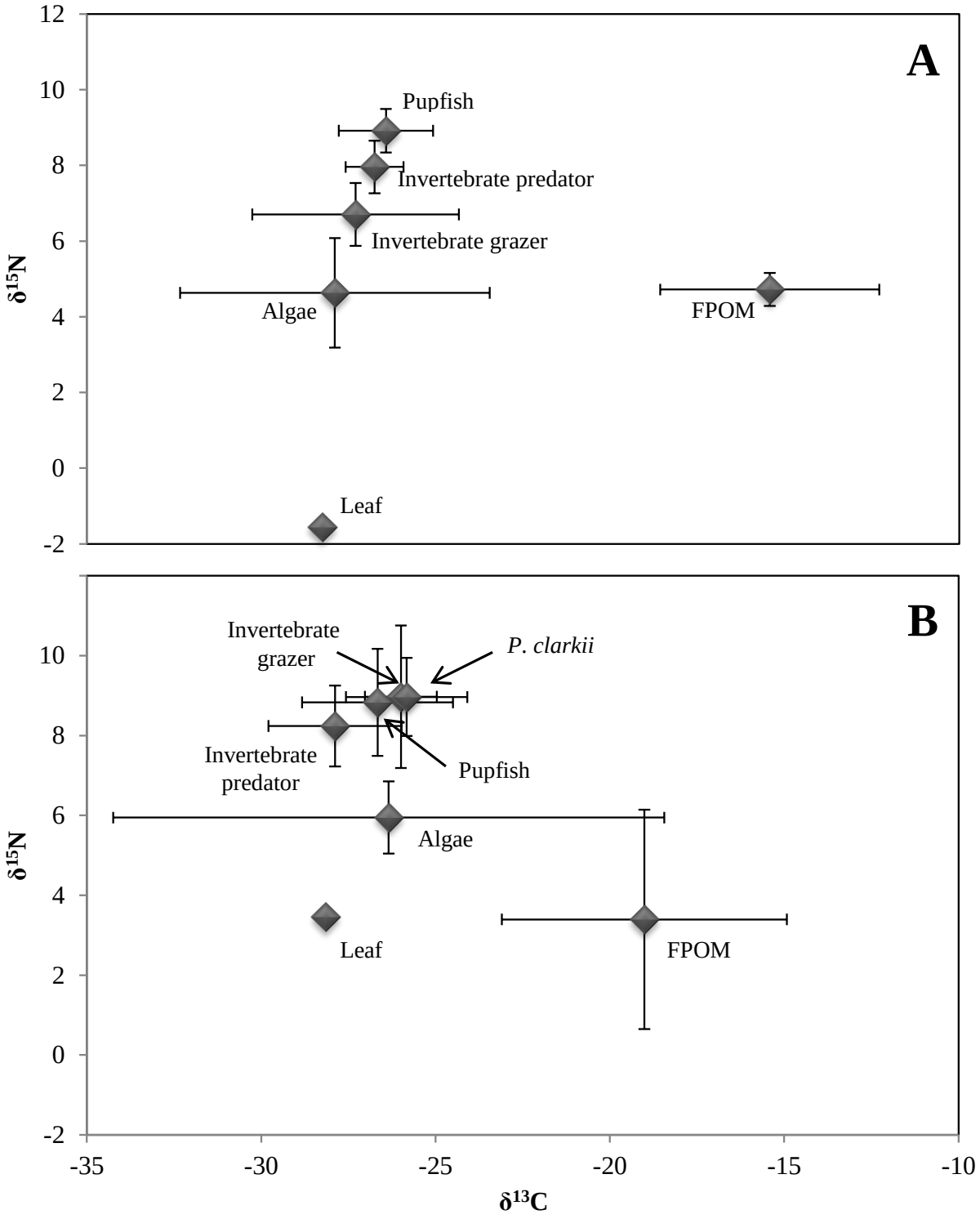

Figure 9: C:N plots for all materials collected in the spring sampling season for crayfish absent (A) and crayfish present (B) springs. Error bars represent ± 1SD from the mean.

Table 7: Mean trophic position values for all materials collected for food web assessment. Values in parentheses represent +/- 1 SE from the mean. N = number of samples contributing to mean. Invertebrate grazer, predatory invertebrate, other consumer, FPOM and leaf litter were either N=1 or N=2.

