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# The Role of Hydrologic Regimes in Driving Morphologic Divergence and the Trait Compositions of Fish Assemblages

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The Role of Hydrologic Regimes in Driving Morphologic Divergence and the Trait  
Compositions of Fish Assemblages

A thesis submitted in partial fulfillment  
of the requirements for the degree of  
Master of Science in Biology

by

Lindsey Bruckerhoff  
Missouri State University  
Bachelor of Science in Biology, 2012

May 2016  
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This thesis is approved for recommendation to the Graduate Council.

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## Abstract

The hydrologic regime is an important determinant of the ecological integrity of a stream. Hydrologic regimes are defined by the magnitude, timing, frequency, rate of change, and duration of high and low flow events and capture long term patterns of variability and predictability of water movement in a stream. Flow regimes influence many aspects of the biophysical environment in lotic systems; therefore organisms have adapted to natural flow patterns. We investigated how fish have adapted to flow regimes at both a population and community level. In the first study presented in this thesis, we hypothesized fish exhibit phenotypic divergence to allow them to persist across gradients of hydrologic variability. We combined a comparative field study and mesocosm experiment to investigate the morphological divergence of *Camptostoma anomalum* (central stonerollers) between streams characterized by highly variable, intermittent flow regimes and streams characterized by relatively stable, groundwater flow regimes and assessed the plastic effects of one component of flow regimes, magnitude (water velocity), on fish morphology. We observed differences in shape between flow regimes likely driven by differences in allometric growth patterns, but observed no morphologic plasticity. The second study included in this thesis investigated the relationships between fish traits and hydrologic metrics and determined how traits are spatially auto-correlated within a stream network. We observed complex relationships between fish traits and hydrology; some traits exhibited different responses in different flow regimes. Trait-hydrology relationships were the strongest in groundwater and runoff streams, but very weak in intermittent streams. Spatial factors described more variability in the distribution of fish traits than hydrologic metrics within and between flow regimes and different types of spatial auto-correlation structured trait patterns across flow regimes. Overall, the results of these studies support the implementation of

environmental-flow standards and contribute new considerations to include in the development of ecological-flow relationships.

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## **List of Published Papers**

Chapter 1: Bruckerhoff LA, Magoulick DD. In preparation. Hydrologic regimes as potential drivers of morphologic divergence in fish. *Journal of Evolutionary Biology*.

Chapter 2: Bruckerhoff LA, Leasure DR, Magoulick DD. In preparation. Trait Composition of Fish Assemblages across Hydrologic Regimes. *Ecohydrology*.



## **Introduction**

Understanding the relationship between organisms and the physical environment is a fundamental goal of ecology. Differences in habitat condition affect species composition, and habitat heterogeneity supports biodiversity (MacArthur and Wilson 1967, Benton et al. 2003, Tews et al. 2004). Habitat can be considered a “templet” for different ecological strategies (Southwood 1977, Townsend 1997) or a regulator of community composition (Wiens 1984). Organisms adapt to deal with environmental pressures at different temporal (“now” or “later”) and spatial (“here” or “elsewhere”) scales, leading to the variety of species traits we observe in nature (Southwood 1977, 1988). Environmental pressures can be viewed as “filters”, and the species present within a defined habitat will be those with traits allowing them to pass through “filters” at hierarchical spatial scales (Menge and Olson 1990, Poff 1997).

In lotic systems, environmental pressures are largely determined by the hydrologic regime (Naiman 2008). Ecologically important components of the hydrologic regime include the magnitude of discharge and frequency, duration, timing, and rate of change of flow events of different magnitude (Poff and Ward 1989, Richter and Baumgartner 1997, Poff et al. 1997). These components influence habitat volume, current velocity, channel geomorphology, substratum stability, suspended sediments, temperature, chemistry, and channel connectivity (Poff and Ward 1989, Jowett and Duncan 1990), which are all important habitat characteristics influencing which species are present (Poff et al. 1997). Because hydrology controls so many attributes of the physical environment, organisms adapt and evolve in response to maintained variation of hydrologic regimes (Lytle and Poff 2004). Therefore, different patterns of flow variability support different communities exhibiting a range of traits (Poff et al. 1997, Pusey 2000, Biggs et al. 2005). Maintaining these natural flow patterns is necessary to sustain stream biota (Bunn and Arthington 2002, Postel and Richter 2003, Poff 2009).

Stream organisms exhibit a combination of life history, behavioral, and morphological adaptations (Lytle and Poff 2004) that provide the most benefit to fitness in response to variability in all components of hydrologic regimes. Energy allocation to reproduction or growth is often timed to take advantage of predictable conditions or avoid mortality during flow disturbance events (Iwasa and Levin 1995, Lytle 2001). Diapause of macroinvertebrates during drought (Hynes 1970), rapid growth and development of aquatic insects (Gray 1981) and riparian vegetation (Karrenberg et al. 2002) following floods or drought, and early spawning of fish before the flooding season (Jensen and Johnson 1999) are all life history adaptations to hydrologic patterns. While life history adaptations take advantage of long term predictability of flow events, behavioral adaptations allow for direct response to disturbance (Lytle and Poff 2004). Belostomatidae are known to retreat from streams during large rainfall events (Lytle 1999) and many species of fish will move out of the main channel as water levels rise (David and Closs 2002) for protection during flood events. Stream organisms also exhibit morphological adaptations to flow. Aquatic macrophytes and many species of riparian plants allocate more biomass to root structures or change physiological processes in preparation for high flow events (Barrat-Segretain 2001, Blom and Voesebeck 1996).

Because hydrology shapes the biogeochemical environment, alterations to natural hydrologic regimes may negatively affect stream fish. Increased high flow events increase erosion and sedimentation (Johnson et al. 1997), change channel shape (Infante 2006), and decrease habitat complexity (Roth et al. 1996). Reduced flows decrease connectivity and access to refuge, spawning, and nursery areas (Junk et al. 1989), and alter interspecific interactions during drought (Lake 2003). Timing of flood events is important, as high flows may be cues for spawning and migration (Junk et al. 1989), but may interrupt spawning for other species (Fausch

and Bestgen 1997, Craven 2010). Large spates can cause mortality of both juvenile and adult fish (Schlossner 1985). Juvenile fish are particularly vulnerable to extreme events due to siltation, inability to inflate air bladder, and reduced habitat stability (Peterson and Jennings 2007, Freeman and Bowen 2001). Due to differences in historic natural flow regimes, similar perturbations may have very different ecological effects in different streams (Poff et al. 1997).

With increased water use for agricultural, residential, and industrial purposes, very few rivers retain their historic hydrologic regime (Postel and Richter 2003). Global climate change will likely exacerbate alterations due to anthropogenic stress, and proactive management efforts are needed (Plamer et al. 2008). Meeting human water demands under changing climatic conditions while retaining the ecological integrity of freshwater systems presents a challenge for water resource managers. Historically, management practices consisted of minimum low/high flow thresholds or focused on requirements of a few target species. As these thresholds do not account for other ecologically important aspects of flow, this approach does not fit the needs of entire aquatic ecosystems (Poff et al. 1997). Providing environmental flows is a growing trend in water management. Environmental flows are defined by the Brisbane Declaration (2007) as the “quantity, quality, and timing of water flows required to sustain freshwater and estuarine ecosystems and the human livelihoods and well-being that depends of these ecosystems”. To implement environmental flows, managers must be able to determine flow standards timely and cost effectively at regional spatial scales. It is impractical to set standards of environmental flow on a site-by-site basis, and therefore a group of international scientists developed a framework for developing environmental flows at a regional level (Poff et al. 2010). The “ecological limits of hydrologic alteration” (ELOHA) method suggests building a foundation of hydrologic knowledge using streamflow data, basin characteristics, climate, and water use data, and then

using this information to group hydrologically similar streams (Poff et al. 2010). Environmental flow standards are then based on the ecological-flow relationships of each classified stream type and tested hypotheses of ecological responses to alteration (Poff et al. 2010).

Traits can be used to describe patterns of community assemblages along hydrological gradients, and thus develop ecological-flow relationships (Poff and Allan 1995, Mims and Olden 2012). Trait based approaches assume that species traits converge when environmental pressures are similar (Southwood 1988). Based on this theory, categorizing species by different traits allows for the study of community assemblages across biogeographic boundaries (Schluter 1986). Several studies have applied trait based approaches to stream communities. Life history traits explain and predict insect assemblages and distributions (Townsend and Hildrew 1994, Poff et al. 2006), and environmental guilds are useful to identify fish species with similar response to disturbance (Welcomme et al. 2006). Hoeinghaus et al. (2007) found using functional groups based on trophic and life history traits useful to characterize fish assemblages at sites differing in habitat condition and stability. Hydrologic variability is strongly related to the functional organization of fish assemblages in Minnesota and Wisconsin streams (Poff and Allan 1995). The three demographic strategies proposed by Winemiller and Rose (1992) explained the distributions of fish along seasonal hydrologic gradients (Tedesco et al. 2008) as well as across intercontinental (Olden and Kennard 2010) and continental (Mims and Olden 2012) spatial scales. These studies, as well as others, emphasize the role of hydrology in shaping fish assemblages and underline the usefulness of trait based approaches in assessing fish communities.

While community composition of traits may differ across flow regimes, some species are able to persist across diverse gradients of hydrologic variability. Species distributed across

heterogeneous environmental conditions (space and/or time) may exhibit intraspecific variation in physiology, morphology, and behavior. Intraspecific variation is commonly documented in fish (reviewed in Robinson and Wilson 1994) and may be the result of a combination of abiotic and biotic selective pressures. Because flow regimes influence abiotic (Poff and Ward 1989, Jowett and Duncan 1990) selection pressures and potentially influence biotic interactions (by influencing community composition), fish species may exhibit divergence across hydrologic gradients.

The objective of this thesis is to develop ecological-flow relationships in the Ozark-Ouachita Interior Highlands. In this thesis, I present two papers with the goal of developing ecological-flow relationships from both a population and community perspective to determine how flow regimes drive both intraspecific variation in traits and differences in community composition of traits. In the first paper, I describe how flow regimes may drive morphological divergence in fish, and in the second paper, I compare relationships between fish life history traits and hydrologic metrics across flow regimes and describe the importance of accounting for spatial auto-correlation when developing ecological-flow relationships. These papers are compiled in this thesis to fit the University of Arkansas Graduate School degree requirements.

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Chapter 1: Hydrologic Regimes as Potential Drivers of Morphologic Divergence in Fish

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## Abstract

Fish often exhibit phenotypic divergence across gradients of abiotic and biotic selective pressures. In streams, many of the known selective pressures driving phenotypic differentiation are largely influenced by hydrologic regimes. Because flow regimes drive so many attributes of lotic systems, we hypothesized fish exhibit phenotypic divergence across gradients of hydrologic variability. We combined a comparative field study and mesocosm experiment to investigate the morphological divergence of *Campostoma anomalum* (central stonerollers) between streams characterized by highly variable, intermittent flow regimes and streams characterized by relatively stable, groundwater flow regimes and assessed the plastic effects of one component of flow regimes, magnitude (water velocity), on fish morphology. We observed differences in shape between flow regimes likely driven by differences in allometric growth patterns. Although we observed differences in morphology across flow regimes in the field, *C. anomalum* did not exhibit morphologic plasticity in response to water velocity alone. This study contributes to the understanding of how complex environmental factors drive phenotypic divergence, and may provide insight into the evolutionary consequences of disrupting natural hydrologic patterns, which are increasingly threatened by climate change and anthropogenic alterations.

Keywords: flow regime, morphology, geometric morphometrics, phenotypic plasticity, genetic divergence

## Introduction

Morphological characteristics of fish are related to ecological functions such as feeding and habitat use (Keast & Webb 1966, Douglas & Matthews 1992, Wood & Bain 1995, Koehl 1996), as well as swimming performance (Vogel 1994). Fish must maximize locomotive capabilities to perform essential tasks such as feeding, avoiding predators, and spawning. It is therefore predicted that morphology should provide the optimal locomotive capabilities for the environment in which a fish lives. Differences in fish morphology often reflect a tradeoff between steady and unsteady locomotive capabilities (Blake 1983, Domenici 2003, Langerhans & Reznick 2010). Swimming in a straight line with constant speed is considered steady swimming, while complicated locomotive patterns with frequent changes in velocity is considered unsteady swimming (Blake 1983). Steady state swimming allows fish to remain in place against current, search for food or mates, cruise to avoid areas with predators or competitors, and migrate over large distances. These locomotive activities are often achieved through streamlined body shapes, narrow caudal peduncle regions, and high caudal fin aspect ratios (Blake 1983, Vogel 1994, Fisher & Hogan 2007, Langerhans & Reznick 2010). Unsteady state swimming is useful when fish have to make frequent fast starts, rapid turns, and quick stops. These complex maneuvers are useful for escaping predators, capturing prey, and navigating through complex habitats (Langerhans 2009). Unsteady state swimming is often associated with deep bodies through the caudal region, median fins, and large caudal fins with low aspect ratio (Blake 1983, Walker 1997, Domenici *et al.* 2008).

Steady and unsteady state swimming capabilities are differentially selected for across biotic and abiotic gradients. Intraspecific variation in morphology reflecting tradeoffs between steady and unsteady state swimming is documented for many fish families worldwide in response to water velocity (Brismead & Fox 2002, Imre *et al.* 2002, Langerhans *et al.* 2003,

Kerfoot & Shaefer 2006, Sidlauskas *et al.* 2006, Langerhans *et al.* 2007), predation pressure (Walker & Bell 2000, Walker *et al.* 2005, Domenici *et al.* 2008, Langerhans 2009, Franssen 2011) and habitat complexity (Ehlinger & Wilson 1988, Robinson & Wilson 1994, Schluter 2000, Hendry *et al.* 2002, Hendry & Taylor 2004). These works suggest steady state swimming characteristics are favored in open habitats, high velocity waters, and relatively low predation risk environments while unsteady state swimming characteristics are favored in complex habitats, low velocity flows, and environments with high predation pressure (Langerhans & Reznick 2010).

Flow regimes likely influence known selective pressures (habitat complexity, velocity, biotic interactions), as well as other unknown pressures influencing fish morphology. Many of the biophysical attributes and functioning of lotic systems are largely determined by hydrologic regimes (Naiman *et al.* 2008). The hydrologic regime of a stream is defined by the magnitude of discharge and frequency, duration, timing, and rate of change of flow events (Poff & Ward 1989, Richter *et al.* 1997, Poff *et al.* 1997). Flow regimes influence habitat volume, current velocity, channel geomorphology, substratum stability, suspended sediments, temperature, chemistry, and channel connectivity (Poff & Ward 1989, Jowett & Duncan 1990). Because hydrology controls many attributes of the physical environment, organisms adapt and evolve in response to maintained hydrologic regimes (Lytle & Poff 2004). Flow regimes may therefore promote morphologic divergence in fish across gradients of hydrologic variability.

Morphologic divergence between populations may result from a combination of phenotypic plasticity and genetic divergence. Plasticity is commonly documented in North American freshwater fish (Robinson & Parson 2002) and is documented in response to gradients of water velocity in salmonids (Imre *et al.* 2002, Peres-Neto & Magnan 2004, Keeley *et al.* 2007,

Fischer-Rousseau *et al.* 2010). Phenotypic plasticity may allow organisms to persist in heterogeneous habitats (Via *et al.* 1995, Valladares *et al.* 2005), therefore selection may favor phenotypic plasticity in variable environments (Bradshaw 1965, De Jong 1995). Some streams experience highly variable flow regimes in which phenotypic plasticity in morphology may be under selection. Although plasticity can be both adaptive and non-adaptive (reviewed in Ghalambor *et al.* 2007), understanding the role of plasticity in driving phenotypic variation helps us understand how the environment drives adaptation and phenotypic diversity, and further, patterns of speciation and evolution across variable environments (Langerhans 2008, Schluter 2000).

Because flow regimes influence many attributes of lotic systems, including known drivers of fish morphology (habitat, water velocity, and biotic interactions), we predicted fish populations would exhibit morphological differences across hydrologic gradients. We combined a comparative field study and mesocosm experiment to determine: (1) Do fish exhibit intraspecific morphological divergence between flow regimes?, (2) What is the nature of morphologic divergence between flow regimes?, and (3) What is the role of phenotypic plasticity in driving morphological divergence in fish between different flow regimes?. We completed a comparison field study to test our hypotheses that fish exhibit morphological divergence across flow regimes and a mesocosm experiment to test our hypotheses that fish from intermittent streams would exhibit phenotypic plasticity in response to water flow. Contrary to other studies that only consider “flow regime” in terms of water velocity or Reynold’s number, we define flow regime in terms of the magnitude, duration, frequency, rate of change, and timing of flow events (Poff *et al.* 1997). As we do not believe previous observed differences in morphology due to water velocity alone could explain differences in morphology between flow

regimes, we designed this study to determine if there are broad differences in shape between streams experiencing low hydrologic variability (groundwater streams) and streams experiencing high hydrologic variability (intermittent streams). By investigating morphological divergence across flow regimes, we contribute to existing theory of phenotypic divergence across complex environmental gradients. This study contributes to the understanding of how hydrologic regimes drive intraspecific variation, and may provide insight into the evolutionary consequences of disrupting natural hydrologic patterns, which are increasingly threatened by climate change and anthropogenic alterations.

## **Materials and Methods**

### *Study System and Organism*

We investigated morphological variability of *Campostoma anomalum* (central stoneroller) in the Ozark Highlands and Boston Mountains of Arkansas and Missouri (USEPA 2010). This region is hydrologically diverse; some streams have a large groundwater influence with low hydrologic variability, while others are largely influenced by runoff surface water and experience predictable flow events associated with seasonal weather changes. Other streams in the study area experience extreme drying and unpredictable changes in flow. *C. anomalum* is a widespread and abundant (Pflieger 1975) grazer in the study region found across these diverse hydrologic gradients.

### *Field Collections*

We collected *C. anomalum* from 10 groundwater flashy and 10 intermittent flashy streams in the White River drainage basin of Arkansas and Missouri (Figure 1). The hydrologic regime classification of these streams were derived from Leasure et al. (2016). This flow classification scheme produced seven flow classes representing a gradient of hydrologic

variability in terms of all components of the flow regime (magnitude, duration, timing frequency, and rate of change between high and low flow events). We chose two flow regimes that represented extreme endpoints of this gradient of variability. Groundwater flashy streams (referred to as groundwater streams in this paper) are characterized as having low daily flow variability, mean daily discharge ranging from 4.3 to 905 cfs, and never dry up completely, while intermittent flashy streams (referred to as intermittent streams in this paper) are dry 1-3 months each year, have at least six low flow spells each year, and have mean daily discharge ranging from 2.8 to 8.7cfs (Leasure *et al.* 2016). Stream sites within each hydrologic regime were selected based on criteria of having drainage areas smaller than 200 km<sup>2</sup> so that all streams represented small headwater streams, were at least 10 km (shortest aquatic route) from other sampling sites so sites were spatially segregated, and had a probability of belonging to the flow regime higher than 0.5 (Leasure *et al.* 2016). Sampling locations were clumped within each flow regime as results of the landscape factors driving hydrologic variability in the system. Although the aim of this study is to investigate flow regimes, results of this study may be related to other landscape factors used to classify flow regimes.

