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Diversity and Dispersal Among Eastern Continental Divide Headwater Stream Fishes in Gwinnett County, Georgia.

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Acknowledgements

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Abstract

The eastern continental divide that bisects Georgia runs through Gwinnett County, separating headwater streams of the western Chattahoochee River watershed from headwater streams of the eastern Ocmulgee and Oconee River watersheds. This landscape feature was used to test hypotheses regarding headwater habitat, fish diversity and gene flow. Headwater habitats are dominant components of river network ecosystems delivering vital ecosystem services and biodiversity. Three headwater streams, one in the Chattahoochee watershed and two in the Oconee watershed, on Gwinnett County Park property, were sampled for differences in physical and chemical properties and fish inter- and intraspecific diversity. Our results suggest the headwater habitats are each distinguished by unique physical and temporal features. Initial hypotheses regarding expectations of fish diversity based on habitat type were not supported. However, hypotheses regarding the impact of gene flow on genetic diversity among headwater stream species were supported. Gene flow estimates and phylogenetic analyses among three well-sampled species observed in these headwater streams, *Semotilus atromaculatus*, *Notropis lutipinnis*, and *Nocomis leptocephalus*, suggest the Eastern Continental Divide acts as a barrier for gene flow for some species. Our findings highlight a proposed methodology for headwater stream analysis that combines habitat heterogeneity with community and species-level measures of diversity.

Keywords: headwater stream, fish, eastern continental divide, diversity, gene flow

INTRODUCTION

River systems are often considered linear habitats with movement, both active and passive, defined by upstream dispersal and downstream flow. This perspective is demonstrated by the influential River Continuum Concept (RCC) (Vannote et al. 1980), which suggests upstream headwater habitats (first and second order streams) have unique features not seen in downstream habitats (third and greater order streams); one of which is lower species diversity. While this linear view is important for defining simple features of river systems largely based on habitat size, it ignores the non-linear network structure of the river system. When the network structure of the river system is considered, headwater streams become a dominant feature with some estimates suggesting headwaters contribute over 70% of the total stream length in most river systems (Richardson and Danehy 2007). From the river network perspective, rather than

simple small species-poor habitats, headwaters are large, complex and potentially species rich habitats that provide initial input for downstream physical and biological processes (Gomi et al. 2002).

From a strictly linear perspective it is easy to see why headwaters would be considered species-poor habitats. Headwaters are by definition smaller habitats with less intrinsic habitat heterogeneity compared to larger stream sections. Habitat heterogeneity is known to be a key driver in stream community-level measures of species diversity (Roa-Fuentes et al. 2020, Heino and Mykra 2008). Therefore, from a strictly linear perspective, lower diversity measures expected in first and second order streams can be largely explained by constraints on variation in habitats due to smaller "living space". The living space itself can be defined in physical terms related to depth, width, velocity, substrate type and structure, as well as chemical terms related to dissolved oxygen and conductance. Alternatively, a network perspective expands the scope of the headwater stream from simple, upstream-downstream components to include horizontal across-headwater habitat components. With this perspective, headwater habitats and biodiversity have a much more dynamic and potentially important role to play in overall diversity within the river system.

The headwater habitat is generally defined as having narrower and shallower channels with greater organic input from coarse particulate matter and higher levels of dissolved oxygen. Constancy of flow also distinguishes first and second order streams from larger stream class categories. Many headwater streams are characterized as intermittent and some are virtually unmapped within river systems due to the intermittence of flow, highlighting the temporal dynamism of headwater streams. Given the scale of the contribution to the river network, these headwater features can vary greatly across the network system (Meyer et al. 2007) providing a potential range of habitat types unequaled in larger stream categories. One of the most relevant features of headwater habitats is the influence of the surrounding terrestrial environment which has a greater influence in smaller streams due to the greater relative contribution to organic and inorganic input. The headwaters collectively act as a primary point source for the aquatic-terrestrial interface with significant impacts on downstream biogeochemical characteristics (Richardson 2019, Tonkin et al. 2017). As point source habitats, headwaters are also sensitive to environmental flux, both natural and man-made.