Season	Site	Pupfish	N	Crayfish	N	Invertebrate Grazer	Predatory Invertebrate	Other Consumer	FPOM	Leaf Litter
Fall	Big Spring	2.23	1	1.97 (±0.12)	7	2.21	1.78	-	0.82	-
	Jackrabbit Spring	2.67 (±0.16)	10	2.06 (±0.09)	12	2.08	2.08	-	0.85	-
	South Scruggs Spring	2.19 (±0.15)	3	2.03 (±0.15)	4	1.48 (±0.25)	1.39	1.08 (±0.02)	0.44 (±0.12)	-
	North Scruggs Spring	1.89 (±0.12)	3	-	0	1.15	0.87 (±0.17)	-	0.21	-1.76
	School Spring	2.45	1	-	0	1.70	1.50	-	0.89 (±0.03)	-0.43
	Indian Spring	2.93	1	-	0	-	2.28	-	-	-
Winter	Big Spring	1.95	1	1.74 (±0.07)	7	-	2.06		-0.16	0.78
	Jackrabbit Spring	2.43 (±0.08)	18	2.15 (±0.10)	9	2.06	2.28	1.42	0.89	-0.20
	South Scruggs Spring	1.79 (±0.19)	11	1.77 (±0.14)	5	1.51 (±0.26)	1.53 (±0.16)	-	-	-1.36 (±0.77)
	North Scruggs Spring	2.54 (±0.12)	8	-	0	1.99 (±0.31)	1.95 (±0.55)	-	1.22 (±0.54)	0.10 (±0.01)
	School Spring	2.33 (±0.19)	10	-	0	1.84 (±0.17)	1.88 (±0.61)	-	0.87 (±0.08)	0.08 (±0.10)
	Indian Spring	2.87 (±0.09)	16	-	0	2.49	2.56	-	1.09	-0.07
Spring	Big Spring	1.36 (±0.57)	5	1.69 (±0.08)	2	2.17	1.60	-	-	-
	Jackrabbit Spring	2.22 (±0.10)	4	1.99 (±0.10)	24	2.03	2.17	-	-0.49	-0.49
	South Scruggs Spring	-	0	1.85 (±0.07)	13	1.50	1.46 (±0.02)	-	0.21 (±0.09)	0.46 (±0.32)
	North Scruggs Spring	1.97 (±0.30)	6	-	0	0.78	1.80	-	0.69 (±0.24)	0.69 (±0.24)
	School Spring	-	0	-	0	1.90	2.18	-	1.07	1.07
	Indian Spring	2.12 (±0.15)	10	-	0	1.28	1.72	-	-	-

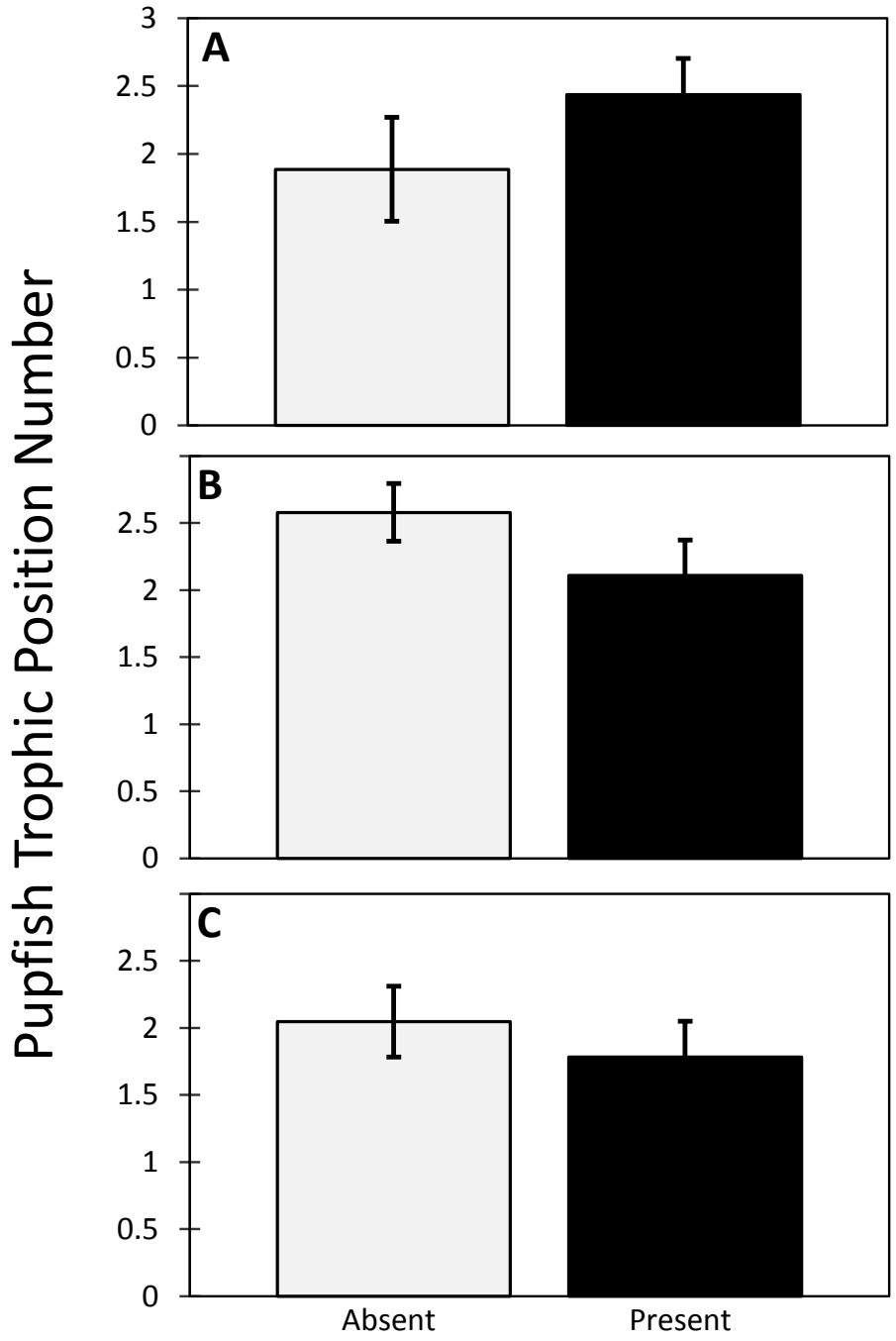

Figure 10: Pupfish trophic position number means for crayfish absent versus crayfish present springs by season. Error bars show (± 1 SE) and A = fall, B = winter, C = spring.