Thirty fish (63-131mm) were collected from each stream during the summer of 2014 using a Smith-Root LR-24 backpack electrofisher. We kept the first 30 fish with a standard length greater than 60 mm for analysis to limit our analysis to sexually mature adults. Males and females were kept for analysis, as we were interested in population level response to flow and it is difficult to discriminate between sexes without dissection. The left side of each fish was photographed in the field using a Sony Cyber-shot DSC-H55 mounted to a stand attached to a platform. All fish were oriented in the same manner in the same location on the platform for each photo.



### *Phenotypic Plasticity*

We compared the potential for phenotypic plasticity in morphology between individuals from groundwater streams and intermittent streams. A 2x2 fully factorial mesocosm experiment was conducted to determine the effects of source population and water velocity (flow induced plasticity) on *C. anomalum* morphology. Although we were interested in the effects of flow regimes on morphology, we focused on water velocity because it is difficult to mimic all components of flow regimes in a mesocosm. A transplant experiment is ideal for assessing patterns of local adaptations between environments (Kawecki & Ebert 2004), but a transplant experiment was not feasible during the current study. This experiment was designed to test for a plastic response in fish from groundwater and intermittent streams to one component of flow regimes, water velocity, known to be associated with morphological plasticity in fish. We compared the relative contributions of source populations and water velocity to fish morphology. We interpreted a significant effect of the flow treatments to indicate plastic response of fish morphology to water velocity.

The two levels of source population were groundwater and intermittent streams, and the two treatments were high and low velocity. We used oval polyethylene tanks (1.26 m x 0.84 m x 0.49 m, volume = 0.52 m<sup>3</sup>) with a mixture of gravel (<0.003 m) and pebble (0.003 m – 0.06 m) substrate as mesocosms. Partitions in the mesocosms served to create a constant, circular flow path to simulate a flowing stream. Mesocosms were located in a temperature and humidity controlled greenhouse at the University of Arkansas. High flow treatments were created in the mesocosms by two Rio Plus Aqua Power Head 2100 pumps and one Fluvial MSF 205 filter pump, while low flow treatments had a single filter pump. High flow treatments had an average flow velocity of 0.29 m/s (0.23-0.36 m/s) and low flow treatments had an average flow velocity of 0.04 m/s (0.01-0.07 m/s).

*C. anomalum* young of the year were collected using a seine from Cherry Creek, an intermittent stream ( $n=70$ , mean length =  $31 \pm 0.24$  mm SE) and from Clear Creek (Figure 1), a groundwater stream ( $n=70$ , mean length=  $28 \pm 0.29$  mm SE) on May 21 2014. Fish were held in holding tanks until introduction to the mesocosms on May 28 2014. Initial SL and mass were measured for each fish before introduction into the mesocosms. Treatments were randomly assigned to 28 mesocosms and five fish from each population were randomly assigned to mesocosms. Each of the four treatments (2 source populations x 2 water velocity treatments) had seven replicate mesocosms. Five fish per mesocosm represented natural densities observed in streams in the study area (Magoulick, D. D, unpublished data). Fish remained in the mesocosms under constant flow (high or low) for 15 weeks. On September 19-20 2015, fish were removed from mesocosms, length and mass measurements were obtained, and fish were photographed in the same manner as the field comparisons.

#### *Geometric Morphometric Analysis*

Morphological variation was assessed using landmark based geometric morphometric analysis. This method utilizes landmark coordinates, outline curves, surfaces, and multivariate statistics to examine shape variation and covariation with other variables (Rohlf & Marcus 1993). A single author (Bruckerhoff, L. A.) digitized 10 landmarks (Figure 2) on digital images of each specimen in tpsDig (Rohlf 2004). We used these landmarks because we believed they captured aspects of overall shape to test for broad differences in morphology and because these landmarks could be consistently placed on each photo.

Generalized Procrustes Analysis (GPA, Gower 1975) was used to superimpose and rescale the position of landmarks of all specimens ( $n=592$ ) onto one coordinate system and remove non-shape variation using tpsRelw (Rohlf 1994). GPA utilizes least-squares estimates to

translate all specimen centroids to origin, scale them to a common unit, and rotate so all landmarks are aligned (Rohlf 1990). GPA was performed separately for field and mesocosm experiments. We used tpsSmall (Rohlf 2015) to determine if shape variation was small and therefore tangent space approximation was appropriate. We used the superimposed landmark configurations produced by GPA and projected into Procrustes tangent space (Rohlf, 1990) to calculate partial warps using tpsRelw (Rohlf 1994). Partial warps are derived from the bending energy matrix of superimposed configurations and can be used to analyze variation in shape.

### *Statistical Analysis*

Statistical analyses were conducted using Stata14 (StataCorp 2015) and thin plate spline deformation graphs were created in R (R Core Team 2014) using the shapes package (Dryden 2014). We used centroid size as a measure of body size for all analyses as it was highly correlated with standard length ( $p < 0.001$ ,  $R^2 = 0.982$ ). Centroid size is the square root of the sum of squared distances of all landmarks from their centroid. Because we were most interested in size independent morphology, we included centroid size as a covariate in statistical models to control for shape variation due to body size. Centroid size was log transformed to improve normality in all analyses. Partial warps (shape variables), including 14 non-affine and 2 affine components of shape, were used as dependent variables in all multivariate models. All F-values were calculated using Wilk's lambda and effect size was calculated using partial eta squared ( $\eta_p^2$ ).

### *Field Comparisons*

We used multivariate analysis of covariance (MANCOVA) and discriminant function analysis (DFA) to determine if morphology differed between flow regimes and principal component analysis (PCA) to visualize multivariate distribution of shape variables between flow

regimes. We used DFA to determine the effect of flow regime on morphology. This method tests the ability to correctly classify individuals into groundwater and intermittent streams, with the null hypotheses that 50% can be classified by chance. MANCOVA was used to determine the combined effects of flow regimes, size, and site on fish morphology. The MANCOVA model included shape variables as dependent variables, centroid size as a covariate, flow regime (to test for the effect of intermittent and groundwater flow regimes), and site nested within flow regimes (to test for the effect of site within the regimes). Fixed factors included flow regime and site nested within flow regimes. Although we planned *a priori* to treat site nested within flow regimes as a random factor, we had to treat this effect as fixed due to the negative matrix determinant preventing the use of matrix algebra required in MANCOVA. We tested for multivariate normality, homogeneity of variance and covariance, and heterogeneity of slopes. All non-significant interactions were dropped from the final model. Due to a significant flow regime by centroid interaction, we used the permutational procedure described in Piras *et al.* (2011) using the common slope function in R to test the hypothesis that individuals from different flow regimes differ in their allometric trajectories. This procedure compares the distance between the vectors of coefficients resulting from the multivariate regression of shape variables on log centroid size. Permutation of the residuals from the regression model are used to test the statistical significance of the vector distances between groups (flow regimes). To determine the nature of morphological divergence between flow regimes, we used canonical axes derived from MANCOVA to produce thin-plate splines and deformation plots. These plots show the differences in shape between flow regimes while controlling for size, site, and the interaction between size and flow regime.

### *Phenotypic Plasticity*

We also used MANCOVA to determine the contribution of source population and flow treatment on *C. anomalum* morphology. The model included shape variables as dependent variables, flow treatment (to test for plasticity between high and low flow treatments) and population (to test for the effect of groundwater flashy and intermittent flashy populations) as fixed factors, and centroid size as a covariate. Heterogeneity of slopes was tested and all non-significant interactions were dropped from the final model

### **Results**

Relative warp scores derived from PCA of shape variables were variable within flow regimes, exhibiting minor separation across the 1<sup>st</sup>, 2<sup>nd</sup>, and 3<sup>rd</sup> axes (Figure 3). Discriminant function analysis correctly classified 69.93% of individuals from groundwater streams and 63.51% of individuals from intermittent streams ( $F_{16,577} = 8.948$ ,  $p < 0.001$ , Figure 4). Flow regime, site (nested within flow regime), centroid size, and the interaction between centroid and flow regime all had significant effects on body shape of *C. anomalum* (Table 1). Centroid size had the strongest effect on shape ( $\eta_p^2 = 0.343$ ) followed by site nested within flow regime ( $\eta_p^2 = 0.228$ ), the flow regime-centroid interaction ( $\eta_p^2 = 0.070$ ), and flow regime ( $\eta_p^2 = 0.047$ ). The significant effect of the flow-centroid interaction indicates the individuals from groundwater and intermittent streams may have different allometric trajectories (Figure 5). The permutation based common slope test indicated a significant difference ( $p < 0.001$ ) in the allometric trajectories between individuals from intermittent and groundwater streams.

Thin-plate splines revealed differences in head shape, posterior region depth, and dorsal fin insertion points (Figure 6) between flow regimes. Individuals from intermittent streams had relatively smaller heads, deeper caudal fin insertion points and exhibited posterior deepening

compared to individuals from groundwater streams. The dorsal fin insertion points on individuals from groundwater stream were placed further towards the posterior region compared to those from intermittent streams. We also used thin-plate spline deformations to visualize allometric shape difference between flow regimes (Figure 7). These plots reveal more pronounced changes in shape in intermittent populations than groundwater populations, characterized by streamlining with increasing size.

When testing for plastic effects of flow on *C. anomalum* morphology, centroid size had the strongest effect ( $P < 0.001$ ,  $\eta_p^2 = 0.545$ ) followed by population ( $\eta_p^2 = 0.278$ ) and flow treatment ( $\eta_p^2 = 0.171$ ), although neither population nor flow treatment were significant (Table 1). No interaction terms were significant in the MANCOVA model. All fish across treatments grew in the mesocosm experiment, with fish from the groundwater streams (mean change in SL =  $37.164 \pm 1.919$  mm and mass =  $4.892 \pm 0.350$  g) growing longer than fish from the intermittent stream (mean change in SL =  $30.832 \pm 2.318$  mm and mass =  $4.269 \pm 0.373$  g). Fish in the high flow treatment (mean change in SL =  $37.478 \pm 2.007$  mm and mass =  $5.200 \pm 0.311$  g) grew longer and heavier than fish in the low flow treatment (mean change in SL =  $30.993 \pm 2.182$  mm and mass =  $4.028 \pm 0.349$  g).

## **Discussion**

Fish often exhibit phenotypic divergence in response to selective pressures and therefore serve as model organisms in which to investigate phenotypic divergence across complex environmental gradients. Because flow regimes influence a variety of biotic and abiotic factors known to drive phenotypic divergence in fish, the interaction of multiple selection forces may drive complex phenotype- environment relationships across hydrologic gradients. Morphology is directly related to locomotive capabilities required for fish survival and is likely under selective

pressure across hydrologic gradients. We observed complex relationships between morphology, size, and allometric growth patterns of *Campostoma anomalum* inhabiting intermittent and groundwater flow regimes.

Although no previous studies have investigated morphological divergence across classified flow regimes, observed shape differences between flow regimes in the present study fit predictions based on known drivers of morphological divergence in fish. Repeated documentation of morphological divergence across predator regimes, habitat structure and complexity (related to prey availability and type), and water velocity suggest predictable patterns of morphology driven by tradeoffs between steady and unsteady state swimming capabilities (Langerhans & Reznick 2010). Intermittent streams may be considered more complex habitats than groundwater streams, as they are characterized as hydrologically variable streams with dramatic fluctuations in flow. Based on the model proposed by Langerhans & Reznick (2010), if we consider intermittent streams complex environments, fish from our intermittent streams should exhibit more features enhancing unsteady state swimming capabilities. The smaller anterior region and deeper posterior regions of individuals from intermittent streams relative to the fusiform shapes observed in individuals from groundwater populations fit this prediction, although these results must be interpreted keeping in mind the important of size in our study. A large body of literature also suggests fish exhibit intraspecific differences in shape across gradients of water velocity, with relatively more streamline body shapes in high flow (velocity) environments. Meta-analysis revealed significant support for streamlining shape with increased water velocity in over 40 studies (Langerhans 2008). While the relationship between water velocity and fish body shape is well documented, the results from our field collections cannot be directly compared to this existing literature. In streams, water velocity is dynamic; it changes

across short and long temporal scales and the predictability and variability of flow events differs across the landscape. The streams sampled in this study cannot simply be classified as “high” or “low” velocity streams. More work is needed to determine how fish maximize swimming capabilities in temporally variable flow environments.

An interesting result of the study was the importance of body size on fish morphology. We observed a significant interaction between centroid size and flow regime. This interaction could be attributed to different sizes between flow regimes, different allometric trajectories between flow regimes, or a combination of the two. Although we used the same methods to collect the first 30 fish with a standard length over 60mm, mean length and centroid size was higher in groundwater streams than intermittent streams, although there was high variation in size in both flow regimes (Figure 8). Interestingly, we also observed more growth in young of the year from groundwater streams than those from intermittent streams in our mesocosm experiment. Different sizes between flow regimes could be the result of different resource availability between flow regimes or smaller mature adults in the harsher intermittent streams (Winemiller & Rose 1992, Lytle 2001). Allometric trajectories describe how shape changes with changes in body size (not considering development or age). In the size range collected in our study, we observed different allometric trajectories (Table 1, Figure 5). Allometric shape changes are often linked to ontogenetic changes in species that grow larger as they grow older, and can therefore provide insight into the underlying developmental changes driving phenotypic divergence and evolutionary patterns (Gould 1977, McKinney & McNamara 1991, Klingenberg 1998). Flow regimes may be influencing important environmental factors influencing development such as temperature, dissolved oxygen levels, and light penetration, further influencing adult phenotypes. While the current study was not designed to investigate allometric



or ontogenetic shape changes, many new statistical tools and frameworks (Adam & Collyer 2009, Collyer & Adams 2013, Mitteroecker *et al.* 2013) can be utilized in future studies to determine how the magnitude and direction of phenotypic trajectories differ across flow regimes. We observed different patterns of shape change across a relatively small size range of fish (60-130 mm). Comparisons of body shape across different age classes and a wider size range will provide more insight into key developmental stages potentially driving phenotypic differences.

We did not observe evidence of phenotypic plasticity in response to water velocity in neither intermittent or groundwater fish. As in the comparative field study, centroid size described the most variability in fish shape compared to water velocity treatments or flow regime of origin. Our results though, must be interpreted with caution as we may not have captured important developmental cues over the course of our mesocosm experiment. We were not able to simulate complete flow regimes in a mesocosm experiment; therefore we only investigated water velocity (magnitude of flows). The lack of plasticity observed in these experiments could indicate that water velocity (within the ranges we observed) is not an important driver of fish morphology in this system or for *C. anomalum*. Future studies addressing how fish respond to variability in other components of the flow regime (duration, frequency, timing, and rate of change) to fully understand if phenotypic plasticity contributes to fish morphology and if plasticity is more apparent in highly variable streams (intermittent) versus more stable streams (groundwater).

Flow regimes are considered a ‘master variable’ (Power *et al.* 1995) influencing many abiotic and biotic characteristics of streams, and ecologists and water resource managers recognize the importance of sustaining natural flow regimes to protect stream biodiversity and ecosystem functioning (Poff *et al.* 1997, Bunn & Arthington 2002). In order to truly understand

and manage the relationships between flow regimes, biota, and ecosystems, we must first understand how stream organisms have adapted to natural flow patterns. While a large amount of literature documents shifts in community composition along hydrologic gradients, characteristics allowing some species to persist across diverse hydrologic gradients are not well understood. The differences in allometric shape changes between flow regimes and morphological differences between sites across hydrologic gradients observed in the current study represent one of the first documented cases of phenotypic divergence in response to flow regime. The morphological differences observed in this study may be the result of a combination of direct and indirect effects of flow patterns and highlight the importance of understanding how ‘master’ or composite variables act as selective forces. As flow regimes are increasingly altered by climate change and increased anthropogenic water demands, we can expect the selective pressures acting on stream biota will change. Understanding how stream organisms have adapted to natural flow patterns helps us to manage natural flow patterns and eventually understand the evolutionary consequences of flow alteration.