Connectivity among headwater streams will determine the outcome of environmental changes for the headwaters themselves and downstream communities that depend on input from upstream sources. Gene flow, a biological measure of connectivity, among headwater streams impacts many features of river network ecology such as adaptation to environmental changes (Hendry and Taylor 2004), community diversity (Rodriguez-Iturbe et al. 2009), and biological community interactions (Cuddington and Yodzis 2002, Fagan 2002, Pulliam 1988). The network perspective and the RCC raise the question, how connected are headwater streams? The RCC assumes a linear connection between upstream headwaters and downstream high order streams and rivers governed by drift and the colonization cycle (Muller 1974). Assumptions regarding the network perspective, connectedness among headwater streams, are not well developed, though studies of β diversity, or dissimilarity, among headwater stream macroinvertebrate communities suggest connectedness is limited (Finn and Poff 2011, Finn et al. 2011). Given the terminal position of headwaters in river networks, limited connectivity is an easy assumption to make with greater potential for dispersal barriers, particularly for strictly aquatic species.

However, given the importance of headwater stream contributions to the overall network, few studies have tested this assumption, particularly with strictly aquatic species such as stream fish (Fagan 2002, Schmera et al. 2017).

Headwaters fit into the river network as terminal points that define the extent of the watershed. The geographic differences among river watersheds can be defined by direction of flow among headwater streams and is primarily a function of topography. In Georgia there are 14 recognized river systems evenly split across the eastern continental divide that separates water flowing to the Atlantic Ocean in the east and water flowing to the Gulf of Mexico to the south and west. The eastern continental divide (ECD) runs through Gwinnett County and separates the western flowing Chattahoochee watershed from the eastern flowing Oconee and Ocmulgee watersheds. Taking advantage of this geographic feature, we investigated three headwater streams in Gwinnett County parks on either side of the continental divide to test hypotheses related to physical and regional determinants of headwater stream fish community structure, namely: H1) Habitat physical features play a determinant role of the diversity of fish species found in headwater streams, and H2) Regional processes (dispersal and gene flow) impact community and genetic diversity among headwater stream fish communities and populations.

MATERIALS & METHODS

Three headwater streams located on opposite sides of the ECD in Gwinnett County, Georgia were surveyed for the physical characteristics depth (m), width (cm) and dissolved oxygen (mg/L). Rock Springs (34°02'24.2"N, 84°01'24.0"W) is located in the Chattahoochee River basin feeding into the Gulf of Mexico, while Duncan Creek (34°04'21.2"N, 83°54'18.4"W) and Little Mulberry (34°02'44.7"N, 83°53'18.2"W) are both located in the Oconee River basin flowing into the Atlantic Ocean. All streams were first order with substrates consisting primarily of gravel, with medium-sized rocks and fallen trees obstructing all of the streams at points along all transects.
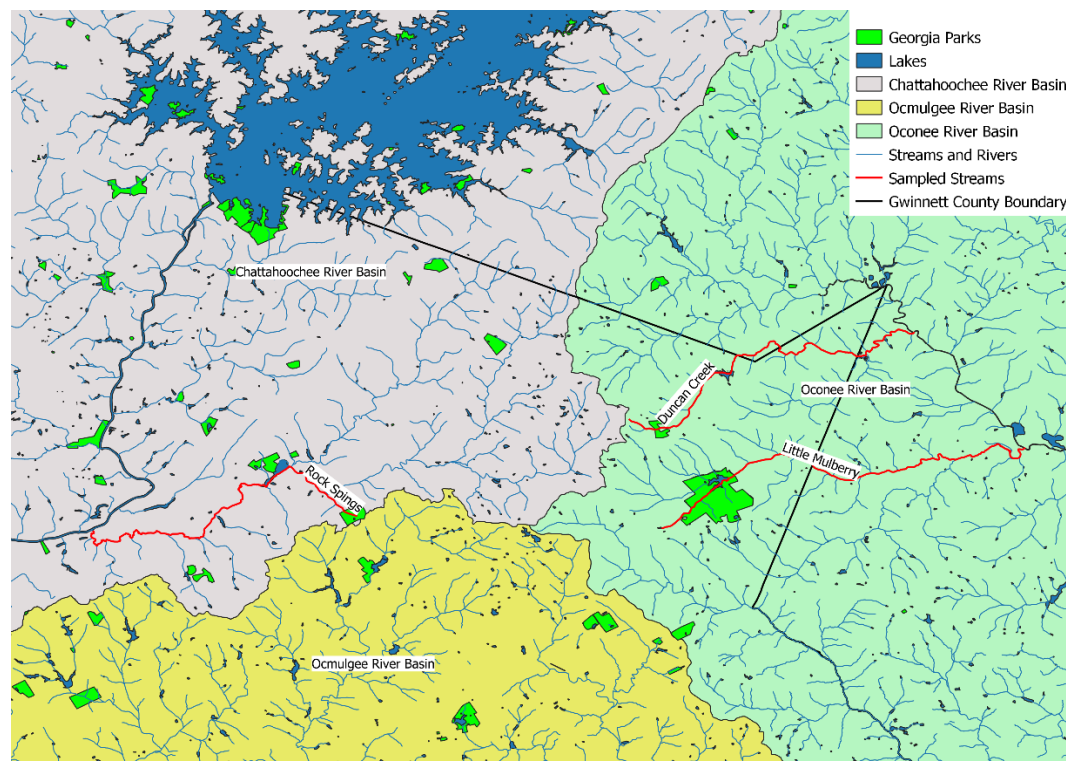
Figure 1: Map of the 3 sampled headwater streams represented in red, with their respective river basins separated east and west by the Eastern Continental Divide.