Table 8: Parameter result of mixed model ANOVA for pupfish trophic position number fixed effects of season, crayfish treatment (present/absent) and the interaction of season and crayfish treatment.

Parameter	**p-value**	**α level**
Season	0.307	0.05
Treatment	0.8	0.05
Interaction	0.273	0.05

Gut Contents

Gut contents of crayfish from Jackrabbit, Big, and South Scruggs Springs were variable across sites and season. The mean percent composition of crayfish guts are presented in Figure 11. Fall gut contents for Jackrabbit and South Scruggs Springs contained all six types of material, with detritus a major portion for the latter. Big Spring crayfish guts were dominated by macrophytes and woody material was scarce in all fall samples. Jackrabbit and Big Spring showed a shift toward animal based materials in the winter samples, while South Scruggs shifted to detritus dominated contents, with woody material sparse again. A more even distribution of materials returned in spring samples except for South Scruggs Spring, which lacked any algae or woody material and contained somewhat even amounts of detritus, macrophytes and sediment.

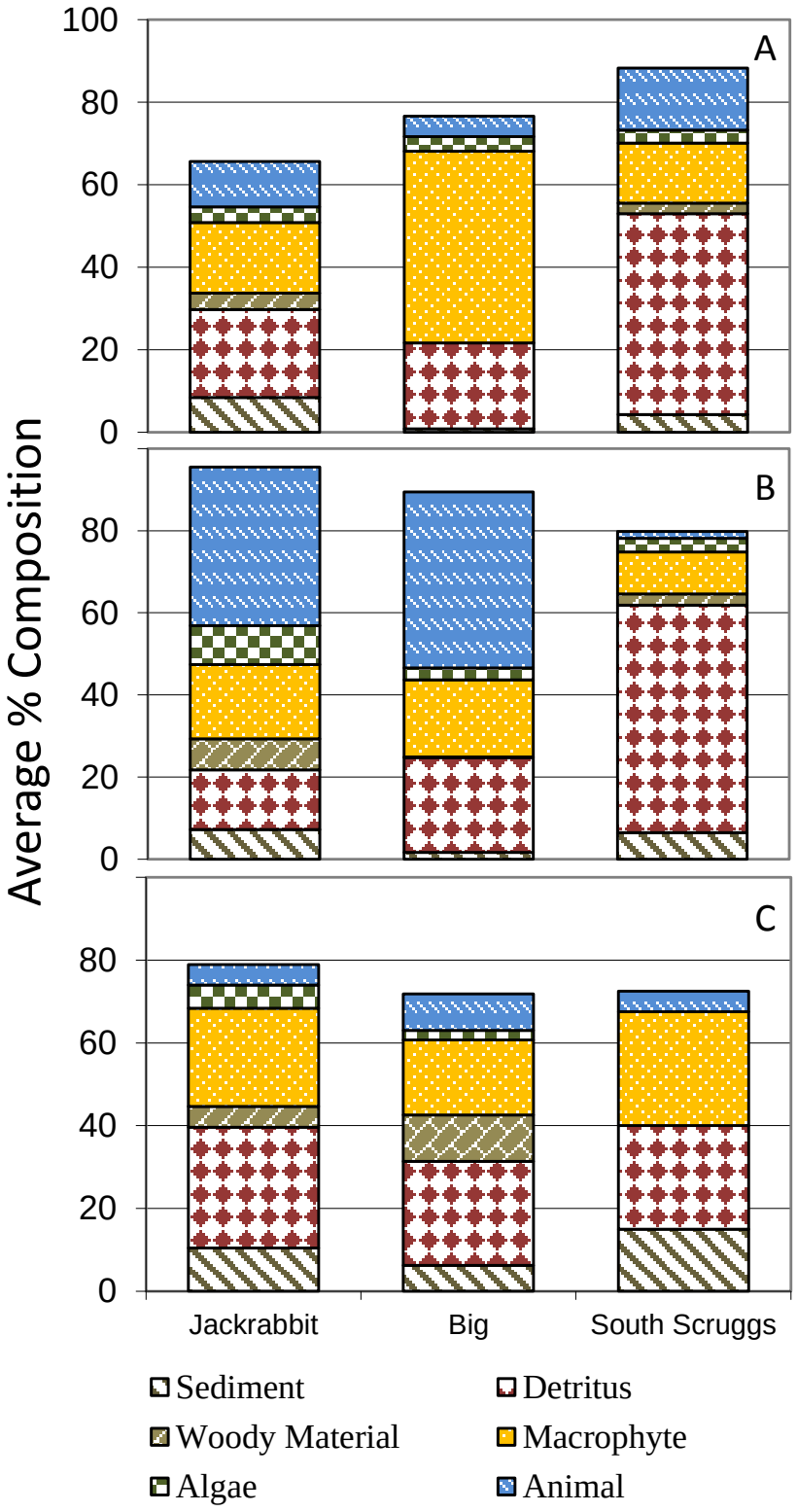

Figure 11: Stacked graph representing mean percent composition of the six stomach content categories (sediment, woody material, detritus, macrophytes, algae and animal material) from crayfish guts from crayfish present springs separated by season. A=fall, B=winter, C=spring.