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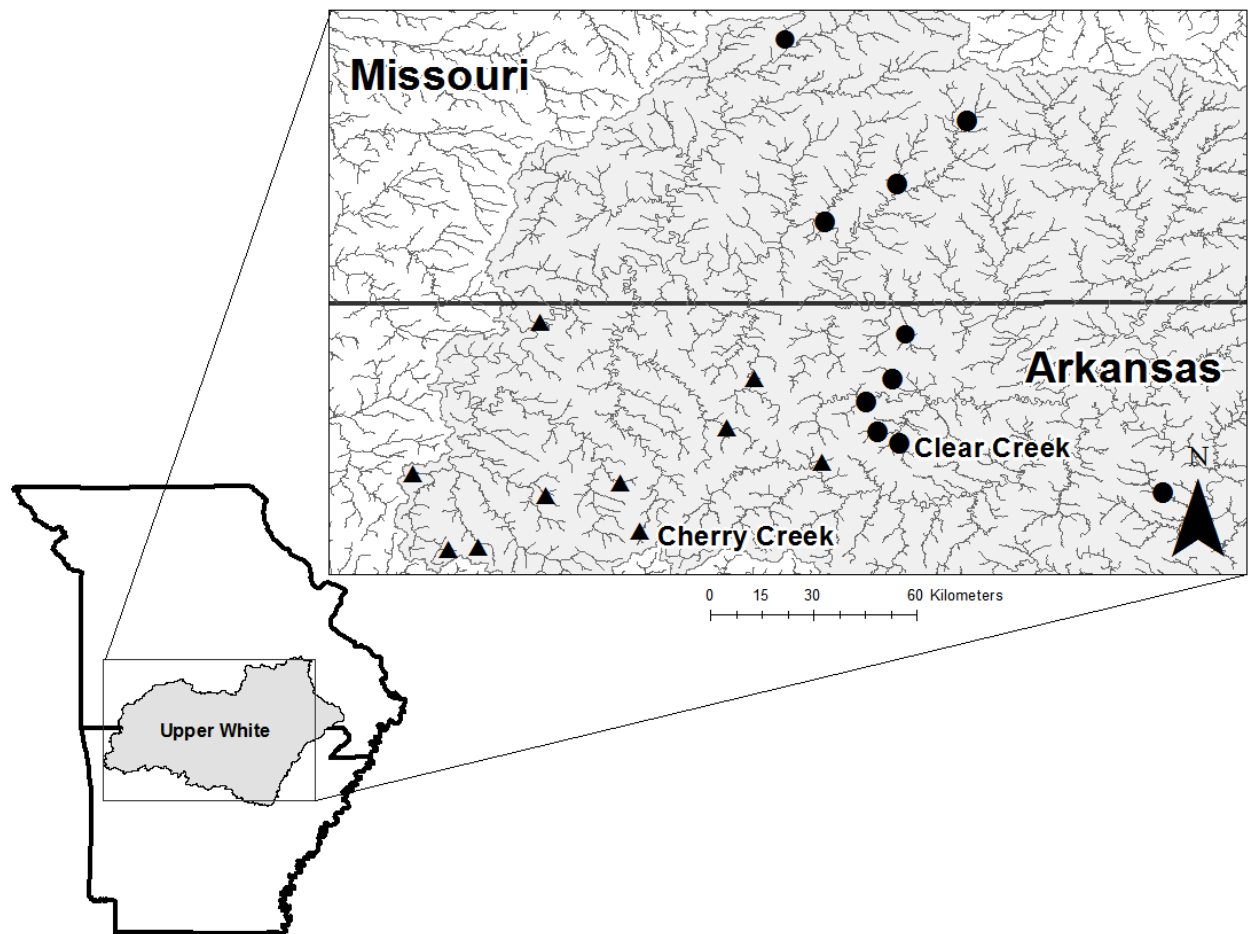
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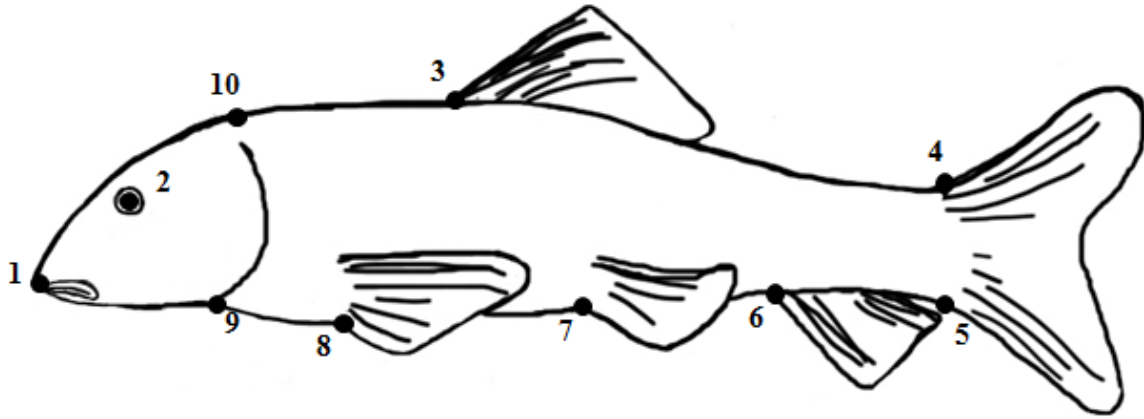
## Tables and Figures

**Table 1.** Results from field comparisons of body shape variation between flow regimes and genetic divergence and plasticity experiment. (a) MANCOVA model using shape variables collected from individuals occupying groundwater and intermittent streams. (b) MANCOVA model using source population and flow treatment as factors.

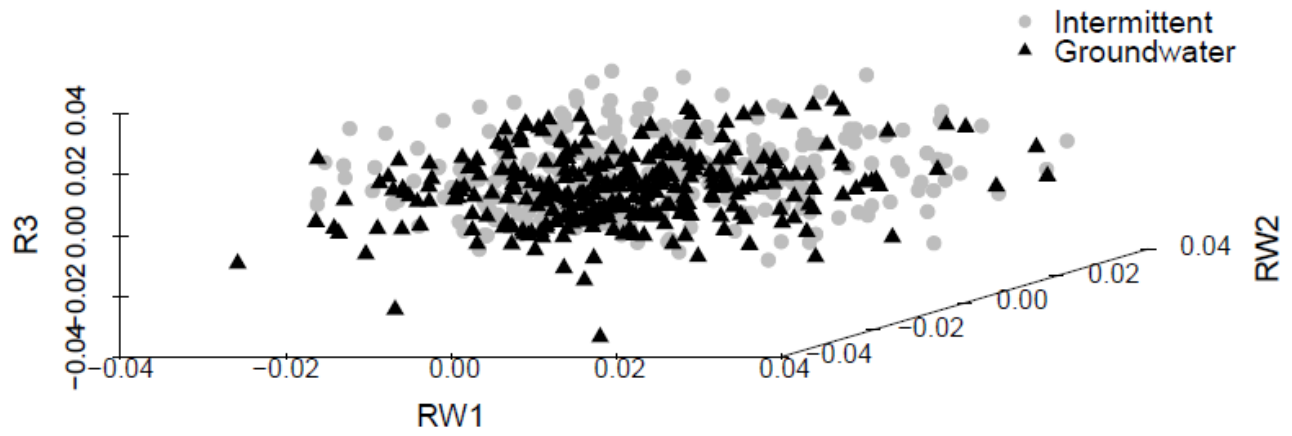
Source	Wilk's $\Lambda$	$\eta_p^2$	df	F Ratio	P
<b>(a) Flow Regimes</b>					
Centroid	0.657	0.343	16, 557	18.21	<0.001
Flow Regime	0.953	0.047	16, 557	1.72	0.040
Site (Flow Regime)	0.049	0.228	288,6732	6.90	<0.001
Flow x Centroid	0.952	0.070	288,6732	1.76	0.034
<b>(b) Plasticity</b>					
Centroid	0.411	0.545	16, 69	6.18	<0.001
Population	0.723	0.278	16, 69	1.65	0.077
Flow Treatment	0.830	0.171	16, 69	0.89	0.587



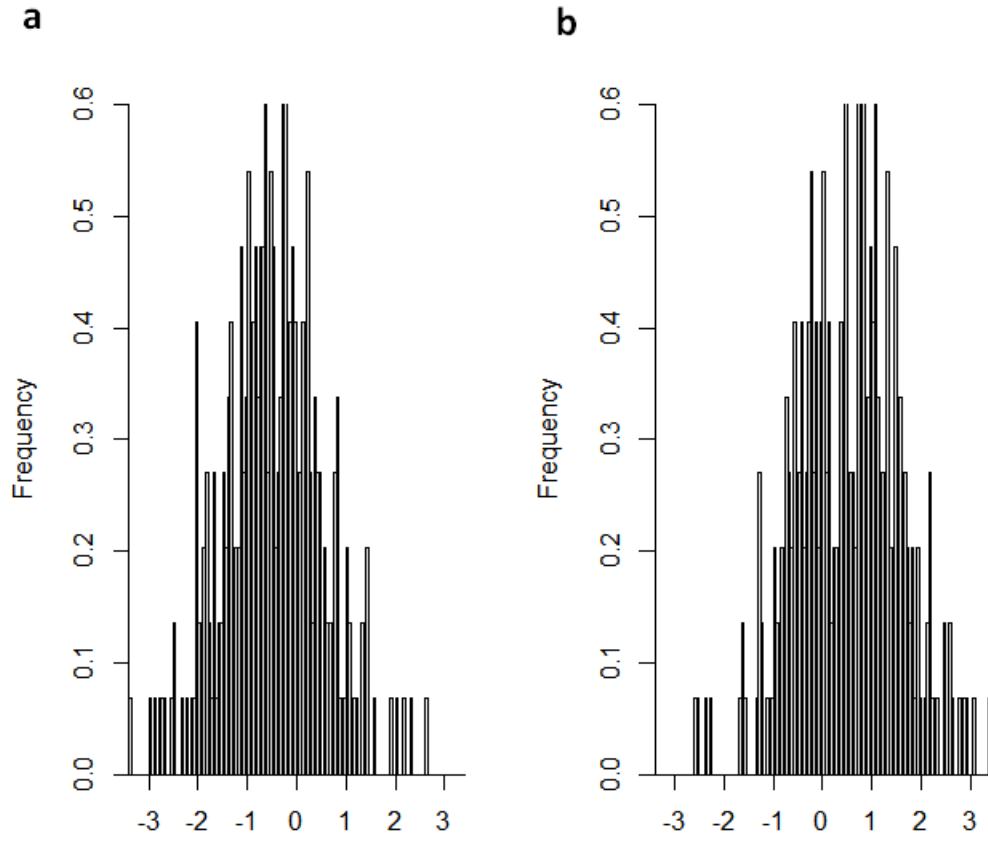
**Figure 1.** Location map of the 10 groundwater (●) and 10 intermittent (▲) stream populations sampled for field comparisons in the White River drainage basin. Cherry Creek and Clear Creek were sampled for the mesocosm experiment.



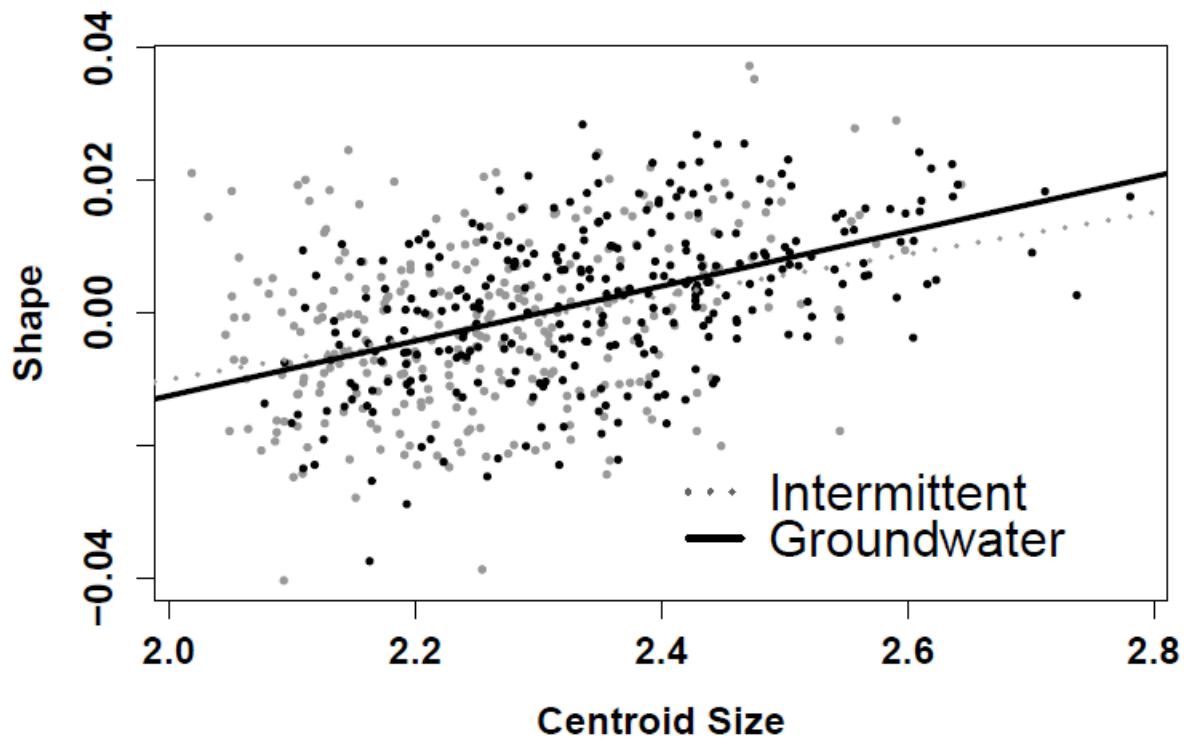
**Figure 2.** Landmark coordinates used for geometric morphometric analysis of body shape.



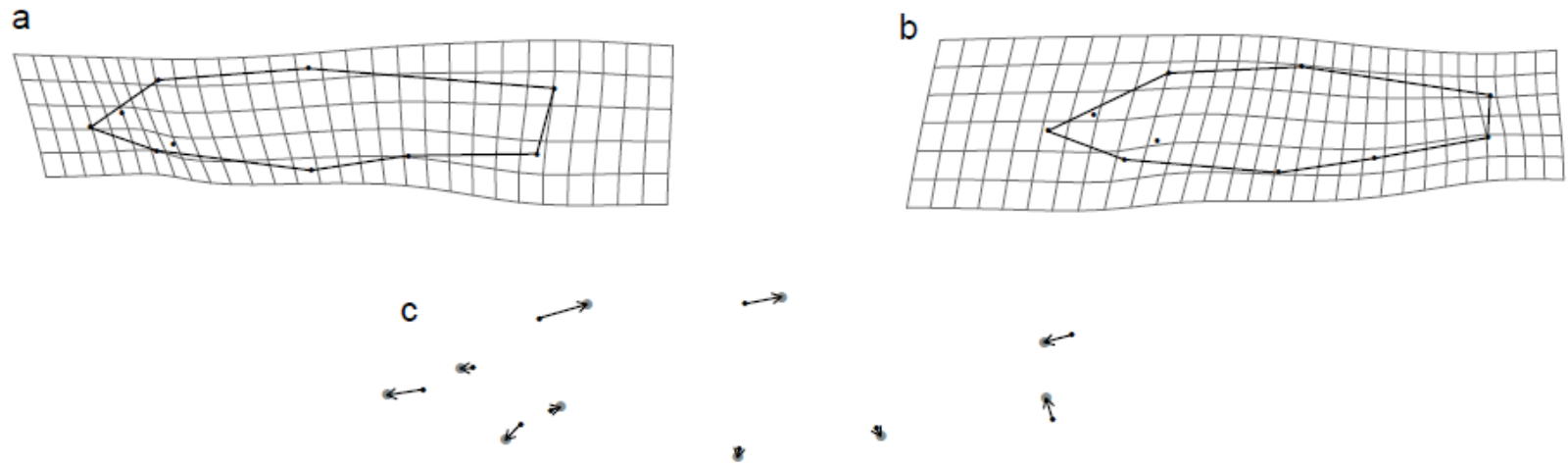
**Figure 3.** Individual scores on the first three relative warps scores derived from principle components analysis of partial warp scores (shape variables) for *Campostoma anomalum* from groundwater and intermittent streams.



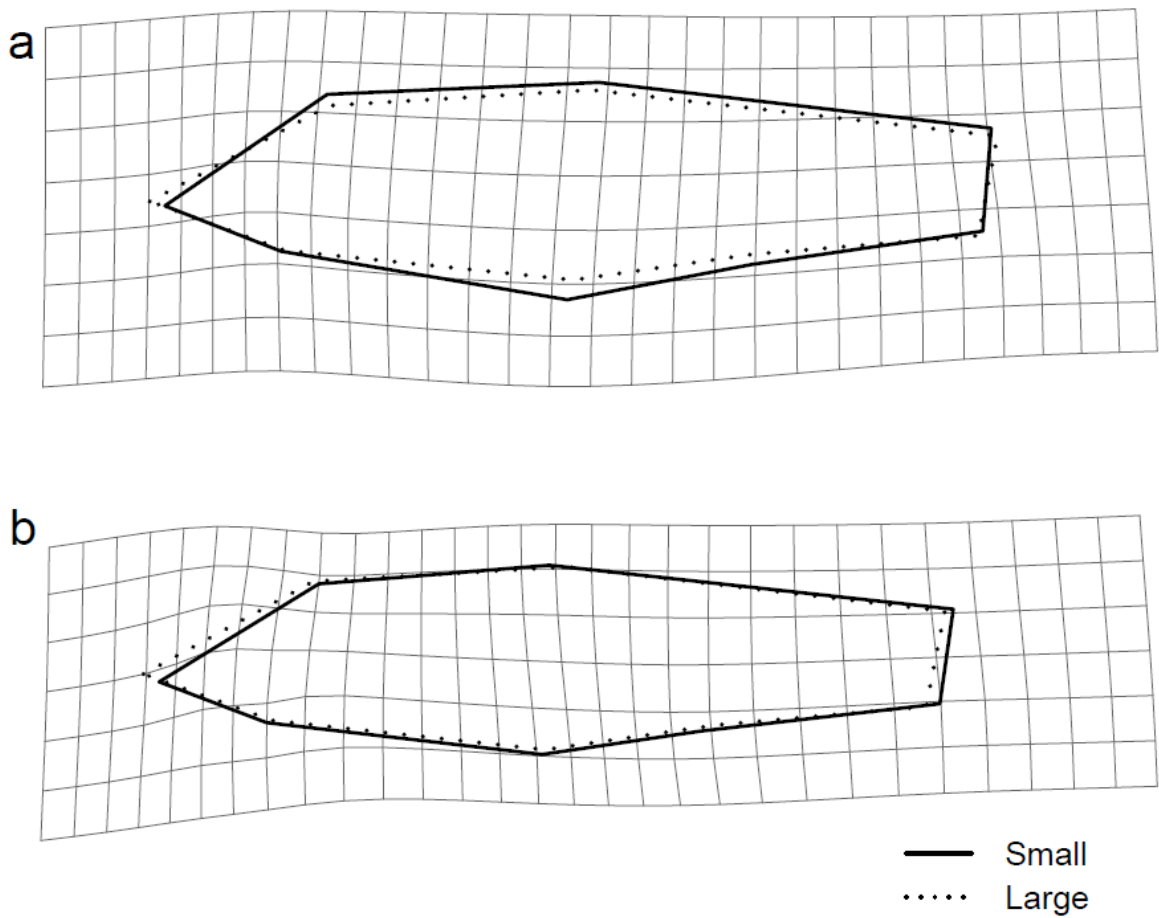
**Figure 4.** Histograms of individual scores on the single discriminant function derived from discriminant function analysis (DFA). DFA correctly classified 70% of individuals from groundwater streams and 64% of individuals from intermittent streams ( $F_{16,577} = 8.948$ ,  $p < 0.001$ ).



**Figure 5.** Plot showing the significant interaction (Interaction between shape ( $F= 1.76$ ,  $p=0.034$ ) between fish shape variables and centroid size from MANCOVA. Shape is represented by the discriminant function used in MANCOVA.