Samples were collected in February and September of 2021 and taken along a downstream 100-150 meter transect of each headwater. The flow rate was not directly measured among the three streams, however, there were no significant obstructions to flow for either sample date. Measurements were collected by three teams of Georgia Gwinnett College students working in tandem, with measurements taken every 5-10 steps. Depth was measured at stream midpoint, half distance of midpoint and bank, and bank using standard meter sticks. Width was measured by taking the distance from water edge to edge with tape measures. A handheld YSI ProQuatro (Yellow Springs, OH) was used to measure dissolved oxygen, with the meter probe left completely submerged for 10-15 seconds until a stable reading could be recorded. Statistical analyses (t-test, ANOVA, Tukey HSD, linear regression) of physical variables were conducted with R statistical language (R Core Team 2020).

Headwater stream fish community and genetic diversity samples were collected with 7-10 baited minnow traps placed in each stream and left in place for twenty-four hours. Captured fish were sight identified by morphology using previously collected photographic samples of known headwater stream fish, and 3-5 fish from each trap were randomly selected for DNA sampling. IACUC-approved procedures were used to collect caudal fin tissue samples (< 1cm.) from a subset of captured fish. Tissue samples were placed in centrifuge tubes with absolute ethanol and chilled within 24 hours of collection. Shannon index of diversity (H') and t-test, with Bonferroni correction, analyses of fish communities were conducted according to methods described in Nangendo et al. (2002). Beta diversity among the fish communities was evaluated with Sorensen's dissimilarity

index (β_{sor}), and beta diversity components nestedness (β_{sne}) and turnover (β_{sim}) according to procedures described in Herrera-Perez et al. (2019). Permitting for data collection in the parks was provided by Mark Patterson, Deputy Department Director, Gwinnett County (permit # 72000).

The Chelex method (Chelex 100 Resin, BIO-RAD, Hercules, CA) was used for extraction of genomic DNA from tissue samples (Walsh et al. 1991). Genomic DNA was used to amplify a region of the cytochrome oxidase subunit one (CO1) gene using primers: CO1_VF2_t1-5' TGT AAA ACG ACG GCC AGT CAA CCA ACC ACA AAG ACA TTG GCA C 3' and CO1_FISHR2_T1-5' CAG GAA ACA GCT ATG ACA CTT CAG GGT GAC CGA AGA ATC AGA A '3 (Ivanova et al. 2007). PCR reactions (25 µl) consisted of: 1 µl template DNA, 12.5 µl of GoTaq® Master Mix (Promega™); 1 µl of each 10 µM forward and reverse primer; and 9.5 µl of nuclease-free water. All PCR reactions were carried out on an Eppendorf Nexus (Eppendorf North America, Inc., Happauge, NY) thermocycler using the following protocol: 95°C for 2 min, then 35 cycles of 95°C for 30 s, 52°C for 45 s, 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products matching target size were cleaned (ExoSAP-IT™) and submitted to Eurofins Genomics (Louisville, KY) for Sanger sequencing.