Discussion

Pupfish appear to be enduring despite the challenges they face and crayfish are not posing an immediate threat. The macroinvertebrate community, however, does not have the same outlook. Springs containing crayfish consistently had fewer species and contained little to no endemic springsnails (Table 2, 3, Figure 5). Animal material was present in 93% of crayfish stomachs analyzed, with one gut filled entirely with native springsnails and native riffle beetle larvae. The small nature of the endemic invertebrates makes them susceptible to predation by the invasive crayfish. The Refuge expends time, energy and funds in an attempt to control and eradicate *Procamabrus clarkii* from the refuge, and the current study attests to the need for continued work.

Invertebrate Abundance and Diversity

The invasive *Procambarus clarkii* appears to be having the biggest impact upon the invertebrate communities within Ash Meadows NWR. Marked patterns were apparent within crayfish present springs in regards to richness of species and with particular invertebrates either missing or absent from populations.

Shannon diversity and Simpson's diversity indices were both used in this study in an effort to thoroughly quantify and assess invertebrate communities. Both indices assess abundance and evenness of samples, but Shannon diversity looks at the likelihood that a single individual will belong to a certain species while Simpson's index corresponds to the number of pairs drawn to obtain two organisms of the same species. There was only slight variability between treatments for each index (Table 3) due to the uneven nature of the samples, specifically the dominance by either Chironomids or *Tryonia* or *Pyrgulopsis* snails. Looking at taxa

richness alone, there were marked differences. The three crayfish present springs consistently had lower richness although Jackrabbit should not be included in this due to its past drying and the probable elimination of any and all snails. This result is not unusual considering other literature which found that invasive crayfish can and do negatively affect taxon richness and densities (Charlebois and Lamberti 1996, Stenroth and Nyström 2003, McCarthy et al. 2006, Correia and Anastácio 2008, Nilsson et al. 2012). Specifically, McCarthy et al. (2006) found that the Rusty Crayfish had the greatest negative impacts to Gastropoda, Diptera, Amphipoda and Ephemeroptera. In a separate study, Nilsson et al. (2012) found parallel results where invasive crayfish reduced densities of Amphipoda and Mollusca. These taxa are important parts of the communities in AMNWR springs. Also, the Red Swamp Crayfish significantly decreased Simpson Diversity of invertebrates in a mesocosm experiment (Correia and Anastácio 2008). At this point, the picture is not clear enough to say whether this difference in richness is due to crayfish alone as other non-native higher level consumers are found along with the crayfish. Mosquitofish (*Gambusia affinis*) are found in all three crayfish present springs, sailfin mollies (*Poecilia latipinna*) are found in both Jackrabbit and Big and largemouth bass have been found in Big Spring. Each of these species can be insectivores or piscivores and contribute to differences in the invertebrate community. Further site evaluations and addition of other organisms and springs would add resolution to the influence which the Red Swamp Crayfish or other invasive organisms have on Ash Meadows invertebrate communities.

Another interesting pattern was most discernible between North and South Scruggs specifically, in regards to some of the native, endemic invertebrates. These two springs run parallel to each other and are in very close proximity. However, South Scruggs contains crayfish while North does not and it was evident from the raw numbers that South Scruggs had lower

abundances of both *Stenelmis* beetles and the small bodied *Tryonia* and *Pyrgulopsis* snails. These sites were clearly separated on the MDS plot (Figure 5), having less than 60% similarity. In regards to habitat, these sites were similar in depth, flow, substrate score and % vegetation cover (4.5 cm vs. 5.5 cm, 0.82 m/s^2 vs. 0.89 m/s^2, 1.293 vs. 1.292 and 27% vs. 40% for North Scruggs vs. South Scruggs, respectively). It is also noteworthy that endemic snail taxa were absent from the site in Big Spring, a site with crayfish. Big Spring has historically been inhabited by the sportinggoods tryonia (*Tryonia angulata*) (Hershler1999) but no springsnails were detected during sampling. The sample site was well downstream of the spring head, where springs snails are often in their highest numbers (Hershler and Sada 2002), but the site was also littered with crayfish. Other springs on the refuge are inhabited by both crayfish and native springsnails (Fairbanks, Crystal and others) and study expansion to include such springs could provide more concrete data for management.

Indian Spring demonstrated interesting patterns of diversity indices. Here, Shannon and Simpson's index were more variable across seasons compared to all other sites (Table 3), but this is most likely due to its recent restoration status. Its change from Dipteran dominated to a more even distribution between Dipterans, Amphipods, Gastropods and Coleopterans demonstrates successional patterns and gives some insight into the procession of a site's community after restoration and removal of crayfish. All endemic taxa showed increases in abundance and *M. tuberculata* numbers remained relatively low compared to the native springs snails (Table 2). Further monitoring of this spring for any return of crayfish and further shifts in the community would help refuge managers to plan further restoration efforts for other springs.

Jackrabbit Spring was still included in the invertebrate analysis even though it had dried completely in the past and thus its aquatic community composition may have been altered (Pister

1974, Williams and Sada 1985, Ash Meadows staff, personal communication). Still, this spring has had restored flow for over 40 years and does, at times, connect to Big Spring's outflow (Williams and Sada 1985). For these reasons, it was included in all invertebrate analyses.

