

Conservation status, habitat use, and
phylogeography of the imperiled
Tennessee-endemic Egg-mimic Darter
(*Etheostoma pseudovulatum*)

Zachary L. Wolf

Conservation status, habitat use, and phylogeography of the imperiled
Tennessee-endemic Egg-mimic Darter (*Etheostoma pseudovulatum*)

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To the College of Graduate Studies:

We are submitting a thesis written by Zachary L. Wolf entitled “Conservation status, habitat use, and phylogeography of the imperiled Tennessee-endemic Egg-mimic Darter (*Etheostoma pseudovulatum*).” We have examined the final copy of this thesis for form and content. We recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science in Biology.

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ABSTRACT

ZACHARY L. WOLF. Conservation status, habitat use, and phylogeography of the imperiled Tennessee-endemic Egg-mimic Darter (*Etheostoma pseudovulatum*) (under the direction of DR. REBECCA JOHANSEN.)

Restricted to two counties within the Duck River system (Tennessee), *Etheostoma pseudovulatum* is state endangered and has been petitioned for federal listing. In addition to its small range, the mainstem Duck River may be a barrier isolating smaller tributary populations. Despite this, a status survey has not been conducted in two decades and genetic diversity has never been evaluated. Thus, objectives were to: 1) evaluate the current conservation status of *E. pseudovulatum* by describing its current distribution, estimate abundance and population size, assess overall genetic diversity, and evaluate anthropogenic effects within its range; 2) describe its general habitat use; and 3) assess phylogeographic patterns of genetic diversity to evaluate whether the Duck River acts as a barrier to gene flow among tributary populations.

Twenty-five localities representing all historical localities of *E. pseudovulatum* were sampled in spring and fall using standard seining techniques to assess presence and estimate abundance and population size using the Petersen mark-recapture method. Habitat variables were measured and analyzed for association with *E. pseudovulatum* presence, and range-wide genetic diversity was examined using the mitochondrial ND2 gene.

Etheostoma pseudovulatum was present at all 25 localities sampled and abundance estimates ranged from 5 to 258 individuals per 75-meter reach, comparable to those observed historically. The species was significantly associated with low flow, a range of greater depths, and presence of undercut banks, debris, and root wads. Eleven haplotypes were detected (haplotype diversity= 0.624; nucleotide diversity= 0.0054) with one haplotype shared across all

tributaries except Beaverdam Creek, which had a unique assemblage of haplotypes compared to all systems except Little Piney Creek. One individual from Little Piney Creek possessed a haplotype shared with Beaverdam Creek. Haplotypes were recovered in two clades: 1) Beaverdam Creek and two individuals from Little Piney Creek; and 2) the other five tributary populations. These results suggest historical gene flow among all tributaries except Beaverdam Creek, which has potentially undergone long-term isolation. Overall, the species was locally abundant and appears stable, however continued future monitoring with focus on smaller tributaries, which appear most susceptible to extirpation, and on the genetically distinct Beaverdam Creek population is recommended.

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CHAPTER I

INTRODUCTION

The Southeastern United States has the highest diversity of freshwater fishes in North America (Burr and Mayden, 1992; Warren et al., 1997, 2000). Unfortunately, an estimated 28% of fishes from this region are recognized as extinct, endangered, threatened, or vulnerable (Warren et al., 1997, 2000; Jelks et al., 2008). In 2010, the Center for Biological Diversity (an environmental advocacy organization) submitted a petition to the United States Fish and Wildlife Service to review over 400 southeastern aquatic and riparian species for federal protection under the Endangered Species Act. This historic petition generated the need to collect data for these species to determine whether each warranted federal protection. One of the fishes included in the petition and lacking recent study was the Egg-mimic Darter (*Etheostoma pseudovulatum*, Family Percidae, traditionally classified in subgenus *Catonotus* [Page et al., 1992], currently recognized in *Stigmacerca*, a subclade of *Goneaperca* [Near et al., 2011; Harrington and Near, 2015]).

Currently, *E. pseudovulatum* is recognized as endangered by the state of Tennessee, vulnerable by the American Fisheries Society and the International Union for Conservation of Nature (IUCN Red List), and “globally critically imperiled” by NatureServe (Jelks et al., 2008; Withers, 2009; NatureServe, 2013, 2015). A primary reason *E. pseudovulatum* is considered imperiled is because it has a small native range. *Etheostoma pseudovulatum* is endemic to two counties of Tennessee, Dickson and Hickman counties, which are located in the Western Highland Rim physiographic region (Etnier and Starnes, 1993). Within these counties, the species is known from 38 historical localities in six tributary systems (Piney River, Beaverdam Creek, Little Piney Creek, Happy Hollow Creek, Only Creek, and East Fork Wolf Creek; Figure 1) to the Duck River (Tennessee River drainage). Because it is restricted to an area

approximately less than 600 square miles, a disturbance in the area could have a large impact on the species.