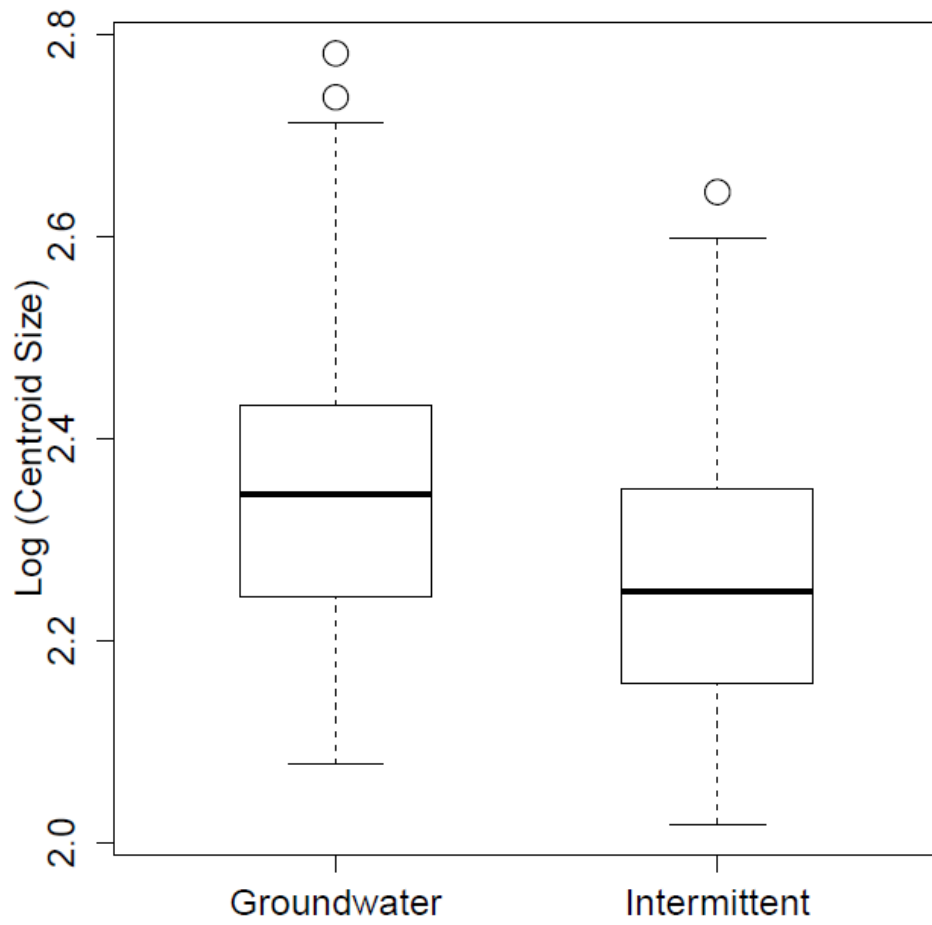


**Figure 6.** Visual representation of morphological variation of *Campostoma anomalum* between flow regimes. Grids are thin-plate spline transformations from the reference configuration of all individuals and flow associated shape (from MANCOVA) in (a) fish from intermittent streams and (b) fish from groundwater streams. Landmark vectors point in the direction landmarks move from intermittent to groundwater populations.



**Figure 7.** Thin-plate spline transformation grids showing shape differences between small (minimum size) and large (maximum size) *Campostoma anomalum* from (a) intermittent and (b) groundwater populations. The small individual represents the mean shape for individuals within the first quartile of centroid size, while the large individual represents the mean shape for individuals within the fourth quartile of centroid size.





**Figure 8.** Boxplots showing distribution of natural log transformed centroid size between fish collected from groundwater and intermittent flow regimes.

## Chapter 2: Trait Composition of Fish Assemblages across Hydrologic Regimes

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## **Abstract**

Establishing ecological-flow relationships is a crucial component of managing lotic systems within an environmental flow framework. Species traits may be useful for developing ecological-flow relationships because they can be used to make comparisons across biogeographical boundaries. Fish traits, such as life history strategies and spawning characteristics, have been linked to hydrologic metrics and classified flow regimes at relatively large spatial scales, but not smaller, management level scales, and the role of spatial autocorrelation in driving trait distributions in stream networks has not been assessed. We used combined fourth-corner and RLQ analysis and mixed moving average spatial stream network (SSN) models to (1) determine the relationship between fish traits and hydrologic metrics within classified flow regimes at a management (state) level spatial scale, (2) determine how traits are spatially auto-correlated within a stream network, and (3) compare the degree of spatial autocorrelation between flow regimes. The strength of relationships between fish traits and hydrologic metrics were relatively strong in groundwater and runoff streams, while relationships were weak in intermittent. Relationships between fish traits and flow metrics were often different between flow regimes. Spatial factors described more variability in the distribution of fish traits than hydrologic metrics within and between flow regimes and different types of spatial autocorrelation structured trait patterns across flow regimes. This study highlights the complex relationships between biota and hydrologic regimes and the importance of considering spatial patterns when developing ecological-flow relationships.

**Keywords:** environmental flows, spatial autocorrelation, streams

## **Introduction**

The biophysical attributes and functioning of lotic systems are largely determined by hydrologic regimes (Naiman et al. 2008). Ecologically important components of hydrologic regimes include the magnitude, frequency, duration, timing, and rate of change of flow events (Poff and Ward 1989, Richter and Baumgartner 1997, Poff et al. 1997). Natural flow regimes are critical for sustaining freshwater ecosystems (Bunn and Arthington 2002, Postel and Richter 2003, Poff 2009), but are increasingly threatened by water demands, landscape changes, and climate change (Postel and Richter 2003, Palmer et al. 2008). Alterations of natural flow regimes can have profound effects on stream geomorphology, habitat complexity (Johnson et al. 1997, Infante 2006, Roth et al. 2006), fish, macroinvertebrate, and riparian vegetation (reviewed in Poff and Zimmerman 2010 and Webb et al. 2013). Therefore, maintaining natural flow patterns is recognized as a priority in water management (Poff et al. 2010).

Environmental flows are defined by the Brisbane Declaration (2007) as the “quantity, quality, and timing of water flows required to sustain freshwater and estuarine ecosystems and the human livelihoods and well-being that depends of these ecosystems”. The idea of environmental flows accounts for the importance of natural flow regimes for sustaining biota, as well as the fact that humans are dependent on water resources. To implement environmental flows, managers must be able to determine flow standards timely and cost effectively at regional spatial scales. Determining flow standards is difficult, as it is based on both quantifying and classifying flow patterns and linking flow patterns to ecological processes (Arthington et al. 2006). A recent framework, the Ecological Limits of Hydrologic Alteration (ELHOA), was developed to aid in the determination and implementation of environmental flows (Poff et al. 2010). This framework is based on grouping of hydrologically similar streams, establishing an understanding of ecological structure and function across natural flow regimes, and assessing

how flow alterations change ecological structure and function (Poff et al. 2010). While there has been much development in quantifying different aspects of stream hydrology (Richter et al. 1996, Olden and Poff 2003) and classifying flow regimes (Falcone et al. 2010, Kennard et al. 2010, McManamay et al. 2012, Olden et al. 2012), the linkages between ecological structure and function to hydrology, specifically classified flow regimes, are often unknown, unclear, or site specific (Sanderson et al. 2012, Davies et al. 2014, McManamay et al. 2015). The difficulty in developing ecological-flow relationships is a major limitation of the implementation of environmental flows (Davies et al. 2014).

Trait based approaches may be useful for developing ecological-flow hypotheses and present several advantages over using taxonomic approaches (Heino et al. 2013). Trait based approaches assume that species traits converge when environmental pressures are similar (Southwood 1988). Based on this theory, categorizing species by different traits allows for the study of community assemblages across biogeographic boundaries with different regional species pools (Schluter 1986, Stutzner et al. 2004). The ability to develop ecological-flow relationships across biogeographic boundaries is important because flow regime classification schemes or management areas are likely to include multiple watersheds with different species pools. Traits are also appropriate for ecological-flow studies because they are likely to represent long-term patterns in assemblage structure (Poff and Allan 1995, Poff et al. 2006, Tedesco et al. 2008). This is important because flow regime classification is based on long term hydrologic records, not short term hydrological events (Kennard et al 2010). Trait based approaches may also be advantageous because the mechanisms driving ecological-flow relationships can be hypothesized more directly than when assessing taxonomic relationships (Southwood 1977, Poff 1997, Heino et al. 2013).

Although there are several advantages to using trait based approaches, methods to directly assess relationships between traits and environmental variables, such as hydrologic metrics, have only recently been developed. We cannot directly measure trait values associated with environmental variables; rather we measure trait values for species. When relating traits to environmental variables, we typically need to combine three different data tables: table R containing environmental variables for each site, table L containing species information (counts, presence absence, abundance, etc.) for each site, and table Q containing trait information for each species. Estimating parameters and testing significance of a matrix representing the relationships between environmental variables and traits (matrix  $\Omega$ , or the fourth-corner matrix) is referred to as the “fourth-corner problem” (Legendre and Harmelin-Vivien 1997). In many trait based studies of lotic systems, traits are represented as the proportion of species at a site exhibiting a binary type trait, or traits are represented as the average trait value of species at each site for quantitative type traits. While representing traits in this manner allow for the use of various statistical methods, information held in the site by species matrix (L) and trait by species matrix (Q) is lost. Relatively new methods (fourth-corner approach and RLQ analysis; Legendre and Harmelin-Vivien 1997 and Dolédec et al. 1996) compute matrix ( $\Omega$ ) for the direct analysis of all three tables, but have been little utilized in the study of lotic systems (but see Tall et al 2006, Díaz et al. 2008, Pease et al. 2012, and Shieh et al. 2012).

Although most studies of trait-environment relationships in streams are through indirect assessment, relationships between hydrology and community trait composition have been documented at several spatial scales. Several hydrologic metrics described the composition of the three demographic life history strategies proposed by Winemiller and Rose (1992) across the continental United States (USA; Mims and Olden 2012). Seasonality of stream flows also

explained the life history composition of fish communities across 39 drainage basins in West Africa (Tedesco et al. 2008). Life history strategies are also related to metrics capturing flow variability at intercontinental scales (Olden and Kennard 2010). While traits have been linked to components of flow regimes (i.e. hydrologic metrics), few studies have assessed relationships between traits and classified flow regimes. Hydrologic classes described trends in the trait composition of fish assemblages across the USA, but more variation in fish traits was explained by other landscape factors (ecoregions and provinces) than hydrology (McManamay et al. 2015). No studies (to our knowledge) document relationships between trait compositions and hydrologic classes at regional, management level spatial scales. Understanding the utility of trait based approaches at smaller spatial scales is important, as water resource allocation and management usually occur at these scales (state level or smaller).

Spatial autocorrelation is an important factor to consider in ecological studies, but has received little attention in the development of ecological-flow relationships. Spatial autocorrelation represents the degree of statistical dependency between random variables based on spatial relationships (Cressie 1993). Traditional geostatistics used to quantify spatial autocorrelation are based on Euclidean distance, which may not always be appropriate for streams because they are organized in hierarchical networks and connected by unidirectional flow. While models based on Euclidean distance may explain some large scale landscape patterns, they do not capture spatial autocorrelation that is dependent on processes occurring within the stream network. Some have used stream distance to qualitatively assess spatial patterns of stream processes (see examples in Ver Hoef and Peterson 2010), but traditional geostatistical models of spatial autocorrelation are not valid when Euclidean distance is replaced by stream distance (Ver Hoef and Peterson 2010). Due to the historical lack of valid models to

represent spatial autocorrelation in streams, the role of spatial factors in driving ecological patterns within a stream network, especially in relation to hydrology, has been largely ignored.

Our objective was to assess the relationships between fish traits, hydrology, and spatial patterns to aid in the development of ecological-flow relationships. Our first goal was to determine the relationship between fish traits, individual hydrologic metrics, and flow regimes at a regional, management level spatial scale. We combined RLQ and fourth-corner analysis (Dray et al. 2014) to analyze overall trends in the trait composition of fish communities across different flow regimes and test bivariate relationships between fish traits, single hydrologic metrics, trait syndromes, and groups of hydrologic metrics. Our second goal was to determine how traits are spatially auto-correlated within classified flow regime stream networks. We used spatial stream network (SSN) models to compare how fish traits are related to hydrologic metrics across different flow regimes and across all regimes combined, and to determine how fish traits are spatially structured within stream networks. For both objectives, we utilized flow metrics used in a recent regional classification of flow regimes to assess if the flow metrics used to classify flow regimes are also ecologically meaningful. This study contributes to the development of ecological-flow relationships across natural flow regimes by determining if fish traits are useful for monitoring changes in fish assemblages. Understanding the relationships between traits and hydrology in natural flow regimes further aids in predicting ecological responses to altered hydrologic regimes across regional, management level spatial scales. In addition, this study is one of the first to consider spatial autocorrelation across stream networks in relation to environmental-flows research.



## Methods

### *Fish Assemblage Data*

We used pre-existing assemblage data from sites located in the Ozark Mountains, Boston Mountains, Arkansas Valley, Ouachita Mountains, and South Central Plains of Arkansas (Figure 1) compiled in the Arkansas GAP Analysis (ARGAP) dataset (MORAP 2009). The ARGAP dataset includes point observations of fish species at over 7,700 sampling points located over 3,700 stream segments across Arkansas. The point observations were collected by government agencies, academic institutions, and museums between 1927 and 2009 using a variety of sampling methods (electrofishing, seines, rotenone, visual counts, or other methods). Due to the variety of collection methods and sampling periods, we only included whether a species was present or absent at each site in our assemblage dataset.

In order to calculate hydrologic metrics, determine flow regime classification, calculate landscape variables associated with hydrologic alteration (see Site Selection), and model spatial autocorrelation within the stream network, point observations had to line up with stream segments included in the National Hydrology Dataset (NHD Plus, Version 2; McKay et al. 2002). The ARGAP point observations were associated with stream segments in a network custom built by delineating several digital elevation models (DEMs; MORAP 2009) that did not align with NHD Plus. We used the Snap Points to Features tool (GME 0.72; Beyer, 2012) with a snap tolerance of 30m to align point observations with the NHD Plus stream segments. To validate point observations were snapped to the correct stream segment, we compared drainage areas calculated from the NHD Plus DEM of pre and post snapped points. Incorrectly snapped points were manually moved to the correct stream segment, and points in which the correct stream segment could not be identified were dropped from analysis. Observation points within the

NHD Plus stream network were then moved to the most downstream node of each segment. Presence/absence data for all observation points were combined at each node, so each site observation point represented all species present or absent at all sampling locations along the stream segment. Observations were compiled in this manner so the scale of the assemblage data matched the hydrologic data (stream segments).

### *Site Selection*

Because we were interested in associations between fish traits and natural flow regimes, we only included sites with minimal flow alteration. We selected six variables (total road density, developed land cover, pasture and cropland cover, dam density, dam storage, and density of National Pollutant Discharge Elimination Systems) to set criteria for selecting sites with minimal flow alteration. We choose these metrics because they are included in the calculation of the Hydrologic Disturbance Index (Falcone et al. 2010), represent potential drivers of flow alteration in the study area, and may affect fish assemblages through other mechanisms in addition to flow alteration. Only sites with values lower than the median for all six metrics were kept for analysis. We further reduced sites to those sampled after 1980 to keep data contemporary. Because all sites were not sampled the same number of times, we only included those sampled at least three times and with more than three species present (n=286, Figure 1) to minimize bias due to sampling intensity. We chose three species as a cutoff based on our knowledge of the study region, where less than three species is uncommon in small headwater streams (D. D. Magoulick and L.A. Bruckerhoff pers. obs).

### *Hydrologic Data*

Hydrologic metric calculation and flow regime classification were based on the classification scheme and hydrologic modeling framework of Leasure et al. (2016). We grouped

the seven flow regime classes from Leasure et al. (2016) into three broad flow regime classes, groundwater (n=38), intermittent (n=204), and runoff (n=44) (Figure 1), due to the lack of representation of reference sites in our dataset across all seven flow regimes. Groundwater streams on average have less than two low flow spells (flow less than 5% mean daily flow) per year, never experience complete drying, and experience less variability in flows than runoff or intermittent streams. Runoff streams average more low flow spells, lower base flows, and more zero flow days per year than groundwater streams, but less daily flow variability than intermittent streams. Intermittent streams are characterized by relatively small drainage areas, more variability than both runoff and groundwater streams, and extreme drying lasting up to three months each year.

### *Fish Traits*

We used the Fish Traits database (Frimpong and Angermeier 2009) to assign trait values to fish species. Several species were not included in the Fish Traits database, or had incomplete trait data. For these species (*Etheostoma artesia*, *Etheostoma fragi*, *Etheostoma uniporum*, and *Percina fulvitaeria*), we assigned trait values first by searching the literature for trait information. If information for a species was not available, we used trait information from the closest phylogenetic relative (based on the most recent cladogram available) to fill in missing data.

We included reproductive and life history traits in our analysis (Table 2). Although the Fish Traits database includes a variety of trophic, life history, reproductive, habitat preference, and other traits, we choose to only focus on life history and reproductive traits because broad trends between these traits and hydrology are documented at large spatial scales (Olden and Kennard 2010, Mims and Olden 2012, McManamay et al. 2015). Species with common ancestry likely share similar traits, so it is necessary to account for phylogenetic effects to ensure

independence of traits among species (Gittleman and Luh 1992). We used generalized linear models (Gaussian, poisson, and binomial) to remove variation in traits described by phylogeny (McManamay et al. 2015). We used family number (Nelson 2006), which describes the phylogenetic position of families relative to other families (Frimpong and Angermeier, 2009) to represent phylogenetic relationships in the models. We used deviance residuals from all trait models in all following analyses to represent trait values corrected for phylogeny (McManamay et al. 2015).

We assigned each species to one of the three life history strategies described by Winemiller and Rose (1992). Because we did not have all the data necessary to assign species to life history strategies in the same manner as Winemiller and Rose (1992), we calculated the Euclidean distance between each species in multivariate space and endpoints representing the three life history strategies (equilibrium, periodic, and opportunistic). We compared life history strategy assignments based on endpoints described in McManamay et al. (2015) and Mims et al. (2010). For example, the opportunistic endpoint was defined by Mims et al. (2010) using minimum fecundity, minimum juvenile investment, and minimum maturation size, and McManamay et al. (2015) defined the opportunistic endpoint as the maximum value for serial spawning and season length, and minimum values for maximum length, age at maturation, longevity, fecundity, and parental care. Assignments to life history groups were the same for 61% of species using both endpoints. For species in which assignments were not the same, we used life history strategy classifications presented in other published works or our knowledge of the local fish fauna to make the final life history classification.

### *Statistical Analysis*

We utilized two analytical methods to investigate the relationships between fish traits and hydrology: combined RLQ and fourth-corner analysis and SSN modeling. We used RLQ and fourth-corner analysis to examine relationships between single traits, groups of traits, single hydrologic metrics, and groups of hydrologic metrics while preserving information about community composition and species traits at each site. SSN models were used to analyze the relationships between each trait and hydrologic metrics while accounting for different patterns of spatial autocorrelation within the stream network. We completed all analyses separate for each flow regime, as well as on all hydrologic regimes combined to address how ecological flow relationships differ across flow regimes. We used the 11 flow metrics used to classify flow regimes in the study region (Leasure et al. 2016, Table 1) as explanatory variables in analyses. This set of 11 metrics was reduced to five (MA4, FH7, DL4, TA1, RA3; Table 1) after metrics that were highly correlated at our sites were removed to avoid multicollinearity in models. All hydrologic metrics were  $\log(x + 1)$  transformed to improve normality.

### *RLQ and Fourth-Corner Analysis*

We combined RLQ and fourth corner analysis (Dray et al. 2014) to examine the relationships among three data tables: table R containing 5 hydrologic metrics for 286 sites, table L containing 102 species for 286 sites, and table Q ( $s \times t$ ) containing 6 traits (corrected for phylogeny) for each species. We choose to only include age at maturity, season, and spawning classification (open spawners, brood hiders, open guarders, and nest guarders) in this analysis. We included age at maturity because it was highly correlated with maximum total length, fecundity, and longevity. Substrate indifferent spawners were not included in this analysis because only one species, *Gambusia affinis*, exhibited this spawning mode. We did not include

life history strategies in the analysis to avoid redundancy because the assignment to life history groups was determined by trait values included in this analysis.