The lack of the springsnails and lower species richness raises concern for the future outlook of these invertebrate populations. The Warm Springs Complex in particular, where the habitat available is small and isolated, is especially vulnerable. The macroinvertebrate community data, combined with particular evidence from the crayfish gut contents highlight some grim consequences of crayfish invasion in Ash Meadows NWR springs.

Gut Contents

Given the presence of both sharp edged mandibles and a set of gastric teeth between the cardiac and pyloric stomachs of crayfishes, food items found in stomachs are highly masticated and difficult to identify to lower taxonomic units. This prevents fine scale resolution of diets based solely on gut contents. However, gut contents can add lines of support to hypotheses of resource use. When used independently, the common method of gut content analysis will give a biased estimate of diet considering that animal material breaks down at a faster rate compared to plant materials. Gut contents are merely a snapshot view of recent diet choices and must be analyzed with caution. This quality makes stable isotopes and gut contents the perfect pair and interdependent on one another. Stable isotopes can give insight to assimilation of resources that are consumed. In this study, gut contents were used in two ways: 1) to accompany stable isotope data and clarify isotopic signatures and 2) alone in an effort to assess whether crayfish were predominantly feeding on particular food sources.

Crayfish guts collected here were dominated by macrophytes, detritus and animal material (Figure 11). Animal material was comprised entirely of invertebrate body parts, snail, crayfish and fish parts. Woody material and algae were encountered only occasionally or in small amounts in the guts. The data give a snapshot view of what resources crayfish seek out and it is interesting to note that 20 of the 62 stomachs (32%) contained either fish scales, fin rays or vertebrae, though species determination was not possible. One gut was comprised almost entirely of fish parts (82%). Others have documented the crayfishes ability to readily consume fish (Parkyn et al. 2001, Correia 2003, Thomas 2011) as well as pupfish directly (Rogowski and Stockwell 2006). A separate study, focusing on *Orconectes* crayfish, pinpointed that animal matter made up 42% of the diet, and that fish, in particular, made up 12% indicating that animal protein is an important component of crayfish diet (Taylor and Soucek 2010). Not only was there concrete evidence that crayfish will consume fish but one particular crayfish gut from South Scruggs Spring was filled almost entirely with native *Pyrgulopsis* snails, whole and crushed. At least 3 other guts had fragments of snail shells in them. Such evidence suggests that crayfish can and will readily eat a multitude of these native snails and could be having direct negative effects on these organisms in the springs. Thin-shelled snails appear to be easy prey for crayfish. Alexander and Covich (1991) and Nyström and Pérez (1998) found that crayfish fed on the common pond snail in accordance to selective foraging theory. In both studies, the smaller, thinner shelled snails were preferred. In Africa, *P. clarkii* was introduced purposefully as a control agent for schistosomiasis carrying snails (Hofkin et al. 1991) and elsewhere it has been implicated as a control agent for zebra mussels (Perry et al. 2000). The thin shelled native springsnails may be even more at risk where *M. tuberculata* is sympatric and crayfish are also present. Though not tested in the current study, this kind of interaction might bring about more

predation on the native snails due to the thicker shelled red-rimmed melania being a less desirable prey to crayfish. Based upon previous literature and the evidence found here, these crayfish certainly have the ability to pose a direct threat to the native fish and invertebrate fauna of the Ash Meadows springs.

Fish Abundance and condition

Though evidence of direct predation on fish was found in crayfish guts, it was not possible to identify the species. Thus no concrete evidence was available to demonstrate that *Procambarus clarkii* poses an immediate impact to Ash Meadows pupfish species. This was evident with analysis of fish abundance, condition as well as stable isotope and trophic position analysis.

Two sampling methods (minnow trap and seine) were used to assess pupfish abundance in sites with and without invasive crayfish. Given the diversity in channel morphology across sites, neither method was uniformly effective across all sites. South Scruggs regularly had low pupfish capture rates, with the spring season sampling resulting in no pupfish (Table 5). This particular site was a challenge due to its undercut and overgrown banks and heavy algae cover, which provide adequate refuge for pupfish to avoid being scared into the seine. Other issues with capture efficiency came from funnel trap use. Low numbers of pupfish were captured in Indian Spring during the fall season due to incorrect trap placement and, for unexplained reasons; no pupfish were captured by funnel traps in May (Table 4). No obvious biological pattern could be discerned in overall pupfish abundance between crayfish present and absent springs (t-test, $p>0.05$) for either method. Future study could include more extensive sampling of the spring outflow to possibly detect a stronger interaction effect.

The lack of a crayfish effect on pupfish is a surprising result given that literature has implicated that invasive crayfish can be indirectly responsible for fish population declines (Miller et al. 1992, Nyström 2002, Light 2005) and crayfish have been observed feeding on the small, non-native mollies and mosquitofish in traps (personal observation) and *Gambusia holbrooki* has been found in *Procambarus clarkii* stomach contents (Correia 2003). Rogowski and Stockwell (2006) found that crayfish will readily feed on adult pupfish in experimental settings and can cause a reduction in population size. It is well known that crayfish will readily consume fish eggs (Ilhéu et al. 2007) and this behavior has been linked to fish declines within the Death Valley area of the Southwest (Stolz and Naiman 1978). While not able to be identified to species, fish parts were found in the stomachs of 20 of the crayfishes collected in South Scruggs, Jackrabbit, and Big Springs. At this time however, data suggests that crayfish do not have a direct effect on pupfish abundance. Future studies could incorporate mark recapture methods to obtain better fish abundance estimates and incorporate different locations within springs.