All *CO1* sequences were viewed using CHROMAS 2.6.6 (Technelysium Pty Ltd, South Brisbane, AU). GenBank's BLASTn tool (http://www.ncbi.nlm.nih.gov/BLAST/) was used to perform similarity searches for all sequences in order to support or reject morphological identification of specimens. Sequence alignment was conducted using the MEGA x (Kumar et al. 2018) MUSCLE method with default parameters. Uncalled bases and alignment inconsistencies were resolved by analysis of chromatogram files and manual manipulation of sequence alignments.

Alignments were analyzed using neighbor joining and maximum likelihood methods in MEGA x. Tree topologies were compared among phylogenetic methods for consensus. Maximum likelihood analyses for all sequence alignments, nucleotide, and amino acid, were conducted using best fit substitution models based on lowest BIC, AIC, and highest log likelihood scores in MEGA x. Sequence differentiation, diversity, and gene flow statistics were generated using DnaSP v6 (Rozas et al. 2017).

RESULTS

Significant differences were observed between all three headwater stream sample sites for all physical variables analyzed, with the exception of the February depth measurement ($F_{(2,274)} = 2.13$, $p > 0.12$, Table I). Significant mean depth measurement differences were observed in September between DC and the other two headwater streams LM (Tukey HSD $p < 0.001$) and RS (Tukey HSD $p < 0.001$). Significant stream width differences were consistent across sample dates, February ($F_{(2,215)} = 48.5$, $p < 0.001$) and September ($F_{(2,174)} = 20.0$, $p < 0.001$), with greater mean width for Little Mulberry compared to Duncan Creek and Rock Springs. Differences in dissolved oxygen (DO, mg/L) among the three sites varied by sample date. Significant differences among all sites in February ($F_{(2,248)} = 684.6$, $p < 0.001$) revealed DO values in the following rank order, from highest to lowest: Rock Springs, Little Mulberry, Duncan Creek. In September ($F_{(2,207)} = 167.5$, $p < 0.001$) DO values were observed in the following rank order, from highest to lowest: Little Mulberry, Duncan Creek, Rock Springs. The seasonal difference in mean DO value was greatest for Rock Springs (15.1 mg/L in Feb. and 6.3 mg/L in Sept.,

a difference of 8.8 mg/L), resulting in Rock Springs alternating from highest to lowest values for DO among the sample sites.

Table I. The physical characteristics of the three Gwinnett County Park headwater stream samples. The sample sites are listed in the first column. The top row lists the physical data and dates of collection. Asterisks indicate levels of significance (ANOVA) and superscripts post-hoc significance (Tukey HSD) of differences among sites per month sampled.

	Depth Feb. (cm) *ns*	Depth Sept. (cm) ***	Width Feb. (cm) ***	Width Sept. (cm) ***	DO Feb. (mg/L) ***	DO Sept. (mg/L) ***
Duncan Creek	24.6	26.6^a	199.2^b	195.8^b	7.3^c	6.8^b
Little Mulberry	20.5	14.1^b	318.4^a	270.4^a	10.0^b	7.6^a
Rock Springs	21.2	13.5^b	186.6^b	169.5^b	15.1^a	6.3^c

The only consistent seasonal difference within the parks for the physical characteristics measured was for DO (Table II) which showed significantly lower values in September compared to February, which is not surprising given the known negative relationship between DO and temperature. Stream depth and width decreased for Little Mulberry and Rock Springs in September, though the decrease was not statistically significant for Rock Springs. Duncan Creek appeared relatively seasonally stable for depth and width, and differences in DO, though statistically significant, were much lower in magnitude (0.5 mg/L) than the differences observed for Little Mulberry (2.4 mg/L) and Rock Springs (8.8 mg/L). Little Mulberry appeared to be the least seasonally stable stream environment with significant seasonal differences for all variables measured.

Table II. P-values for t-test analyzing seasonal differences for physical characteristics sampled at the three Gwinnett County Park headwater stream sites are shown below.