RLQ analysis involves the separate ordination of each table, and then combining these separate analyses to identify the main relationships between trait syndromes and environmental variables (Dray et al. 2014). Using the relationships between hydrologic metrics and fish traits, as well as fish traits and species occurrences developed by separate analysis of each matrix, RLQ calculates a new matrix ( $\Omega$ ) describing the main association between traits and metrics (Dray and Legendre 2008). Fourth corner analysis tests for significant bivariate relationships between single traits and single metrics. These relationships are based on cells in matrix  $\Omega$ . Both of these approaches were combined using the framework suggested by Dray et al. (2014) to allow further comparisons between trait syndromes and groups of hydrologic metrics. Analysis was performed in R (R Core Team 2014) using the ade4 package (Dray and Dufour 2007).

We performed RLQ analysis to assess and visualize broad relationships between hydrologic gradients and trait syndromes in each flow regime and across all flow regimes combined. Correspondence analysis (COA) was performed on the species table (L). The row and column weights from this test were then used in subsequent analysis to weigh sites by the number of species observed and species by the number of sites in which they were observed. The hydrologic metric table (R) and traits table (Q) were then analyzed by PCA weighted by species and sites (derived from COA). RLQ analysis was then performed on the scores of these three separate ordinations. We computed multivariate significance tests based on the total co-inertia of the RLQ analysis using Monte Carlo tests (49999 permutations using Model 6 framework described below).

Fourth corner analysis was then used to assess bivariate relationships among single traits and single hydrologic metrics. As recommended by Dray et al. (2014), randomization procedures followed the Model 6 framework (Braak et al. 2012). Model 6 is a method of controlling for type I error rates during randomized permutations to deal with the different statistical units. Permutations were performed on the sites to test the null hypothesis that the distribution of species is not influenced by hydrologic variables when holding traits constant. Permutations were then performed on the species to test the null hypothesis that species compositions are not influenced by species traits when holding hydrologic variables constant. Each permutation test was sequentially corrected using the false discovery rate method. We used a large number of permutations ( $n=49999$ ) to have adequate power in the corrected tests (Dray et al. 2014). Relationships between traits and hydrologic metrics were considered significant if the largest p-value of the two permutation tests (Model 2 and Model 6) was lower than  $\alpha$  (0.05). Correlation coefficients were used to measure the strength of the relationships between each trait and each metric.

RLQ and fourth-corner analysis were then combined by using the fourth-corner method to test for associations between individual traits and sample scores derived from RLQ (representing groups of hydrologic metrics) and individual hydrologic metrics and species scores derived from RLQ (representing trait syndromes). As with the bivariate fourth-corner analysis, we used the Model 6 framework for randomization tests and false discovery rate method to test significance (Dray et al. 2014).

### *Spatial Stream Network Models*

New valid geostatistical methods are available to model spatial autocorrelation in stream networks (Cressie et al. 2006, Ver Hoef et al. 2006). SSN models are based on moving average

constructions and use multiple covariance matrices representing different spatial patterns in stream networks, such as branching, connectivity, and flow direction (Ver Hoef et al. 2006, Peterson and Ver Hoef 2010). These different spatial patterns are captured in three covariance models: “tail-up”, “tail-down”, and classic Euclidean distance. Tail-up covariance matrices are based on hydrologic distance between sites and only allow spatial autocorrelation to occur between flow connected sites (water must move from one site to the other). Tail-up models also include spatial weights assigned to each confluence to account for differences between stream segments, such as watershed area or discharge. Tail-down covariance matrices are also based on hydrologic distance, but they allow correlation between flow un-connected sites (within the same network, but not connected by flow) as well as flow -connected sites. Combining three covariance structures allows models to simultaneously account for multiple spatial patterns within stream networks and across the landscape (Peterson and Ver Hoef 2010).

We used a mixed moving-average SSN models (Peterson and Ver Hoef 2010, Ver Hoef et al. 2006, Cressie et al. 2006) to determine the relationship between hydrologic metrics and fish traits, and the degree of spatial autocorrelation in the distribution of fish traits at multiple spatial scales. We developed mixed models for age at maturity, season, spawning characteristics (open spawners, brood hiders, open substrate guarders, indifferent spawners, and nest guarders), and life history strategies (equilibrium, periodic, and opportunistic) for each flow regime and all sites combined. Each model included all three classes of spatial covariance structures (TU, TD, and EUC) and five hydrologic metrics:

$$\mathbf{y}_{\text{trait}} = \mathbf{X}_{\text{hydrology}}\boldsymbol{\beta} + \sigma_{\text{EUC}}\mathbf{z}_{\text{EUC}} + \sigma_{\text{TD}}\mathbf{z}_{\text{TD}} + \sigma_{\text{TU}}\mathbf{z}_{\text{TU}} + \sigma_{\text{NUG}}\mathbf{z}_{\text{NUG}} \quad (1)$$

where matrix  $\mathbf{X}$  contains the five hydrologic metrics, vector  $\boldsymbol{\beta}$  describes the relationship between the hydrologic metrics and the trait,  $\mathbf{z}_{\text{EUC}}$ ,  $\mathbf{z}_{\text{TD}}$ ,  $\mathbf{z}_{\text{TU}}$ , and  $\mathbf{z}_{\text{NUG}}$  are matrices containing the



autocorrelation values for the EUC, TD, and TU models and nugget effect, and  $\sigma_{\text{EUC}}$ ,  $\sigma_{\text{TD}}$ ,  $\sigma_{\text{TU}}$ , and  $\sigma_{\text{NUG}}$  are the variance components (Peterson and Ver Hoef 2010). We included a mix of covariance structures as recommended by Peterson and Ver Hoef (2010) to account for multiple spatial patterns of autocorrelation and to allow for more autocorrelation among flow-connected sites. All spatial models were executed using the SSN (Ver Hoef et al. 2014) package in R (R Core Team 2014).

We used the STARS toolset (Peterson and Ver Hoef 2014) to generate and format feature geometry, attribute data, and topological relationships necessary for SSN modeling. We created a landscape network (LSN) from the National Stream Internet (NSI; Nagel et al. 2015) dataset, which includes a reconditioned version of the flowlines (stream lines) from NHDPlusV2 (McKay et al. 2002). The reconditioned flowlines do not include any uninitialized features (isolated stream segments, canals or ditches, braided channels), diverging stream segments, converging streams, complex confluences, outlets or sinks, or other types of topological errors that are not permitted in SSN modeling. We used the STARS toolset to snap our point observation data to the LSN, calculate upstream distances among edges and sites, calculate spatial weights based on watershed area, calculate the additive function values, and create a Spatial Stream Network (.ssn) object. The .ssn object contained the spatial, attribute, and topological information needed to create the mixed moving-average SSN models.

We included the same traits included in the RLQ and fourth-corner analysis as response variables in our models. Because we create separate spatial models for each response variable, we also included the three life history strategies and substrate indifferent spawners in our analysis. All trait values were average deviance residuals for each trait, and the life history strategies were represented at each site by the proportion of species in each group. We created

spatial models separately for each flow regime and combined across all sites for each of the 11 response variables, for a total of 44 models.

There are a variety of different models available for the EUC (Gaussian, exponential, spherical, Cauchy), TU (linear sill, Epanechnikov, Mariah, exponential, spherical), and TD (linear sill, spherical, exponential, Epanechnikov, Mariah) covariance structures. Descriptions of exponential, linear sill, spherical, and Mariah models are described in Ver Hoef and Peterson (2010) and Epanechnikov models are described in Garreta et al. (2010). We compared all combinations of the covariance structures (100 models) for each response variable across each flow regime and for all regimes combined. Because all models had the same number of parameters, we selected covariance structures for the final models (shown in Table 3) by selecting the models with the lowest root-mean-square-prediction error (RMSPE). Because the models included variance components and all models included the same fixed effects, all final models were fixed using restricted maximum likelihood (REML) for parameter estimation. Diagnostic plots of residuals were used to check assumptions of normality, influence of outliers, and leverage of observations, and scatterplot matrices and variance inflation factors (VIF) were used to assess multicollinearity. Open substrate guarders and substrate indifferent spawners were removed from analysis due to violations of assumptions.

## **Results**

### *Summary of fish traits*

The final assemblage dataset included 102 species from 14 different families across the 286 reference sites. Species exhibited a range of traits with 40 open spawners, 25 brood hidiers, 2 open guarders, 35 nest guarders, and 1 substrate indifferent spawner. Spawning season ranged 1-6.5 months, and age at maturity ranged from 1- 5 years of age. Trait average deviance (corrected

by family number) varied within and between flow regimes, but there were no noticeable differences in most trait values between flow regimes (Figure 2). A notable exception is greater age at maturity and lower length of spawning season in runoff streams relative to groundwater and intermittent streams. Forty-two species were classified as equilibrium strategists, 31 as periodic strategists, and 58 as opportunistic strategists. The proportion of equilibrium strategists per site ranged from 1-100%, the proportion of periodic strategists ranged from 0-50% per site, and the proportion of opportunistic strategists ranged from 0-88%. The proportion of life history strategists was similar across regimes, with equilibrium and opportunistic strategists dominating at most sites (Figure 2).

#### *RLQ and Fourth-Corner*

Separate RLQ analyses for each flow regime and for all regimes combined revealed weak relationships between traits and hydrologic metrics. The first axis accounted for 76, 83, 87, and 81% of the total co-inertia between traits and hydrologic metrics, while the second axes accounted for 14, 15, 7, and 15% for groundwater, runoff, intermittent, and all streams respectively (Table 4). These axes represented only 12-15% of the correlation described by the first axis in all COAs, 61-80% of the variance retained in the first axes of the trait PCAs, and 84-94% of the variance retained in the first axes of the hydrology PCAs (Table 4). Total co-inertia was low for all flow regimes, ranging from 0.04 to 0.08 and the multivariate relationships between traits and hydrologic metrics were not significant in any flow regime or across all regimes combined (Table 4).

Fourth corner-analysis of individual traits and individual hydrologic metrics revealed no significant bivariate relationships in any flow regime, but fourth corner-analysis of individual hydrologic metrics and trait axes derived from RLQ analysis did reveal significant relationships

in runoff and intermittent streams, as well as across all flow regimes combined. In runoff streams, there was a significant positive relationship between DL4 and the first trait axes from RLQ analysis (Model 2 p-value: 0.005, Model 4 p-value: 0.047). The first axis from the trait PCA had the strongest correlation with the first RLQ axis (Table 5). Brood hidiers and open guarders had the highest negative loadings on this axis (-0.595 and -0.407 respectively) and nest guarders had the highest positive loading on this axis (0.577, Table 6, Figure 3). In intermittent streams, MA4 had a significant negative relationship (Model 2 p-value: 0.003, Model 4 p-value: 0.031) and TA1 had a significant positive relationship (Model 2 p-value: 0.008, Model 4 p-value: 0.042) with the first trait RLQ axis. The first axis from the trait PCA was positively correlated with the first RLQ axis (0.741), and brood hidiers (positive relationship) and open spawners (negative relationship) had the highest loading on this axis (Table 6, Figure 4). The second trait PCA axis was negatively correlated with the first RLQ axis (-0.534, Table 5). Open guarders had the strongest positive loading (0.808) on the second PCA axis and brood hidiers had the strongest negative loading (-0.412) on this axis (Table 6, Figure 4). Across all flow regimes, MA4 had a significant negative relationship (Model 2 p-value: 0.003, Model 4 p-value: 0.025) with the first trait axis from RLQ. The first trait PCA axis was positively correlated (0.630, Table 5) with the first RLQ axis, and brood hidiers and open guarders having the highest positive loadings (0.409 and 0.514 respectively, Table 6, Figure 5). The second trait PCA axis was negatively correlated with the first RLQ axis (-0.494, Table 5). Season had the highest positive loading on this axis (0.611) followed by brood hidiers (0.468). Open guarders had the strongest negative relationship (-0.493) with the second trait axis (Table 6, Figure 5).

### *Spatial Stream Network Models*

Spatial covariance components described more variability than hydrologic metrics in all models (Table 7, Figure 6). The amount of variability described by the different covariance structures varied between flow regimes, between traits within flow regimes, and within traits between flow regimes (Figure 6). TU models described the largest proportion of variability in groundwater streams, while a mixture of TU and EUC models accounted for the most variability in intermittent streams and across all flow regimes combined. EUC models described the most variability in runoff streams, although there was a large amount of unexplained variance for three traits. In groundwater streams, TU models described the most variability for season, equilibrium strategists, opportunistic strategist, and nest guarders, EUC models described the largest proportion of variance for periodic strategists, open spawners, and brood hidiers, and TD models described the largest proportion of variance for age at maturity. EUC models described the most variability in runoff streams for age at maturity, season, periodic strategists, and brood hidiers. TD models described the largest proportion of variability for open substrate spawners, while neither spatial components nor hydrology explained more variability than the nugget for opportunistic and equilibrium strategists and nest guarders in runoff streams. In intermittent streams, EUC models described the most variation for age at maturity, season, and brood hidiers, while TU models described the largest proportion of variance for all three life history strategists. Across all flow regimes combined, TU models described the most variability for age at maturity, equilibrium strategists, and opportunistic strategists, while EUC distance had the most explanatory power for season and brood hidiers.

Fish life history and spawning traits exhibited different linear relationships with hydrology across flow regimes, although the generalized  $R^2$  values were low across all models (0.010- 0.450, Table 7, Figure 7). Age at maturity had a positive relationship with DL4 in

groundwater streams ( $T= 4.648$   $p\text{-value} < 0.001$ , Figure 7f) and across all regimes combined ( $T= 2.922$   $p\text{-value}= 0.004$ , Figure 7g). In groundwater streams, age at maturity also had a positive relationship with FH7 ( $T= 2.089$ ,  $p\text{-value}= 0.045$ , Figure 7e) and negative relationship with RA3 ( $T= -2.311$ ,  $p\text{-value}= 0.027$ , Figure 7g). The length of spawning season had a significant positive relationship with MA4 ( $T= 2.521$ ,  $p\text{-value}= 0.012$ , Figure 7i) and significant negative relationship with RA3 ( $T= -2.006$ ,  $p\text{-value}= 0.046$ , Figure 7j) across all regimes. The proportion of equilibrium strategists had a positive relationship ( $T= 2.924$ ,  $p\text{-value}= 0.006$ , Figure 7k) with DL4, while the proportion of opportunistic strategists had a negative relationship ( $T= -2.773$ ,  $p\text{-value}= 0.009$ , Figure 7l) with DL4 in groundwater streams. Across all regimes combined, the proportion of opportunistic strategists had a negative relationship with FH7 ( $T= -2.696$ ,  $p\text{-value}= 0.007$ , Figure 7m). Periodic strategists only had a significant relationship in runoff streams, exhibiting a positive relationship with DL4 ( $T= 3.213$ ,  $p\text{-value}=0.003$ , Figure 7n). Open substrate spawners had a significant positive relationship ( $T= 2.171$ ,  $p\text{-value}= 0.031$ , Figure 7o) with MA4 across all flow regimes combined. Brood hidiers had significant positive relationships with MA4 ( $T= 2.675$ ,  $p\text{-value} = 0.011$ , Figure 7c) in runoff streams, but a negative relationship with MA4 ( $T= -2.793$ ,  $p\text{-value}= 0.006$ , Figure 7a) in intermittent streams. Brood hidiers also had a negative relationship with DL4 ( $T= -2.672$ ,  $p\text{-value}= 0.008$ , Figure 7b) in intermittent streams and across all flow regimes ( $T= -3.049$ ,  $p\text{-value}= 0.003$ , Figure 7d). Nest guarders exhibited a positive relationship with DL4 across all flow regimes combined ( $T= 2.805$ ,  $p\text{-value} = 0.005$ , Figure 7p).

## **Discussion**

We assessed the relationships between traits and the hydrologic metrics used to classify flow regimes at a regional scale to compare relationships between fish traits and hydrologic metrics across flow regimes. We also addressed the role of spatial autocorrelation in driving

these relationships and compared how spatial patterns in fish traits differ across flow regimes. The two analytical methods we used have rarely been utilized in stream ecology, especially in ecological flows research. These analytical approaches both indicated that relationships between hydrologic metrics and fish traits differ across flow regimes, but these relationships are weak in intermittent streams and across all flow regimes combined. Interestingly, fish traits and life history strategies exhibited stronger relationships with hydrologic metrics in groundwater and runoff streams than in intermittent streams. Fish traits also exhibited different spatial autocorrelation patterns across flow regimes. The combined weak relationships between traits and metrics and large amount of variance explained by spatial models indicate that other factors, such as landscape variables or in-stream processes, need to be considered when developing ecological-flow hypotheses.

Both combined RLQ and fourth-corner analysis and SSN models indicated two hydrologic metrics are important predictors of fish trait distributions in our study system. Magnitude of 30 day minimum flows (DL4) and variability of daily flows (MA4) had more significant relationships with fish traits than any other metric included in this study. DL4 was important across all streams combined and in all flow regimes for six traits, including all three life history strategies, age at maturity, brood hiders, and nest guarders. These relationships may indicate that magnitude of extended minimum flows is an important driver of fish community structure across all flow regimes throughout the study area and that low flow conditions are important regardless of flow regime. MA4 had relationships with spawning traits, including length of spawning season, brood hiders, and open spawners. The relationships spanned across runoff and intermittent streams and were apparent across all flow regimes combined, but there were no relationships with MA4 in groundwater streams. Variability of daily flows may not be

an important driver of fish community structure in groundwater streams, where flow conditions are relatively stable, but is important in runoff and intermittent stream that experience higher flow variability.

Combined RLQ and fourth-corner analysis provided visualization of differences in community structure across flow regimes and revealed complex relationships between hydrology and fish traits. While we did not observe any significant bivariate relationships, we did observe significant relationships between trait axes representing groups of traits and single hydrologic metrics (DL4, MA4, and TA1) in runoff and intermittent streams, as well as across all flow regimes combined. While DL4 and MA4 were also important metrics in SSN models, the significant relationship between TA1 (constancy) and fish traits was only revealed in combined RLQ and fourth-corner analysis. The significant trait axes derived from RLQ analysis represent combinations of traits with different (positive or negative) relationships with flow metrics. As with other ordination based analyses, these relationships are difficult to interpret. While it is difficult to untangle the relationships between the metrics and fish trait axes, these relationships help identify flow metrics that are important in structuring fish communities. The significant relationships of trait axes with hydrologic metrics also supports the use of trait syndromes, such as life history strategies, when developing ecological-flow hypotheses.