The mixed model ANOVA found no significant differences between crayfish absent and crayfish present springs for pupfish condition (K) which is in line with previous findings of other research on crayfish-fish interactions. Juvenile trout survival, length and weight were unaffected by invasive *P. leniusculus* during an enclosure experiment (Stenroth and Nyström 2003). Wilson et al. (2004) found negligible effects on catches of smallmouth and rock bass, but did, however, find a negative correlation between bluegill and pumpkin seed catch to invasive *O. rusticus* catch. Contrary to these findings, Flannelmouth Suckers and Gila Chub from the Colorado River show decreased growth rates in experimental tanks with the crayfish *Orconectes virilis* (Carpenter 2005). Unfortunately, these studies did not assess fish condition factor. Few studies have assessed fish condition in relation to an introduced crayfish. A recent study on the White

Sands pupfish, *Cyprinodon tularosa*, demonstrated reduced growth rates but showed no difference in condition when exposed to *O. virilis* in mesocosms (Rogowski and Stockwell 2006). Condition factor length and weight data is easy to collect and is a measure of reproductive health. It can be influenced by environmental conditions, food availability and seasonality. However, the near constant water temperatures of the springs within Ash Meadows may reduce such variability and thus make condition factor a less useful measure. Further investigations could assess population growth rates.

Stable Isotopes

Stable isotopes are a fundamental addition to the inference of trophic position and food web dynamics because they can be used to trace movement and assimilation of certain nutrients within an organism (Gannes et al. 1997) and because they accumulate in tissues over time, the results are time integrated rather than a basic snapshot (Fry and Sherr, 1984, Vander Zanden et al. 1998, Vander Zanden et al. 1999). The C:N plots demonstrated such food web dynamics and illustrate that many organisms in this study system are competing for the same trophic niche of primary consumer. The food web becomes quite tangled at this level when the invasive crayfish are involved (Figures 7, 8, 9). In winter and spring, pupfish, crayfish, invertebrate predators and invertebrate grazers all share similar $\delta^{15}N$ levels and levels are only slightly separated in fall. Kennedy et al. (2005) came to similar conclusions after an assessment of Jackrabbit Spring. Here they found similar overlap at the primary consumer level between crayfish, *M. tuberculata* and Ash Meadows Amargosa Pupfish. Pupfish are common consumers of algae (Naiman 1975) as are crayfish but $\delta^{13}C$ values in some instances do not reflect use of the tested algae as a food source. In most cases the grazers are enriched by 3-8‰ and this is well above the predicted average enrichment of <1‰ (Vander Zanden and Rasmussen 2001, Post 2002). The change in

the heavy isotope of Carbon ($\delta^{13}C$) is small (0.2 – 1.1‰) with trophic level changes (Peterson and Fry 1987, France and Peters 1997, Vander Zanden and Rasmussen 2001, Post 2002) and in this study it might be that consumers are utilizing a different form of algae than the filamentous type that was analyzed. Kennedy (2005) suggested that consumers were feeding upon microalgae which tend to be more enriched in the heavy ^{13}C isotope (Finlay et al. 1999, Trudeau and Rasmussen 2003). This type of algae is challenging to collect since it is either found in small amounts on surfaces of rocks and vegetation or is mixed with other organic matter and thus not a pure sample. This type of biofilm was observed at many sampling sites in this study, but for the reasons listed, it was not collected. In the future, it may be advantageous to add such a material to stable isotope analyses conducted at Ash Meadows NWR.

Trophic position of pupfish varied with both season and treatment as well as within spring, but not with any regularity. The trophic positions of pupfish from crayfish absent springs in the winter season were higher than any other instance (Table 7), but did not indicate a change in trophic steps. These numbers are not indicating that pupfish in crayfish absent springs are feeding at a higher trophic step (i.e. secondary consumer, predator) versus those pupfish in crayfish present springs. This would require pupfish in crayfish absent springs to be at trophic level 3 versus trophic level 2. This result merely suggests that different dietary resources (i.e. more animal vs. plant matter) are more abundant and utilized more efficiently by pupfish in crayfish absent springs and thus they are able to reach a higher trophic position number within trophic step 2. Trophic position values were overall variable even within a stream (Table 7). Some pupfish, particularly from crayfish present springs, trophic position numbers resulted in below baseline (<1) values which contributes to the overall lower trophic position mean. At this

point, I cannot explain the reason behind these pupfish having such a low trophic position number.

Trophic position analyses for this study were confounded by the fact that material for pupfish stable isotope analysis was difficult to obtain. A minimum of 0.25mg is required for a reliable isotopic signature reading. Due to the small body size of the pupfish, in many instances, all fin clips collected at a site had to be combined in order to reach the minimum amount of material. This meant that some samples were made up of 2, 3 or even up to 5 separate fin clips and thus represented an average for those pupfish at that site on that occasion. Fin clip sampling for pupfish was necessary due to their endangered status and has been shown to be a reliable, non-lethal method (Jardine et al. 2005, Kelly et al. 2006), but it does create complications in this case. More resolution in differences in trophic position and inferred diet of pupfishes could be provided with the use of muscle tissue from pupfish instead of fin clips because assimilation rates can vary between tissue types (Jardine et al. 2005). In the future, multiple trapping events will be needed to obtain more substantial material for stable isotope analysis.