Another reason *E. pseudovulatum* is considered at risk is because it requires specific breeding habitat that is sensitive to anthropogenic stressors, primarily increased siltation. Males of *E. pseudovulatum* and other egg-clustering darters (clade *Goneaperca*; Near et al., 2011; Harrington and Near, 2015) clear out a cavity underneath a flat rock and court females who lay eggs on the underside of the rock, in which the male guards and maintains the eggs until hatching (Page, 1974, 1983, 1985; Page and Bart, 1989; Bart and Page, 1991; Page et al., 1992; Ceas and Page, 1995). When excess sediment is washed into a stream, it can fill in interstitial spaces and crevices beneath rocks that *E. pseudovulatum* uses for nest sites. Not only does siltation reduce the availability of spawning habitat, it also reduces the overall availability of habitat that is used by *E. pseudovulatum* and other benthic species, such as aquatic macroinvertebrates, which are the primary component of most darter diets (Ceas and Page, 1995; Helfman et al., 2009). Additionally, siltation causes an increase in suspended solids which can: reduce water clarity, thereby reducing the ability to search for food and mates; decrease photosynthetic productivity from reduced light availability; smother eggs and other organisms; and have harmful physiological impacts on organisms, such as disrupting gill formation in juvenile fishes (Sutherland, 2005, 2007; Sutherland and Meyer, 2007; Helfman et al., 2009).

Despite conservation concerns, only two studies have focused on *E. pseudovulatum*. Page et al. (1992) described *E. pseudovulatum*, elevating it from *E. neopterum*, and discussed possible phylogenetic relationships of these and other members of the *E. squamiceps* species group (subclade *Stigmacerca*; Near et al., 2011; Harrington and Near, 2015), which includes *E. chienense*, *E. corona*, *E. crossopterum*, *E. forbesi*, *E. neopterum*, *E. nigripinne*, *E. olivaceum*, *E.*

oophylax, *E. pseudovulatum*, and *E. squamiceps*. However, information on the ecology and habitat use was generalized to *Stigmacerca* and not specific to *E. pseudovulatum*. Additionally, Page et al. (1992) noted variation in morphology among the Piney River, Beaverdam Creek, and Little Piney River populations. Most noteworthy was the coloration patterns of the second dorsal fin of nuptial males from Little Piney Creek that showed variation from the other populations, but no subspecific descriptions were applied due to low sample sizes and, “lack of concordance in character variation.”

Ceas and Page (1995) conducted the first and only status survey of *E. pseudovulatum*, providing a thorough investigation of the relative abundance and distribution of the species. They found *E. pseudovulatum* in all tributaries except Little Piney Creek. However, they noted that recent efforts by others that utilized rotenone resulted in the capture of five specimens from this creek. They concluded that the overall population of *E. pseudovulatum* was relatively stable, but habitat degradation and its extremely small range made the species vulnerable to local extirpation, especially in the smallest tributary systems.

Habitat use has been broadly described for all members of *Stigmacerca* (Page et al., 1992; Ceas and Page, 1995) with species typically associated with cool, small, headwater streams in slow-flowing pools with nearby cover, such as slab rocks, woody debris, undercut banks, exposed root wads, or mats of aquatic vegetation (Page et al., 1992; Ceas and Page, 1995). However, habitat used by *E. pseudovulatum* has never been quantified specifically, which is important information when implementing conservation management strategies (Warren et al., 1997; Albanese et al., 2013; Compton and Taylor, 2013).

Understanding patterns of genetic variation among species has become one of the most important tools in forming conservation management plans for imperiled taxa (Powers et al.,

2004; George et al., 2006; Turner and Robison, 2006; George et al., 2009; Fluker et al., 2011). Such data can help determine proper actions for managing species of conservation concern and how to prioritize those actions, such as prioritizing management of subspecific populations with low genetic diversity (George et al., 2006) or recognizing populations as newly discovered species that should be managed independently (Blanton and Jenkins, 2008). The overall genetic diversity of *E. pseudovulatum* has not been estimated despite noted variation in morphology among tributary systems (Page et al., 1992), which suggests there may be significant genetic variation among populations.