Fish can be classified based on life history traits following the tri-continuum model proposed by Winemiller and Rose (1992). The tri-continuum represents trade-offs between parameters of survival, fecundity, and the onset and duration of reproduction (Winemiller 1989, Blanck et al 2007), and the model was extended to relate life history strategies with disturbance regimes (Winemiller 2005). Periodic strategists mature slowly, have large clutch sizes, high fecundity with low larval and juvenile survivorship, and often have synchronous spawning



events. Periodic strategists are predicted to inhabit environments with large scale, predictable temporal variation, and reproductive events coincide with periods of favorable conditions for growth and survival (Boyce 1979). Opportunistic strategists mature early and have rapid larval growth. These fish also have small clutch sizes, but reproduce frequently and often have extended spawning seasons, leading to rapid population turnover rates. Opportunistic strategists are predicted to live in highly disturbed and variable systems in which they can quickly repopulate after disturbance. Equilibrium strategists can be considered traditional “k” strategists (Pianka 1970), as they are predicted to be resource limited and influenced by density dependent factors. Equilibrium strategists have large eggs, small clutches of robust juveniles, and practice parental care.

Most of the relationships between hydrologic metrics and life history strategies observed in the current study are consistent with previous studies and predictions based on life history theory. Opportunistic and equilibrium strategists exhibited opposite responses to the magnitude of 30 day low flows in groundwater streams, with opportunistic strategists having a negative relationship and equilibrium strategists having a positive relationship. Based on our results, the proportion of opportunistic species is predicted to increase, while the proportion of equilibrium strategists will decrease with lower, more dramatic 30 day low flow events in groundwater streams. This decoupling of responses between equilibrium and opportunistic strategists was also observed by McManamany and Frimpong (2015) across multiple hydrologic metrics for fish communities in the US. This relationship is also consistent with predictions of the model proposed by Winemiller (2005), which predicts opportunistic strategists to occupy disturbed, harsh conditions and equilibrium strategists to be less tolerant to disturbance. Inconsistent with predictions based on life history theory and previous observations (Mims and Olden 2012),

opportunistic strategists also had a negative relationship with the frequency of high flow events across all flow regimes. Although opportunistic strategists are predicted to favor harsh environments, the negative relationship with the frequency of high flow events may indicate that opportunistic strategists are not able to handle extreme conditions of all components of the flow regime. Periodic strategists only exhibited a significant relationship with a hydrologic metric (DL4) in runoff streams, which experience moderate levels of variability driven by predictable, seasonal changes in flow. Life history theory predicts periodic strategists favor predictably variable environments, such as the runoff streams in the current study. Although hydrology was important for periodic strategists in runoff streams, we did not observe any relationships with metrics related to timing or predictability of flow events as would be expected based on life history theory. We also did not observe any significant relationships between flow metrics and life history strategies in intermittent streams, and only equilibrium and opportunistic strategists exhibited relationships in groundwater streams

While most of the relationships between life history strategies and hydrology fit predictions based on life history theory, we observed complex relationships between hydrology and fish traits across flow regimes. Age at maturity exhibited the strongest relationship with hydrology in groundwater streams. The negative relationship between age at maturity and fall rate and positive relationship with duration of minimum 30 day flows (which also held true across all flow regimes) support predictions based on life history theory, as slower growing, long lived species are expected to inhabit less variable environments. Length of spawning season only exhibited significant relationships with hydrology across all sites combined. The lack of relationships between hydrology and length of spawning season in classified flow regimes may be due to regional environmental differences influencing spawning season. In our spatial models,

Euclidean distance described a large (45-94%) proportion of variance in intermittent and runoff streams, and across all flow regimes combined. Similarly, McManamay et al. (2015) found length of spawning season had a higher affiliation with regions than hydrology across the USA, with length of spawning season varying along a longitudinal gradient. Spawning modes also exhibited mixed relationships with hydrology. As with length of spawning season, nest guarders and open spawners only exhibited relationships with hydrology across all flow regimes combined. Brood hiders consistently had a negative relationship with duration of minimum 30 day flows, but opposite relationships with variability in daily flows in runoff and intermittent streams.

The observed opposite trait and life history strategy responses to hydrology across flow regimes and importance of different metrics across flow regimes may have important consequences for the development of ecological-flow relationships. Many documented flow-ecology relationships are based on changes in community structure after flow alteration (Freeman et al. 2001, Humphries et al. 2002, Poff and Zimmerman 2010, Webb et al. 2013, and Rolls and Arthington 2014), but few studies have compared ecological-flow relationships across classified flow regimes (except see Poff and Allan 1995, Monk et al. 2006, Chinnayakanahalli et al. 2011, Mims and Olden 2012, and McManamay et al. 2015). Similar to the current study, Mims and Olden (2012) observed predicted relationships between hydrologic metrics and fish life history strategies across all flow regimes combined. However, within classified flow regimes, Mims and Olden (2012, Appendix B) observed relationships that differed between regimes and did not fit predictions based on life history theory. While we may expect different components of flow regimes (hydrologic metrics) to have varying degrees of ecological relevance across flow regimes, opposite trait responses between flow regimes is an unexpected

result. More studies are needed to further explore these inverse relationships and develop explanations for why trait responses may differ across flow regimes.

We observed stronger relationships between hydrologic metrics and fish life history traits in groundwater and runoff streams relative to intermittent streams. This result was unexpected, as one might predict stronger relationships in intermittent flow regimes which are characterized by more hydrologic variability than groundwater streams. The lack of strong relationships between hydrology and life history traits in intermittent streams could be due to lack of distinct populations in intermittent streams. Intermittent streams in this study are largely comprised of small headwater streams that frequently experience extreme drying events. Similar to Schlosser's (1987) conceptual model, fish may move into intermittent streams to exploit previously inaccessible resources, complete life history events, or utilize refugia (Hall 1972, Schlosser 1995), but move back into stable groundwater or runoff streams during extreme high flow or drying events. Emigration out of stream reaches is positively related to intermittency in Virginia streams (Albanese et al. 2004) and high extinction rates and nested community structure are characteristic of hydrologically variable headwater streams in our study area (Taylor and Warren 2001). The fish communities typically observed in intermittent streams may represent a nested subset of communities in the groundwater and runoff streams. If this is the case, community relationships with hydrology are likely driven by conditions in runoff and groundwater streams.

Overall, the relationships between flow metrics and fish traits and life history strategies were weak and spatial covariance components described more variability than hydrology. This finding suggests that large scale landscape factors and small scale in-stream processes are important drivers of community structure and need to be included in the development of ecological-flow relationships. SSN models are useful for determining what environmental

variables may be important in addition to flow. For example, in the current study 65% of the variation in the distribution of opportunistic strategists across all flow regimes combined was described by the tail-up covariance component. Although the proportion of opportunistic strategists varied across the entire study area (Fig. 8), there is small scale clumping between flow connected sites. The large proportion of variability captured by the tail-up covariance component may suggest in-stream characteristics or processes dependent on flow connectivity and direction, such as water chemistry, sediment, or temperature, may be influencing the distribution of opportunistic strategists. The spatial weights included in tail-up models may also indicate that changes occurring within the stream network at confluences, such as changes in discharge, are also important factors influencing the distribution of opportunistic strategists. For length of spawning season in runoff streams, Euclidean distance described the largest proportion of variation (93%), indicating relatively large scale landscape factors, such as ecoregions, may be driving length of spawning season in runoff streams (Figure 9).

In addition to providing insight into potential important covariates, SSN models can help researchers understand patterns of spatial autocorrelation related to sample design and how the location of sample sites influences ecological-flow relationships. Studies utilizing compilations of existing data, such as the current study, often exhibit spatial clumping or segregation of sites, which violates assumptions of independence required for most statistical models. Failure to account for the lack of independence due to spatial autocorrelation may lead to elevated type I error rates (Legendre 1993). SSN models accommodate for the lack of independence due to spatial autocorrelation and provide a means to determine how response variables are spatially auto-correlated within stream networks. In the current study, groundwater streams were clumped in the Ouachita Mountains and tail-up models described a large proportion of variability for

several traits (length of spawning season, proportion of equilibrium and opportunistic strategists, and nest guarders, Figure 6) in these streams. In runoff streams, which were more spatially segregated across the study area, variability in several traits (age at maturity, length at spawning season, and brood hiders, Figure 6) was predominantly explained by Euclidean distance. The importance of different covariance structures between these flow regimes is likely related to the spatial location of sampling sites. The inclusion of spatial covariance components allowed the models to pick up this spatial autocorrelation due to sample locations. Accounting for spatial autocorrelation is important in environmental flows research, as hydrologic or biological data may only be available at spatially clustered sites (e.g. USGS stream gauges).

Although spatial covariance components helped explain patterns of variation in the current study, the relationships between fish traits and life history strategies and hydrologic metrics were weak. These weak relationships may be the result of a combination of the spatial scale of the study and the trait values assigned to each species. The spatial scale of flow regime classification schemes may influence ecological-flow relationships. The current study utilized a flow regime classification scheme across the Ozark-Ouachita Interior Highlands (Leasure et al. 2016). Similar studies (Mims and Olden 2012 and McManamay et al. 2015) exhibiting stronger relationships between fish trait and life history strategy distributions across flow regimes were completed at much larger spatial scales (continental USA). Not only can flow regime classification schemes vary depending on the spatial scale of classification (McManamay et al. 2012), but trait values may also exhibit less variation at smaller spatial scales. Stronger relationships between fish traits and hydrology may be observed at large spatial scales that include highly heterogeneous environments (e.g. deserts, mountains, coastal plains, temperate forests) and large species pools with high trait variation than relationships assessed at smaller

spatial scales (e.g. within temperate forests alone) with smaller species pools and potentially less trait variation. In addition, trait values assigned to each species may also affect the strength of relationships with hydrology. In the current study, each species was assigned single trait values documented in the Fish Trait database (Frimpong and Angermeier 2009). Intraspecific or regional variations in trait values were not accounted for by using this national database. Relationships between fish traits and hydrology would be more accurately assessed with measured trait values within the study area.

This study provides new important considerations for the development of ecological-flow relationships necessary for the implementation of environmental flow standards. First, some flow metrics may influence community structure within a study region regardless of flow regime classification. We identified two metrics that repeatedly exhibited relationships with fish traits. Identifying important metrics such as these may help set management priorities before or during the development of flow classification schemes. Secondly, movement between classified flow regimes should be considered when assessing ecological flow relationships. We believe fish movement may be driving the relatively strong relationships with hydrology observed in groundwater and runoff streams relative to intermittent streams in this study. The movement of individuals between stream reaches has likely not been considered in other studies developing ecological-flow relationships in classified flow regimes due to the larger spatial scale of previous studies. It may not be appropriate to consider observations of a community sampled in a tributary independent of a downstream community in a different flow regime. Third, spatial autocorrelation is important and needs to be considered in the development of ecological-flow relationships. SSN models can help describe spatial patterns due to sample design and spatial autocorrelation of hydrologic patterns or response variables. Considering ecological-flow

relationships within a spatial context may potentially improve the interpretation of observed relationships, help identify important environmental covariate, and develop new ecological flow hypotheses.

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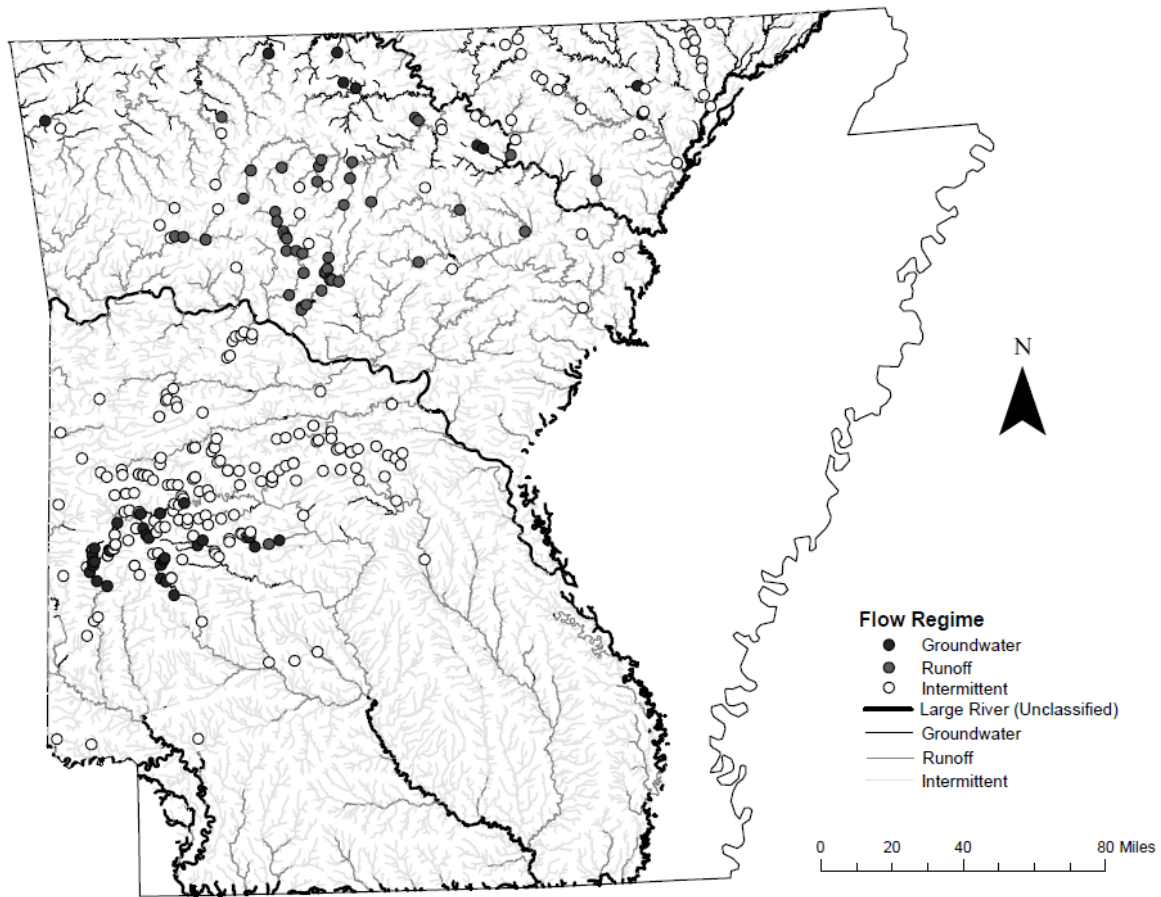
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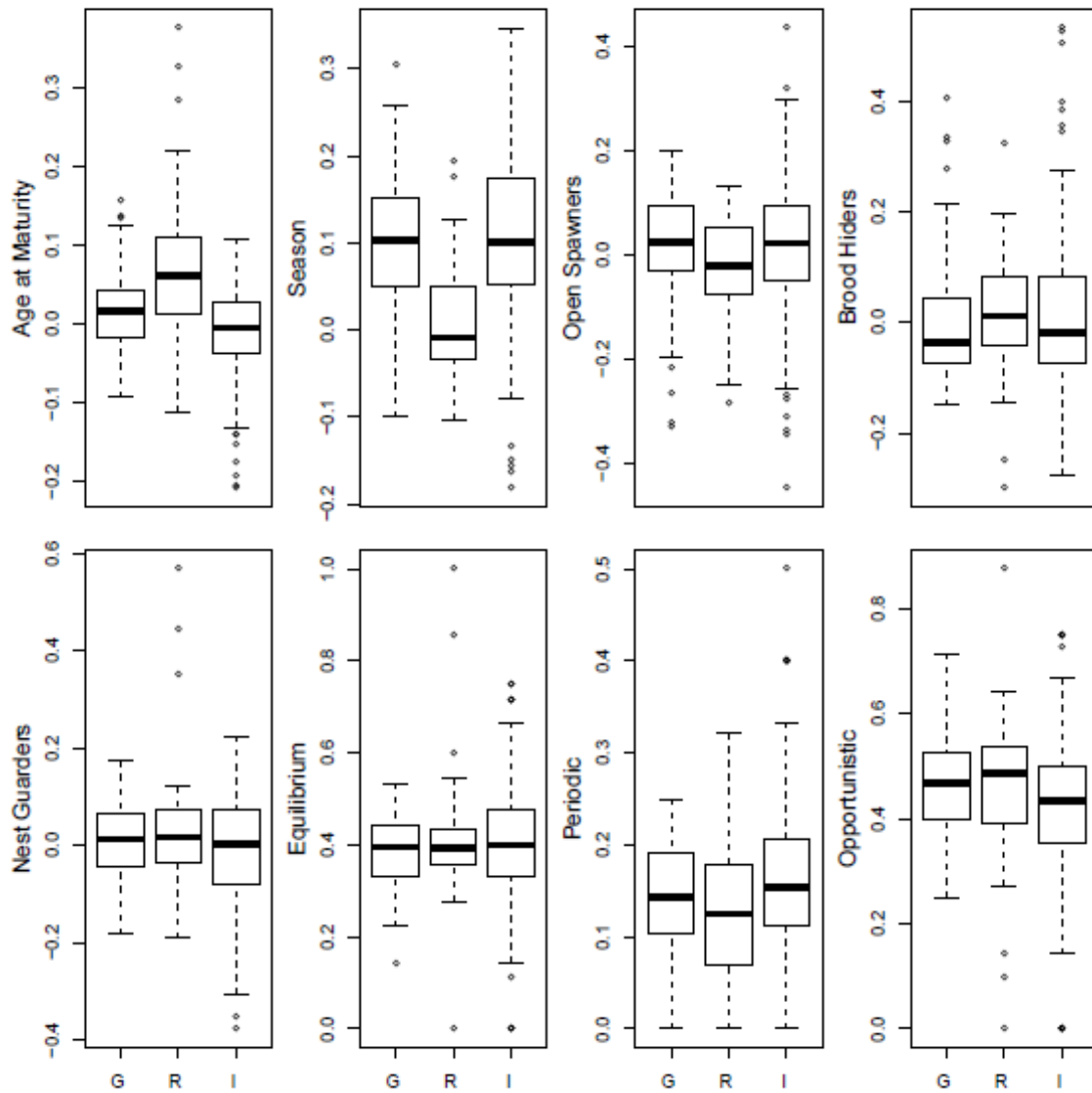
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## Tables and Figures

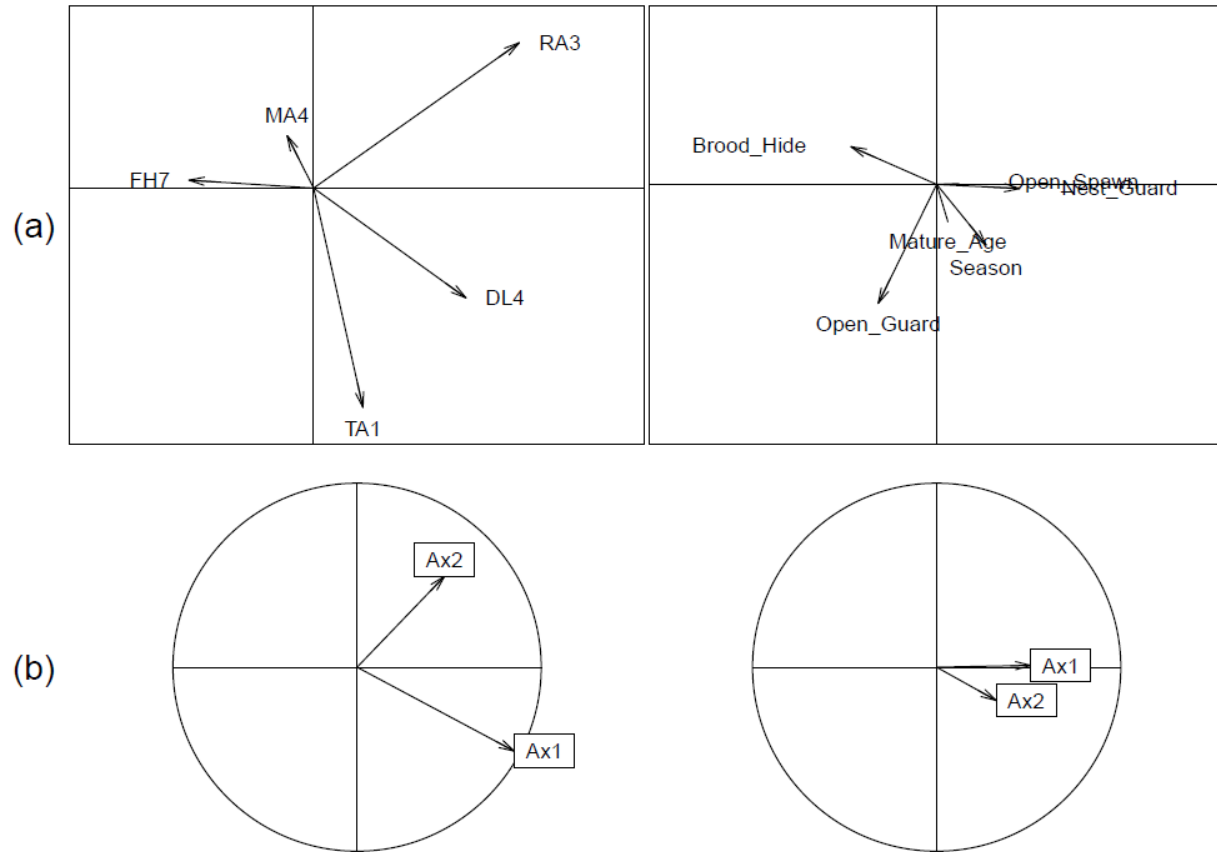


**Figure 1.** Sample locations representing unaltered or natural flow regimes in groundwater, runoff, and intermittent streams. All sample locations are located in the Ozark-Ouachita Interior Highlands of Arkansas.