Evidence from the mixed-model ANOVA reflects the variation in trophic position and shows no difference in pupfish trophic position according to treatment (Figure 10, Table 8). Again this result could be affected by the removal of single value samples from analysis. School, Indian and Big Springs were not used in the fall, Big Spring was not used in winter and the spring samples from South Scruggs and School Springs were destroyed during the isotope analysis procedure. Regardless of these missing samples, pupfish in crayfish absent springs obtained higher trophic positions than those pupfish in crayfish present springs, and a broad-scale t-test revealed that trophic position was significantly greater ($p=0.005$). More resolution of trophic positions might result from the use of higher trophic steps (e.g. predators).

Habitat

The PCA analysis indicated that the factors having the most weight on site variation were depth and % vegetation cover. These factors made up PC1 (Figure 4) and interestingly, there was a separation of crayfish absent and crayfish present springs. The depth result was not surprising given that both Jackrabbit and Big Springs had higher mean depths (31cm and 36cm respectively). These springs also generally had dense vegetation cover at the stream perimeter, with cover resulting from high amounts of rooted bulrush or other species. Other springs did not share this same pattern of densely vegetated spring perimeters and were generally much more shallow (depth<15cm). The vegetation result is contradictory to the multitude of literature which has shown the negative effects which crayfish can have on macrophytes. Feminella and Resh (1989) found that introduced *P. clarkii* were responsible for mass reductions in pondweed cover within a marsh. A long-term study with the invasive Rusty Crayfish (*Orconectes rusticus*) showed significant reductions in both macrophyte abundance and richness (Wilson et al. 2004). Lodge et al. (1994) documented significant declines in macrophytes within enclosures with *O. rusticus* compared to exclosures. The PCA results could be confounded by other conditions, however. Two springs in particular may have had lower macrophyte levels due to their restoration status. Indian Spring had recently been renovated and was completed in April 2011. Renovation involved initial translocation of endemic invertebrates and pupfish and then redirection of spring outflow to a temporary channel while the original channel was desiccated for more than a year to ensure removal of crayfish. After spring flow was restored to the original channel, new substrate was added as well as revegetation of the bank. Invertebrates were re-introduced in stages, while algae and vegetation were allowed to recolonize naturally (Ash Meadows Staff, personal communication). These restoration efforts could have affected its

vegetation and algal community levels and thus affect the % cover. School Spring is also a special case in that its restoration design was similar but rebuilding of the channel included a channel and banks constructed of mortar and boulders in an effort to keep out such vegetation as bulrush and cattails (Weissenfluh 2010). Thus, this component of the analysis is not a reliable explanation of habitat variation. The variability in depth of sites was an unavoidable aspect because of the fact that only three springs within the entire refuge are free of crayfish and those springs are located in the Warm Springs Complex. Springs here are generally low flow, shallow springs and any comparison with other springs on the refuge would result in similar output.

PC2 was most weighted by DO concentrations. Here there was no separation according to crayfish absent or present springs and it was variable across season and sites (Figure 4). Indian Spring tended to have low PC2 scores while School and South Scruggs were on the high end of PC2 during fall and winter. All data was collected during one afternoon for each season, and thus some DO concentrations could be uniquely high for those springs with higher algae cover (School). Data for other springs were located near the midpoint of this axis and thus varied little.

PC3 characterized variability due to a combination of DO and temperature. This was again locally and seasonally variable within the sites and no crayfish absent or present springs separated consistently. Minor variability in temperature between springs or season is not surprising. Ash Meadows springs are warm springs, and the springs surveyed here regularly have temperatures ranging between 27°C and 34°C, which remains rather constant throughout the year with some diel variation. Variability in temperature is not surprising given that the Warm Springs complex (School, Indian, and North and South Scruggs Springs) tend to have temperatures in the 32-34° C range versus Jackrabbit and Big Springs which are generally lower,

26-32°C (Scoppettone et al. 2005). In particular, average temperature in School Spring is higher (33°C, Weissenfluh 2010) versus 26-28°C in Jackrabbit and Big and this difference attributed to some of the variation for PC3. Also, my site temperatures for Jackrabbit and Big Springs are lower due to the downstream gradient which exists in these thermal springs and the fact that my sites were located well downstream of the generally warmer spring source waters. The combination with DO is, as before, probably most attributed to local and seasonal variation, though cooler water does hold more oxygen. This variation seemed to depend on two particular springs, in two different seasons: School Spring in the winter (mean DO = 8.47 mg/L) and South Scruggs in the fall (mean DO = 6.46 mg/L). These two instances were somewhat anomalies since average DO in other springs and even within the respective springs was generally much lower (4-6 mg/L). It is hard to explain these two high points and this could just be attributed to sampler error and not a real difference in spring oxygen levels since these springs are normally within DO levels of 2-6 mg/L (Weissenfluh 2010).