	Depth (Feb./Sept.)	Width (Feb./Sept.)	DO (Feb./Sept.)
Duncan Creek	0.59	0.77	<0.001
Little Mulberry	<0.001	<0.01	<0.001
Rock Springs	<0.001	0.21	<0.001

A total of 632 fish were collected in February (n = 201) and September (n = 431) and identified to morphospecies based on physical characteristics observed in the field. Preliminary analyses conducted by Georgia Gwinnett College (GGC) Ecology (BIOL 3500K) students on these datasets resulted in consistent fish community diversity (Shannon H') (Nangendo et al. 2002) differences among the sample sites with lower diversity for the Duncan Creek site relative to the other two sites, Little Mulberry and Rock Springs. However due to verified misidentification of morphospecies designations using results from DNA barcoding, the subset of fish collected for which DNA data was obtained (n = 161) were used for verifiable analysis of fish community differences among

the sample sites. Eight species were identified through BLAST analysis (Altschul et al. 1990) of sequencing results: *Semotilus atromaculatus* (n=62), *Nocomis leptocephalus* (n=44), *Notropis lutipinnis* (n=28), *Lepomis auritus* (n=10), *Campostoma pauciradii* (n=7), *Ameiurus natalis* (n=5), *Lepomis macrochirus* (n=4), and *Moxostoma rupiscartes* (n=1). *S. atromaculatus*, *N. leptocephalus*, and *N. lutipinnis* were represented from all sample site locations and were by far the most abundant species collected. There was no significant difference observed for fish community diversity between Little Mulberry, which had the highest H' value (1.6), and Rock Springs (Figure 2). Duncan Creek Shannon H' diversity value (1.1) was lower than both Little Mulberry ($p < 0.01$) and Rock Springs ($p < 0.02$). Diversity differences between Little Mulberry and Rock Springs corresponded with consistent stream depth, width and dissolved oxygen differences for the two sites (Table I). However, regression analyses found stream depth to be the only predictor of fish diversity, with greater diversity in shallower streams ($R^2 = 0.82$, $F_{(1,4)} = 18.47$, $p < 0.02$). Analysis of species richness dissimilarity (Sorensen dissimilarity, β_{sor}), or beta diversity (Herrera-Perez et al. 2019), among the three sample sites revealed greater similarity between Duncan Creek and Rock Springs ($\beta_{sor} = 0.2$), than Duncan Creek and Little Mulberry ($\beta_{sor} = 0.4$) and Little Mulberry and Rock Springs ($\beta_{sor} = 0.33$). Partitioning of beta diversity in to component turnover (β_{sim}) and nestedness (β_{sne}) revealed dissimilarity between Duncan Creek and Rock Springs to be entirely due to nestedness, while dissimilarity between Little Mulberry and Rock Springs was entirely due to turnover. The greatest dissimilarity observed between Duncan Creek and Little Mulberry was made up of 62.5% turnover ($\beta_{sim} = 0.25$) and 37.5% nestedness ($\beta_{sne} = 0.15$).
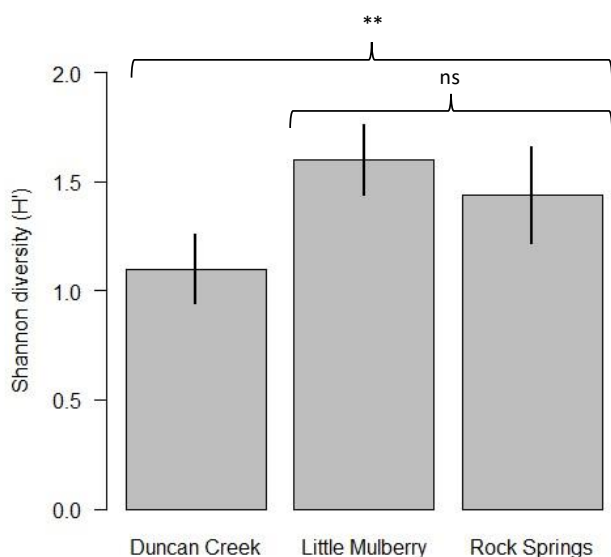

Figure 2. The Shannon index (H') values for the Gwinnett County Park headwater stream sample sites is shown above (+/- 1 SD). Significant differences among the sample sites (t-test of diversity with Bonferroni correction) are indicated by brackets above figure.