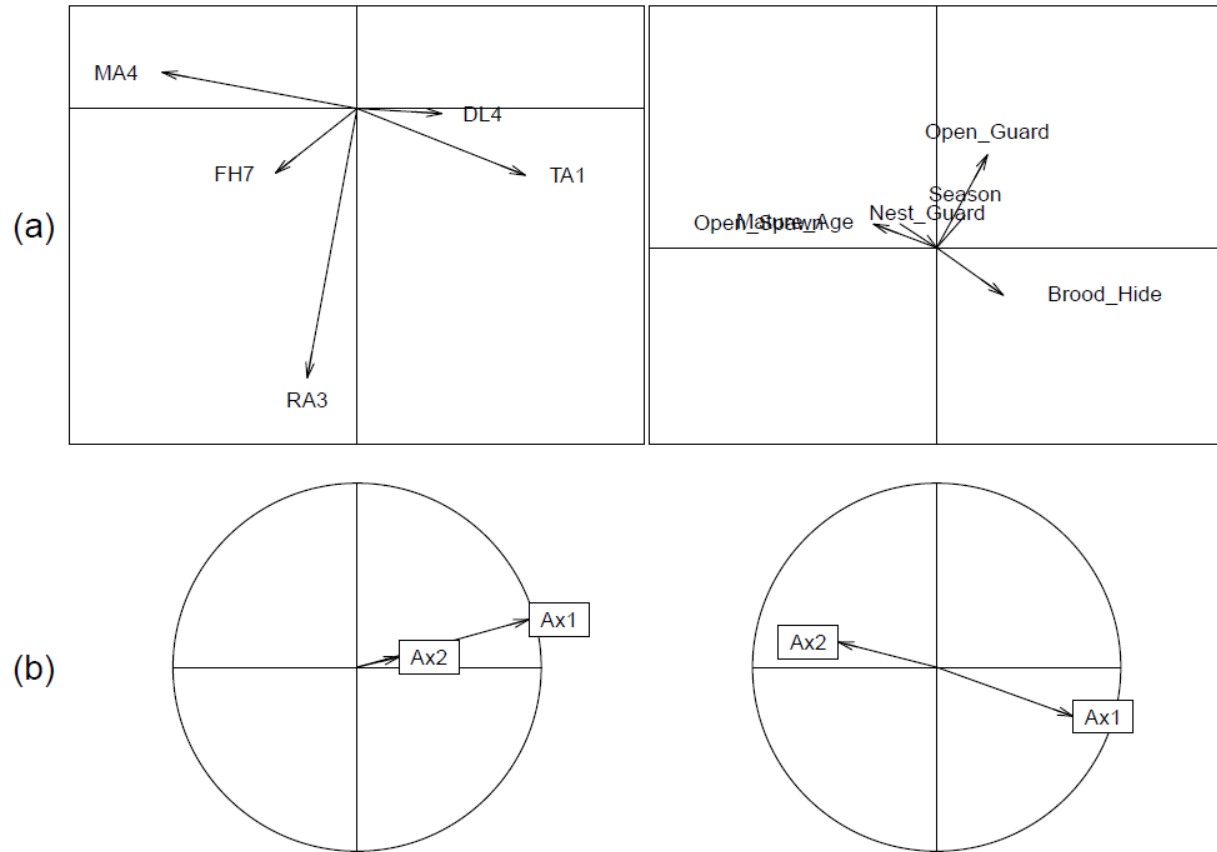


**Figure 2.** Box and Whisker plots of traits (corrected for phylogeny) and proportion of life history strategists within flow regime classes. G- groundwater streams, R- runoff streams, and I- intermittent streams.

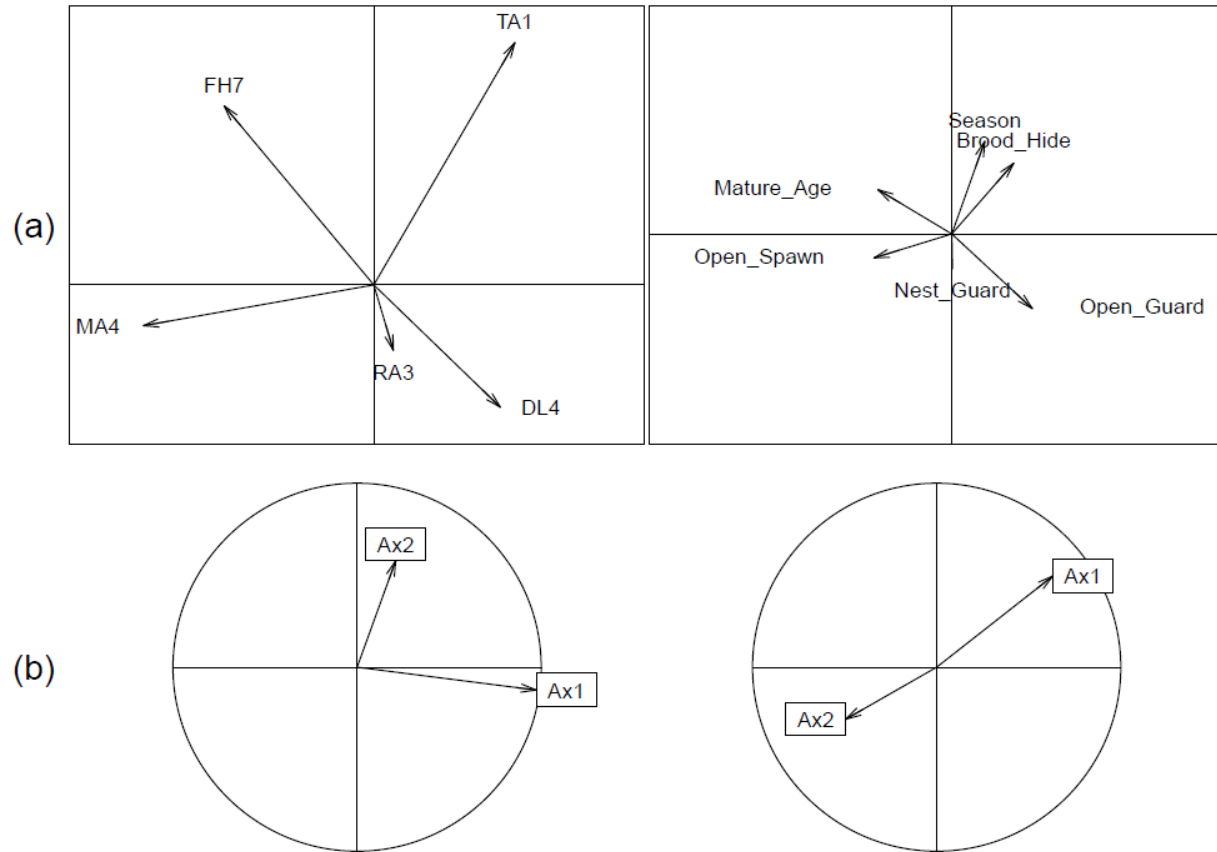




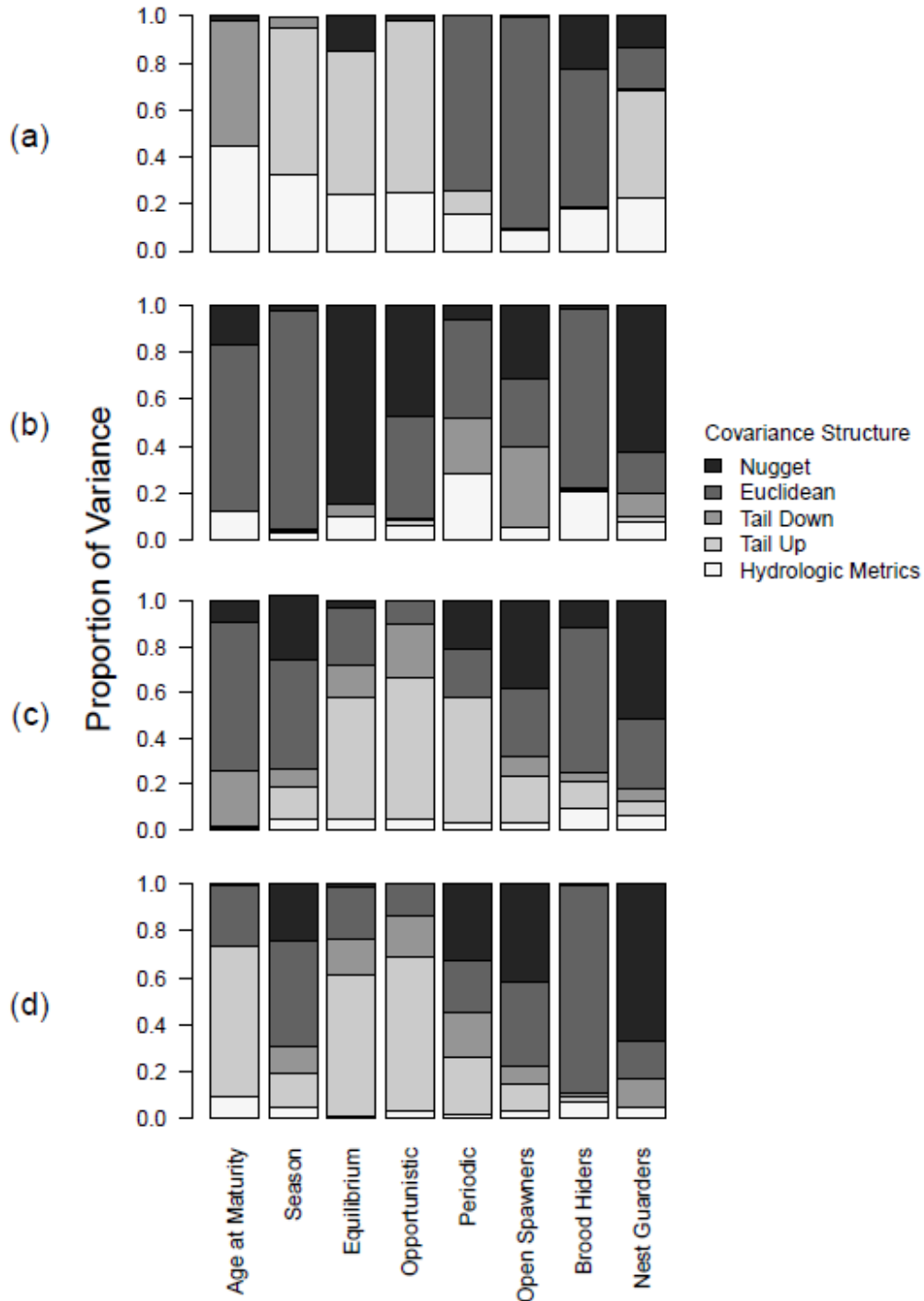
**Figure 3.** Graphical representation of the ordinations of (a) hydrologic metrics and traits and (b) RLQ axes in runoff streams. DL4 had a significant relationship with the first trait axes from RLQ (Model 2 p-value: 0.005, Model 4 p-value: 0.047).



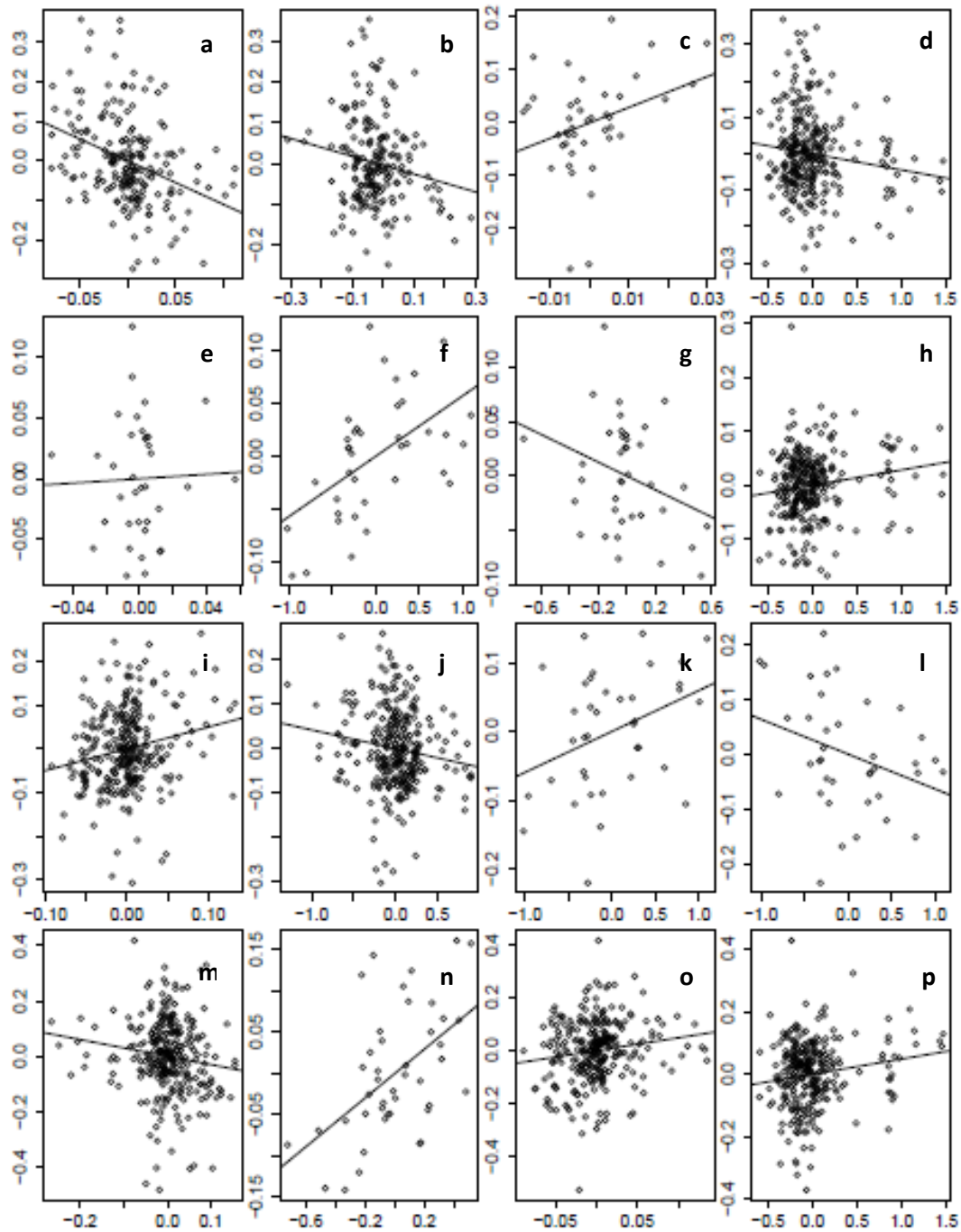
**Figure 4.** Graphical representation of the ordinations of (a) hydrologic metrics and traits and (b) RLQ axes in intermittent streams. MA4 (Model 2 p-value: 0.003, Model 4 p-value: 0.031) and TA1 (Model 2 p-value: 0.008, Model 4 p-value: 0.042) had significant relationships with the first trait axes from RLQ.