Overall, the habitat variability did show a relationship to crayfish treatment (Figure 4), however this variation was not tied to biological patterns caused by crayfish presence. Differences in springs depended upon abiotic factors (depth, temperature and DO) and while these could influence invertebrate communities and fish (Allan 1995) such factors are not dependent upon crayfish presence or absence. Desert pupfish (*Cyprinodon* sp.) are found in many isolated springs in the desert southwest and are no strangers to harsh conditions of higher temperatures, low DO and minimal habitat (Rogowski et al. 2006). The majority of variability in springs resulted from hydraulic differences in springs, differences resulting from restoration status or spring variation between those springs in the Warm Springs Complex and elsewhere on the refuge. But pupfish and other endemics are found in all such habitats. Later studies could

include different crayfish present springs for comparison, though differences would likely be the same given the unique hydrology of the Warm Springs Complex (where the crayfish absent springs are found) to other springs on the refuge.

In conclusion, results from the current study demonstrate that crayfish may not be exhibiting any direct effects to habitat or pupfish abundance, condition or trophic position. Crayfish could be affecting the fish indirectly through competition for resources as made apparent by stable isotope analysis. Direct effects to invertebrate communities were apparent from South Scruggs and Big Spring invertebrate communities. The decreased abundance or even lack of certain species, particularly native springsnails, and the evidence of these organisms in gut contents supports this conclusion. In order to gain more concrete evidence of crayfish influences, future study would be aided by four main additions: 1) incorporating more crayfish present springs, such as those on the northern end of the Refuge to increase samples size for more reliable comparisons and to assess the crayfish-snail interaction, 2) assessing the trophic level of predatory fish (native and non-native) and gaining more materials for pupfish in an effort to compare all springs in all seasons, 3) including more functional groups of invertebrates and other forms of algae to clarify resource use and 4) continued monitoring of those recently restored springs, as the longer these remain without crayfish, the more reliable they can be as "control" conditions. These additions could refine the results found in the current study and give managers of the Refuge direction toward areas that might need more or less attention in terms of crayfish management.

Summary

The multitude of data presented and multitude of literature discussed has illustrated that crayfish can play an important role in food webs elsewhere and within Ash Meadows NWR springs. The springs within Ash Meadows NWR presented a unique opportunity to analyze the food web dynamics in regards to the presence/absence of a non-native introduced crayfish and interactions with the native fish and invertebrate assemblage. Even with its caveats, this study has allowed better understanding of the food web dynamics in springs with and without crayfish. Crayfish did not demonstrate the expected impacts to vegetation and algae. In fact, the vegetation response was actually opposite of the literature and no difference in algae levels was discernible. Furthermore, the data suggests that crayfish may not have a direct effect on pupfish abundance, condition or trophic position. However, the $\delta^{15}N$ and $\delta^{13}C$ data showed considerable overlap at the consumer level. These lines of evidence suggest that fish and crayfish may be competing for the same limited animal resources and thus causing a shift in pupfish diets to more plant based resources. Gut contents revealed the high prevalence of animal materials in some crayfish, while macrophytes and detritus dominated others. A majority of the guts containing animal materials were made up of fish parts and thus crayfish could present more of an impact to small fish (e.g. pupfish) if the opportunity arose. Such evidence supports that crayfish will make animal materials, specifically fish, a major part of their diet if advantageous. The most notable results are evident in invertebrate assemblage differences. Here we see significantly lower richness values ($p<0.05$, Table 3) in crayfish present springs, and the absence of certain native invertebrates, particularly springsnails, in crayfish present springs (Table 2). Habitat variables, specifically substrate, were similar across sites. Substrate is a key factor in invertebrate community composition, and combined with similar flow and only minor temperature variation,

this leaves little other explanation. Also, it is notable that one crayfish gut was filled almost entirely with *Pyrgulopsis* snails. It is well known that crayfish are no strangers to the consumption of small bodied snails. Lodge et al. (1994) document dramatic decreases in snail abundance and diversity in Rusty Crayfish enclosure/exclosure experiments. In another Rusty Crayfish invasion study, snails became almost non-existent compared to pre-invasion data (Wilson et al. 2004). *Procambarus clarkii* will readily consume *Physella* sp. and did so at a higher rate during juvenile stages (Correia et al. 2005). The reduced richness, lack of snails in Big and South Scruggs Springs, combined with the gut contents evidence, suggests that crayfish could be the factor reducing the number of these organisms from the community. The reduced trophic position of pupfish in crayfish present springs could be connected to this indirectly. Though pupfish are generally assumed to consume algae (Naiman 1975), it is also noted in this study that invertebrates were consumed when available. Following studies note similar patterns (Naiman 1976, 1979) and the desert pupfish (*Cyprinodon macularis*) showed preference of animal materials, comprising as much at 56% in some in cases. The author specifically points out the lack of available invertebrates in these study springs. A similar situation is evident in the present study. The reduced invertebrate richness leads to reduce availability of this food source to pupfish and could be influencing the trophic position of those pupfish which occur with crayfish. If pupfish do in fact feed upon invertebrates preferentially when available, those pupfish in crayfish absent springs could have an advantage.

This study highlights the direct and indirect effects which crayfish are exerting on the spring communities of Ash Meadows National Wildlife Refuge. Future management techniques should include the continued efforts to eradicate crayfish from the springs. Such efforts might alleviate some of the competition for resources that was apparent from stable isotope analysis as

well as releasing some endemic invertebrates from predation pressure evident from gut content analysis.

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