A sub-sample of fish collected generated 162 nucleotide sequences used for phylogenetic analysis of a 602bp region of the cytochrome oxidase subunit 1 (CO1) region (Figure 3). One additional *Notropis lutipinnis* sequence from a Chattahoochee River headwater stream in Habersham County, GA was added to the 161 samples collected in the three Gwinnett County parks. Eight headwater stream fish species from four families (Cyprinidae, Centrarchidae, Ictaluridae, Catostomidae) are represented in the analysis, with Centrarchidae serving as an outgroup. Our hypothesis of the eastern continental divide acting as a barrier to gene flow was partially supported with well supported "Eastern" and "Western" clades within the *Semotilus atromaculatus* and *Notropis lutipinnis* groupings. "Eastern" refers to the Atlantic Ocean slope (Duncan Creek and Little Mulberry, Oconee River) and "Western" to the Gulf of Mexico slope (Rock Springs, Chattahoochee River). However, limited directional gene flow across the continental divide was observed with "Eastern" mitotypes RSF33 (*S. atromaculatus*) and RS18 and RS19 (*N. lutipinnis*) found in Rock Springs (Figure 3). The continental divide gene flow hypothesis was not supported for the species *Nocomis leptocephalus* or *Lepomis auratus*. Limited sampling of other species prevented analysis of the hypothesis.

Figure 3. Neighbor-joining phylogenetic analysis (500 bootstrap replicates) of 162 cytochrome oxidase subunit 1 (CO1) nucleotide sequences (602 bp) is shown above. Major clades representing species in the dataset are indicated by species names. "Eastern" and "Western" refer to Oconee and Chattahoochee watersheds, respectively.

Three species, *N. leptocephalus*, *N. lutipinnis*, and *S. atromaculatus*, were chosen based on sample sizes for analyses of genetic differentiation and variation. Genetic differentiation (F_{ST}) among the three sample sites revealed a pattern consistent with the ECD hypothesis for two of the three species. *N. lutipinnis* and *S. atromaculatus*, with low F_{ST} values within catchment sample sites Duncan Creek and Little Mulberry, and high F_{ST} values across catchment sample sites (Table III). For *N. leptocephalus*, F_{ST} values were consistent among the three sample sites and did not support predictions based on the ECD hypothesis for genetic differentiation across catchment sites. Migration estimates

(Nm) varied accordingly, with low migration estimates for *N. lutipinnis* and *S. atromaculatus*, and a relatively high estimate for *N. leptocephalus* (Table III).

Table III. F_{ST} values for the headwater stream populations of *Nocomis leptocephalus* (Bluehead Chub), *Notropis lutipinnis* (Yellowfin Shiner) and *Semotilus atromaculatus* (Creek Chub) as well as the migration estimate (Nm), are shown below. Abbreviations refer to sample sites: Duncan Creek (DC), Little Mulberry (LM), and Rock Springs (RS).

	N. leptocephalus	*N. lutipinnis*	*S. atromaculatus*
DC-LM	0.03	0	0
DC-RS	0.05	0.78	0.88
LM-RS	0.04	0.79	0.88
Nm	7.96	0.73	0.65

Haplotype and nucleotide diversity measures showed distinct patterns across the three sampled sites that were associated with measures of migration (Table IV). Low migration measures for *S. atromaculatus* and *N. lutipinnis* showed low levels of genetic diversity in the eastern Atlantic slope stream communities, Duncan Creek and Little Mulberry, and relatively high measures of genetic diversity in the western Gulf of Mexico slope community Rock Springs. In contrast, the high migration species *N. leptocephalus*, showed no obvious difference in genetic diversity measures, which were all relatively high, across the three communities.

Table IV. Haplotype (Hd) and nucleotide (Pi) diversity measures are shown below for the three species *N. leptocephalus*, *N. lutipinnis* and *S. atromaculatus* in the three sample sites. Migration estimate values (Nm) are shown for each species.

Nocomis leptocephalus, Nm = 7.96	Hd	Pi
Duncan Creek	0.67	0.00183
Little Mulberry	0.63	0.00242
Rock Springs	0.70	0.00317
Notropis lutipinnis, Nm = 0.73		
Duncan Creek	0.22	0.00079
Little Mulberry	0	0
Rock Springs	0.78	0.01799
Semotilus atromaculatus, Nm =0.65		
Duncan Creek	0.12	0.00021
Little Mulberry	0.17	0.00046
Rock Springs	0.70	0.00722