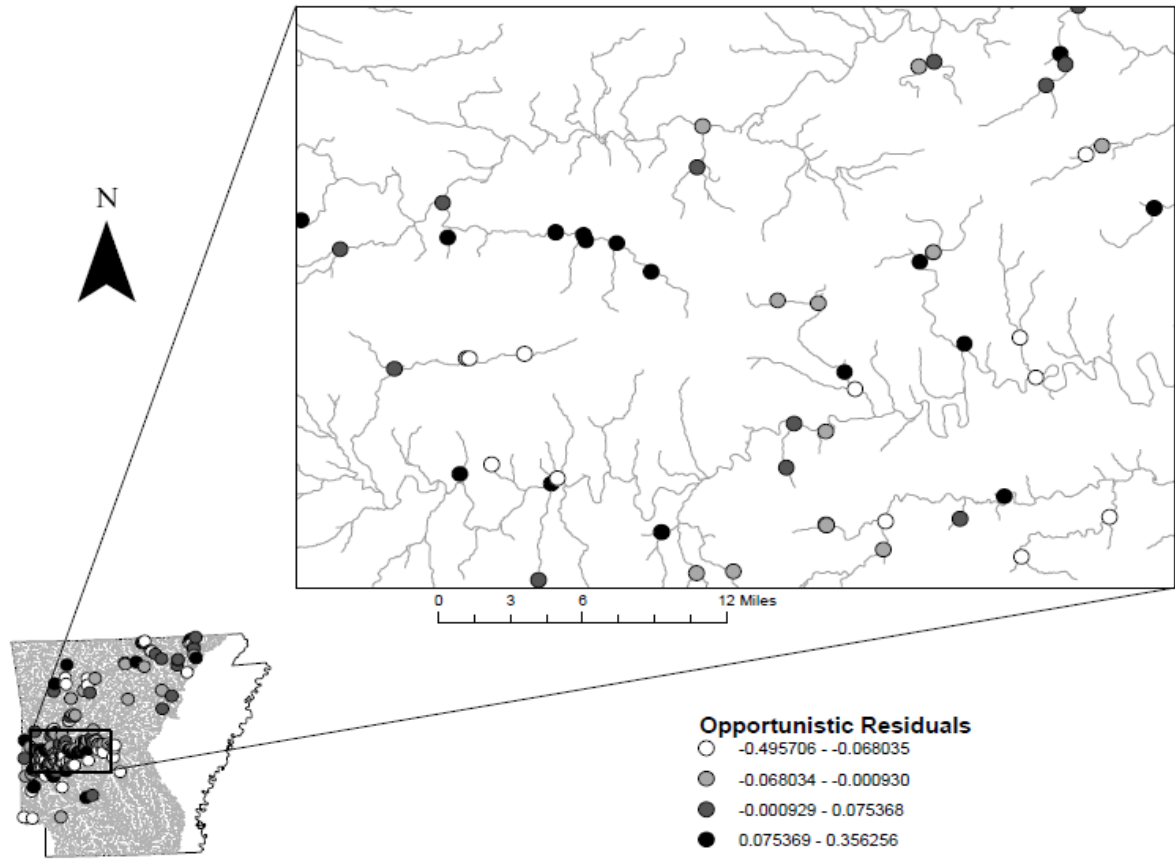
**Figure 5.** Graphical representation of the ordinations of (a) hydrologic metrics and traits and (b) RLQ axes of all sites combined. MA4 (Model 2 p-value: 0.003, Model 4 p-value: 0.025) had significant relationships with the first trait axes from RLQ.



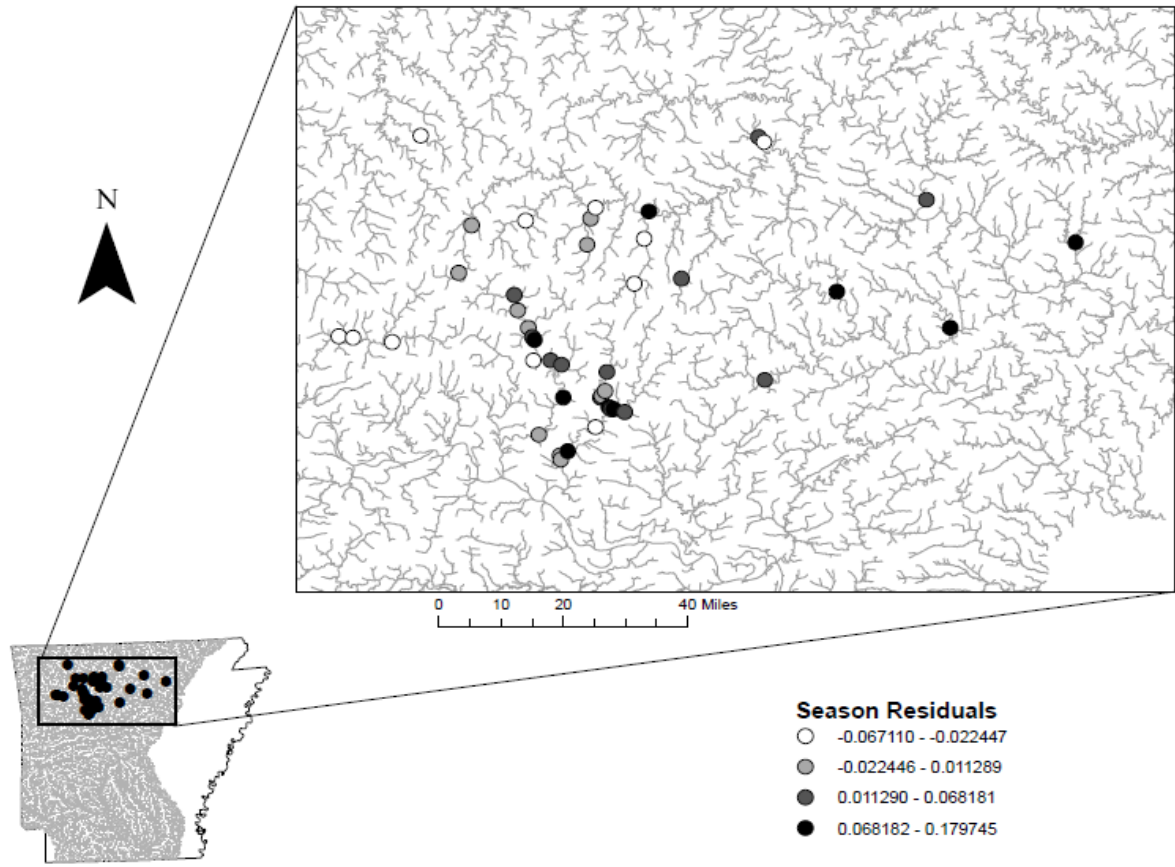
**Figure 6.** Proportion of variance explained for each trait by hydrologic metrics, tail-up, tail-down, and Euclidean covariance structures, and the nugget effect for (a) groundwater, (b) runoff, (c) intermittent, and (d) all sites combined.



**Figure 7.** Partial regressions for significant trait-hydrology relationships for: brood hiders and MA4 (a) and DL4 (b) in intermittent streams, MA4 (c) in runoff streams, and DL4 (d) across all flow regimes; age at maturity and FH7 (e), DL4 (f) and RA3 (g) in groundwater streams and DL4 (h) across all flow regimes; length of spawning season and MA4 (i) and RA3 (j) across all flow regimes; equilibrium strategists and DL4 (k) in groundwater streams; opportunistic strategists and DL4 (l) in groundwater streams and FH7 (m) across all flow regimes; periodic strategists and DL4 (n) in runoff streams; open spawners and MA4 (o) across all flow regimes; and nest guarders and DL4 (p) across all flow regimes.



**Figure 8.** Spatial distribution of residual values (variance unexplained by hydrologic metrics) for the proportion of opportunistic strategists across all flow regimes. Tail-up models described 65% of the variance, indicating small scale or in-stream processes may be influencing the distribution of opportunistic strategists.



**Figure 9.** Spatial distribution of residual values (variance unexplained by hydrologic metrics) for the length of spawning season in runoff streams. Euclidean distance described 93% of the variance, indicating landscape factors may be influencing the length of spawning season.

**Table 1.** Hydrologic metrics used by Leasure et al. (2016) to classify flow regimes in the study area. Bold metrics represent metrics exhibiting minimal correlation and were related to fish traits in the current study.

Code	Metric Name	Definition
MA1	Mean daily flows	Mean daily flow.
<b>MA4</b>	<b>Variability in daily flows 2</b>	Coefficient of variation of the logs in daily flows corresponding to the {5th, 10th, 15th, ..., 85th, 90th, 95th} percentiles.
ML17	Base flow index 1	Seven-day minimum flow divided by mean annual daily flow.
MH14	Median of annual maximum monthly flows	Median of the highest annual daily flow divided by the median annual daily flow averaged across all years.
<b>TA1</b>	<b>Constancy</b>	See Colwell (1974) and Henriksen et al. (2006b, Appendix 5).
FL3	Frequency of low flow spells	Total number of low flow spells (threshold equal to 5% of mean daily flow) divided by the record length in years.
<b>FH7</b>	<b>Flood frequency 1</b>	Mean number of high flow events per year using an upper threshold of 7 times median flow over all years
<b>RA3</b>	<b>Fall rate</b>	Mean rate of negative changes in flow from 1 day to the next.
<b>DL4</b>	<b>Annual minima of 30 day means of daily discharge</b>	Magnitude of minimum annual 30 day flow.
DL18	Number of zero flow days	Mean annual number of days having zero daily flow.
DH4	Annual maxima of 30 day means of daily discharge	Magnitude of maximum annual 30 day flow.



**Table 2.** Traits derived from the Fish Traits database (Frimpong and Angermeier 2009) used to determine life history strategies and used in analyses.

Trait	Data Type	Description
Maximum total length	Quantitative	Maximum total length or asymptotic length in centimeters.
Age at maturity	Quantitative	Mean, median, or modal age at maturity in years averaged across populations for females or males if female data were not available.
Fecundity	Quantitative	Maximum reported fecundity.
Longevity	Quantitative	Longevity in years based on life in the wild or captivity if wild not available.
Serial	Binary	Serial or batch spawners.
Season	Quantitative	Sum of proportions of each month in which spawning occurs.
Open Spawners	Binary	Nonguarders; spawn over open substrates.
Brood Hiders	Binary	Nonguarders; brood hiders.
Open Guardians	Binary	Guarders, open substrate for spawning.
Nest Guardians	Binary	Guarders, nest spawners.
Substrate Indifferent	Binary	Lumping of all bearers, spawning generalists.
Parental Care Index	Ordinal (0-3)	Calculated following McManamay et al. (2015) based on degree of parental investment.

**Table 3.** Covariance structures used in SSN models for each trait in each flow regimes.

Flow regime	Response Variable	Covariance Structure		
		Tail Up	Tail Down	Euclidean
Runoff	Age at Maturity	Exponential	Linear Sill	Gaussian
	Season	Linear Sill	Spherical	Gaussian
	Equilibrium	Linear Sill	Exponential	Exponential
	Periodic	Epanechnikov	Exponential	Gaussian
	Opportunistic	Mariah	Linear Sill	Gaussian
	Open Substrate Spawners	Exponential	Linear Sill	Spherical
	Brood Hiders	Mariah	Linear Sill	Gaussian
	Nest Guardians	Exponential	Epanechnikov	Gaussian
Intermittent	Age at Maturity	Mariah	Linear Sill	Spherical
	Season	Epanechnikov	Linear Sill	Spherical
	Equilibrium	Exponential	Spherical	Spherical
	Periodic	Mariah	Linear Sill	Spherical
	Opportunistic	Mariah	Linear Sill	Gaussian
	Open Substrate Spawners	Spherical	Linear Sill	Spherical
	Brood Hiders	Linear Sill	Linear Sill	Spherical
	Nest Guardians	Linear Sill	Epanechnikov	Gaussian
Groundwater	Age at Maturity	Linear Sill	Linear Sill	Gaussian
	Season	Spherical	Spherical	Exponential
	Equilibrium	Linear Sill	Mariah	Cauchy
	Periodic	Linear Sill	Linear Sill	Gaussian
	Opportunistic	Linear Sill	Epanechnikov	Spherical
	Open Substrate Spawners	Linear Sill	Spherical	Gaussian
	Brood Hiders	Linear Sill	Mariah	Gaussian
	Nest Guardians	Linear Sill	Linear Sill	Cauchy
All Flow Regimes	Age at Maturity	Spherical	Mariah	Cauchy
	Season	Epanechnikov	Linear Sill	Spherical
	Equilibrium	Spherical	Linear Sill	Spherical
	Periodic	Spherical	Linear Sill	Exponential
	Opportunistic	Epanechnikov	Spherical	Cauchy
	Open Substrate Spawners	Epanechnikov	Linear Sill	Spherical
	Brood Hiders	Mariah	Linear Sill	Spherical
	Nest Guardians	Exponential	Linear Sill	Gaussian

**Table 4.** Results from RLQ analysis of each flow regime and across all flow regimes combined including total inertia for each RLQ axis, the percentage of variance retained by the first two RLQ axes relative to separate ordination of species (correspondence analysis), traits (principal components analysis), and hydrologic metrics (principal components analysis), and p-values from Model 6 permutation tests ( $\alpha = 0.05$ ). RLQ analyses were only considered significant if both Model 2 and 4 produced significant p-values.

Flow Regime	Projected inertia (%)		Variance Retained (%)				P value	
	Axis 1	Axis2	Species	Traits	Hydrology	Total Inertia	Model 2	Model 4
Groundwater	76.00	13.62	15.38	60.97	83.58	0.07	< 0.01	0.50
Runoff	82.93	14.83	15.86	62.65	89.58	0.04	0.01	0.42
Intermittent	87.13	7.12	11.78	79.25	93.50	0.08	< 0.01	0.15
All combined	80.46	13.20	14.83	75.44	90.98	0.07	< 0.01	0.33

**Table 5.** Correlation of the first two trait axes with the first two RLQ axes.

Flow Regime	Trait Axis	Correlation with RLQ Axes	
		Axis 1	Axis 2
Groundwater			
	1	0.109	-0.511
	2	0.084	-0.341
Runoff			
	1	0.511	0.010
	2	0.323	-0.180
Intermittent			
	1	0.741	-0.270
	2	-0.534	0.137
All Regimes			
	1	0.630	0.494
	2	-0.494	-0.282

**Table 6.** Loadings of each trait on first two axes of PCA within each flow regime class.

Flow Regime	Trait	Loadings	
		Axis 1	Axis 2
Groundwater			
	Age at Maturity	-0.320	0.209
	Season	0.248	0.282
	Open Spawners	-0.548	0.205
	Brood Hiders	0.578	-0.412
	Open Guardians	0.441	0.808
	Nest Guardians	-0.080	0.108
Runoff			
	Age at Maturity	0.078	-0.261
	Season	0.343	-0.421
	Open Spawners	0.153	0.006
	Brood Hiders	-0.595	0.262
	Open Guardians	-0.407	-0.828
	Nest Guardians	0.577	-0.029
Intermittent			
	Age at Maturity	-0.320	0.209
	Season	0.248	0.282
	Open Spawners	-0.548	0.205
	Brood Hiders	0.578	-0.412
	Open Guardians	0.441	0.808
	Nest Guardians	-0.080	0.108
All Regimes			
	Age at Maturity	-0.488	0.293
	Season	0.218	0.611
	Open Spawners	-0.514	-0.159
	Brood Hiders	0.409	0.468
	Open Guardians	0.514	-0.493
	Nest Guardians	0.005	-0.231

**Table 7.** SSN model results for each flow regime. Parameter estimates, standard error, t-values, p-values, and generalized R<sup>2</sup> are provided for significant hydrologic metric.

Flow Regime	Response	Metric	Estimate	Std. Error	T Value	P Value	Generalized R <sup>2</sup>
Groundwater	Age at Maturity	FH7	0.299	0.143	2.089	0.045	0.450
		DL4	0.062	0.013	4.648	< 0.001	
		RA3	-0.074	0.032	-2.311	0.027	
	Season	---	---	---	---	---	0.326
	Equilibrium	DL4	0.076	0.026	2.924	0.006	0.241
	Opportunistic	DL4	-0.085	0.031	-2.773	0.009	0.247
	Periodic	---	---	---	---	---	0.155
	Open Spawners	---	---	---	---	---	0.089
	Brood Hiders	---	---	---	---	---	0.179
	Nest Guardians	---	---	---	---	---	0.224
Runoff	Age at Maturity	---	---	---	---	---	0.122
	Season	---	---	---	---	---	0.031
	Equilibrium	---	---	---	---	---	0.109
	Opportunistic	---	---	---	---	---	0.063
	Periodic	DL4	0.149	0.046	3.213	0.003	0.283
	Open Spawners	---	---	---	---	---	0.053
	Brood Hiders	MA4	3.770	1.409	2.675	0.011	0.207
	Nest Guardians	---	---	---	---	---	0.075
Intermittent	Age at Maturity	---	---	---	---	---	0.010
	Season	---	---	---	---	---	0.020
	Equilibrium	---	---	---	---	---	0.047
	Opportunistic	---	---	---	---	---	0.048
	Periodic	---	---	---	---	---	0.027
	Open Spawners	---	---	---	---	---	0.034
	Brood Hiders	MA4	-0.811	0.290	-2.793	0.006	0.092
		DL4	-0.126	0.047	-2.672	0.008	
	Nest Guardians	---	---	---	---	---	0.065
All Flow Regimes	Age at Maturity	DL4	0.033	0.011	2.922	0.004	0.097
	Season	MA4	0.421	0.167	2.521	0.012	0.049
		RA3	-0.043	0.022	-2.006	0.046	
	Equilibrium	---	---	---	---	---	0.014
	Opportunistic	FH7	-0.411	0.153	-2.696	0.007	0.036
	Periodic	---	---	---	---	---	0.021
	Open Spawners	MA4	0.474	0.218	2.171	0.031	0.037
	Brood Hiders	DL4	-0.061	0.020	-3.049	0.003	0.075
	Nest Guardians	DL4	0.054	0.019	2.805	0.005	0.047

## **Conclusion**

This thesis presents two papers investigating how hydrologic regimes drive adaptations of stream fish. In the first paper, our objectives were to determine whether or not fish exhibit morphological divergence between hydrologically stable and hydrologically variable streams, how morphology differs between flow regimes, and whether or not fish exhibit plastic morphology in response to one component of flow regimes: water velocity. We observed a significant interactive effect between body size and flow regime, indicating morphology has a different relationship with body size between the two flow regimes. Individuals from intermittent streams had smaller anterior regions and deeper posterior regions relative to the fusiform shapes observed in individuals from groundwater populations, although these results must be interpreted keeping in mind the importance of size in our study. We did not observe any plasticity in morphology in response to water velocity. The morphological differences observed in this study may be the result of a combination of direct and indirect effects of flow patterns and highlight the importance of understanding how ‘master’ or composite variables act as selective forces.

In the second paper, our objective was to compare relationships between fish traits and hydrologic metrics across flow regimes and assess how traits are spatially auto-correlated within flow regimes. The strength of relationships between fish traits and hydrologic metrics were relatively strong in groundwater and runoff streams, while relationships were weak in intermittent streams. Relationships between fish traits and flow metrics were often different between flow regimes and spatial factors describe more variability in the distribution of fish traits than hydrologic metrics. Both large and small, instream patterns of spatial autocorrelation were apparent for traits across flow regimes. Assessing spatial auto-correlation is important in the development of ecological-flow relationships because it can help identify important environmental variables to explain variance and can help researchers account for spatial

patterning of response variables due to sample design. Overall, this study highlights the complex relationships between biota and hydrologic regimes and the importance of considering spatial patterns when developing ecological-flow relationships.



## Appendices

### Research Compliance



UNIVERSITY OF  
ARKANSAS

Office of Research Compliance

#### MEMORANDUM

TO: Dan Magoulick  
FROM: Craig N. Coon, Chairman  
Institutional Animal Care and Use Committee

DATE: May 21, 2014

SUBJECT: IACUC APPROVAL  
Expiration date: May 20, 2016

The Institutional Animal Care and Use Committee (IACUC) has APPROVED protocol 14036: "Trait Composition and Phenotypic Variation of Fish Assemblages Across Hydrologic Gradients". You may begin work immediately

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond May 20, 2016 you must submit a modification for extension. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/aem

cc: Animal Welfare Veterinarian