DISCUSSION

Without an *a priori* expectation of physical differences among headwater sites, we assumed that larger habitats with higher dissolved oxygen values would support greater numbers of fish and fish species. Results suggest the three headwater streams represent distinct habitat types with varying degrees of temporal variation in physical characteristics. As defined by the ECD, the western stream Rock Springs could be characterized as a shallow and narrow stream with extreme seasonal variation in

dissolved oxygen (Table II). Among the two eastern streams Duncan Creek was characterized as the deeper and narrower stream with lower dissolved oxygen values, while Little Mulberry was shallow and wider with higher dissolved oxygen (Table II). The observed physical differences did not support our assumptions regarding absolute number or diversity of species (Figure 2). The only variable that was significantly associated with species diversity was mid-stream depth. However, that relationship was negative (m = -0.04), with diversity higher in shallower streams, like Little Mulberry and Rock Springs, and lower in the deeper Duncan Creek headwater stream, the opposite of our assumptions.

Different data collection dates allowed us to test the temporal variability of the stream physical characteristics across two seasonal timepoints- February and September. Significant differences in observed variation aligned with diversity measures (Table II, Figure 2). Our results suggest temporal variation and diversity are related in the following manner: significant temporal differences for all three measured physical variables for the stream with the highest species diversity (H'), Little Mulberry; significant differences for two of the three variables, depth and dissolved oxygen, for the stream with the second highest H' value, Rock Springs; and significant difference for one of the three variables, dissolved oxygen, for the stream with the lowest H' value, Duncan Creek. High degrees of seasonality paired with predictable seasonal changes can allow different species to occupy the same stretch of stream at different times of year, increasing temporal diversity (Tonkin et al. 2017). Whether our aligned temporal variation and community diversity measures reflect such temporal diversity will require continued sampling for predictability in seasonal changes among the headwater streams.

The temporal variation observed may be a signal of variance in the disturbance regime. As such the association with alpha diversity (H') may reflect the impact of disturbance patterns on community structure/diversity (Finn and Poff 2011, Vellend et al. 2017), an expectation among headwater stream communities (Resh et al. 1988). Clearly, more data is needed, but a hypothesis based on our results would suggest greater temporal variability is associated with greater interspecies diversity (H') at the local scale. Given these results one might conclude depth and temporal stability in physical environment are negatively correlated with fish species diversity. With such a small sample size, conclusions should be met with caution. However, the methodology used to address questions of diversity in headwater streams could be useful given the expected impact of environmental heterogeneity on species diversity, particularly among headwater streams (Altermatt 2013, Finn et al. 2011, Brown and Swan 2010).

As a distinct landscape feature, the ECD was assumed to serve as a barrier to dispersal and gene flow. In general, community differences, or dissimilarities in the form of beta diversity, are predictably maximized among isolated "island" populations, such as headwater streams (Murria et al 2013, Finn et al 2011, Finn and Poff 2011, Herrera-Perez et al. 2019). Following this assumption, we predicted greater community dissimilarity (beta diversity, β_{sor}), and genetic distance (F_{ST}, Dxy) among headwater stream fish species across the ECD in different catchments relative to within catchment communities, specifically Duncan Creek and Little Mulberry. Again, our results did not support our predictions, with a greater beta diversity value within catchment communities, DC-LM β_{sor} = 0.4, relative to across-ECD communities: DC-RS β_{sor} = 0.2, LM-RS β_{sor} = 0.33. Though the beta diversity values we observed are lower than those observed in high altitude Andes headwater streams (Herrera-Perez et al. 2019), our values fall within the

range of those observed in Rocky Mountain headwater stream chironomid communities (Finn and Poff 2011). The observed partitioning of beta diversity with higher observed turnover (β_{sim}) values is a shared characteristic among many headwater stream communities (Herrera-Perez et al. 2019, Zbinden et al. 2017, Rouquette et al 2013, Finn and Poff 2011).

The phylogenetic structure of the fish communities sampled revealed an "eastern-western" bifurcation of the clades representing *S. atromaculatus* and *N. lutipinnis* (Figure 3), with eastern taxa comprised primarily of fish collected from the Atlantic drainage streams Duncan Creek and Little Mulberry and western taxa comprised of fish from the Gulf of Mexico drainage stream Rock Springs (with one additional sample that was collected in Habersham county GA and part of the same Chattahoochee river network). Among the eastern clades for both species, Rock Springs individuals are present with one sample in the eastern *S. atromaculatus* clade and two in the eastern *N. lutipinnis* clade. Whether this represents limited directional dispersal, eastern mitotypes dispersing westward, or incomplete lineage sorting could not be determined. The other well-sampled species, *N. leptocephalus*, showed no phylogenetic structure that would suggest dispersal limitation across the ECD. Dispersal mechanisms for *N. leptocephalus* across the ECD are unknown, but may include phoretic transport of fish eggs (Lovas-Kiss et al. 2020), or bait bucket release.

Among the three well-sampled species, predictable gene flow estimates for *S. atromaculatus* and *N. lutipinnis* contrasted with estimates for *N. leptocephalus*, where relatively high estimates of migration (Nm) and low F_{ST} values were observed (Table III). High F_{ST} values observed for *S. atromaculatus* and *N. lutipinnis* across the ECD reflect differentiation across catchments and are similar to other studies of freshwater fish species (Hughes 2007). From these results one might conclude the ECD acts as a barrier to gene flow for some species but not all. The extent to which this pattern can be generalized requires further sampling across species and headwater stream communities among river networks.

Connectivity among headwater streams is predicted to increase alpha diversity and decrease beta diversity among connected sites (Finn et al. 2011). Given their locations and observed lack of genetic differentiation for the species sampled (Table III), Duncan Creek and Little Mulberry were expected to have higher alpha and lower beta diversity values. Our results gave conflicting signals, with higher alpha diversity for Little Mulberry but the lowest alpha diversity value for Duncan Creek. Additionally, the highest beta diversity value was observed for Duncan Creek-Little Mulberry, indicating greater community differentiation contrary to the connectivity hypothesis. We can conclude from these results that connectivity, as represented by these species, does not impact community-level processes governing diversity. Which raises the question, what factors are impacting the observed diversity differences among these headwater stream communities?

Haplotype and nucleotide diversity, across the three sample sites appeared to be impacted by location relative to the ECD and measures of migration. Lower diversity values were observed for the less migratory species, *S. atromaculatus* and *N. lutipinnis*, in the eastern Atlantic slope drainage sites compared to the western Gulf of Mexico drainage site and the more migratory *N. leptocephalus* . For the Duncan Creek and Little Mulberry communities, migration and dispersal appear to be limiting factors for genetic diversity for some species, with less migration resulting in lower genetic diversity. The relatively higher intraspecies diversity values observed in Rock Springs suggests,

according to this hypothesis, migration among the western slope drainages for *S. atromaculatus* and *N. lutipinnis* might be greater than for the eastern slope drainages.

Higher migration and genetic diversity estimates observed for *N. leptocephalus* support the contention that connectivity is important for local adaptation to varying environmental conditions, a result supported by previous findings in other headwater stream communities (Murria et al. 2013). Given the observed physical and temporal differences among the three sampled sites, local adaptation may play an important role in population persistence in these headwater streams. The combined forces of connectivity, habitat heterogeneity, and dynamism may also play a role in the persistence of local and regional diversity (Finn and Poff 2011, Heino et al. 2005). Theoretical and empirical results have shown that high measures of beta diversity among headwater streams combined with connectivity has a positive impact on regional diversity and population persistence (Morrissey and Kerckhove 2009, Hendry and Taylor 2004). Given the distinct physical and temporal differences observed across the three sample sites in our study (Table I and II), connectivity may be a significant factor for these communities and populations.

Future research should continue to investigate the local-regional diversity predictions along with dispersal and gene flow estimates at larger scales, given the relevance of these ecological and evolutionary forces. Local alpha diversity measures may be lower in headwater communities, but higher beta diversity, or dissimilarity, among headwater communities means regional diversity and ecosystem function can be enhanced with connectivity among headwater streams (Tonkin *et al.* 2017). Headwaters not only comprise the bulk of river networks (Richardson 2019) but also represent major point source nutrient and material contributions to downstream environments (Gomi et al. 2002, Wipfli et al. 2007). Rather than assuming the relatively low local diversity often observed in headwater streams reflects low relative contribution to stream ecosystem structure and function, we contend that a regional or network perspective that incorporates diversity at inter- and intra-population scales (alpha/beta and genetic diversity measures) combined with measures of connectivity would support the conclusion that headwaters are critical habitats that maintain biodiversity and ecosystem function.

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