Additionally, gene flow among populations of headwater-stream adapted fishes may be restricted by large rivers that can act as barriers or filters to dispersal (Starnes and Etnier, 1986; Turner and Trexler, 1998; Powers et al., 2004; George et al., 2006; Turner and Robison, 2006; Hollingsworth and Near, 2009; Fluker et al., 2011; Sterling et al., 2012). Specific habitat requirements, including strict breeding habitat requirements, of *E. pseudovulatum* and other upland-adapted fishes can differ greatly from habitats available in large rivers, thus preventing dispersal of individuals (Starnes and Etnier, 1986; Turner and Trexler, 1998; Turner and Robison, 2006; Fluker et al., 2011, 2014; Sterling et al., 2012). Also, for some fishes larval drift is a major contributor to downstream dispersal, however *E. pseudovulatum* has benthic larvae that do not drift far from nest sites, further restricting potential gene flow (Simon and Wallus, 2005).

The fact that there are no known collections of *E. pseudovulatum* within the mainstem Duck River also suggests that the Duck River acts as a barrier to *E. pseudovulatum* dispersal. If so, then the six tributary system populations could be genetically isolated from each other, which would require specific management practices for each population. Limited dispersal ability

among tributary systems also may result in reduced recolonization potential of extirpated populations.

Objectives:

Plans for managing at-risk species are most effective when equipped with a complete knowledge of the species, including current distribution, abundance, population size, anthropogenic effects, habitat use, and patterns of genetic diversity (Warren et al., 1997; George et al., 2006; Compton and Taylor, 2013). To make an informed decision on whether federal protection is warranted for *Etheostoma pseudovulatum*, estimates of these parameters are needed. Therefore, the objectives of this study were to: 1) evaluate the current conservation status of *E. pseudovulatum* by: i) describing its current distribution in relation to its historical range, ii) estimating abundance and population size, iii) assessing overall genetic diversity, and iv) evaluating anthropogenic effects within its range; 2) describe general habitat use of *E. pseudovulatum*; and 3) assess phylogeographic patterns of genetic diversity to evaluate whether the Duck River acts as a potential barrier to gene flow among tributary populations.

MATERIALS AND METHODS

Localities Examined

Historical localities of *Etheostoma pseudovulatum* were identified from published literature and museum records. Museums surveyed include the Illinois Natural History Survey, University of Michigan Museum of Zoology, University of Tennessee – Etnier Ichthyological Research Collection, Yale University Peabody Museum, Auburn University Museum of Natural History, Cornell University Museum of Vertebrates, University of Kansas Biodiversity Institute – Specimens, Harvard University Museum of Comparative Zoology, North Carolina State Museum of Natural Sciences, Ohio State University – Fish Division, Tulane University Museum of Natural History – Royal D. Suttkus Fish Collection, University of Alabama Ichthyological Collection, National Museum of Natural History, and the Smithsonian Institution (accessed through the Fishnet2 Portal, www.fishnet2.net, 9/15/2013). Additional museums surveyed include Southern Illinois University Carbondale, University of Florida, University of Southern Mississippi, Field Museum of Natural History, American Museum of Natural History, Academy of Natural Sciences of Drexel University, Mississippi Museum of Natural Science, and Austin Peay State University David H. Snyder Museum of Zoology. All historical localities were georeferenced using GEOLocate (Rios and Bart, 2010) and a Tennessee Gazetteer (DeLorme, 2010). Other members of *Stigmacerca* were also surveyed and records with localities situated within the known range of *E. pseudovulatum* were included in the study (six localities that included records of *E. squamiceps*, stemming from collections prior to the description of *E. neopteron* or *E. pseudovulatum*, were included in this study).

Thirty-eight historical localities were identified for *E. pseudovulatum* (Figure 1).

However, only 25 localities were sampled throughout this study because some historical localities were adjacent to others, dry at the time of survey, or too deep to sample safely and effectively. One locality sampled was added by this study for purposes of ease of access to make up for two mainstem Piney River localities that were not sampled due to high flow and difficult accessibility. A map of localities sampled can be seen in Figure 2, and Appendix A provides specific locality information.

Field Collection

Field collections were conducted in 2014 once during the spring (March through May; *Etheostoma pseudovulatum* breeding season) and once during the fall (August through December; non-breeding season) to gauge seasonal variation and deliver robust estimates of abundance, population size, and habitat use. Only two localities were not sampled twice throughout the course of this study: 1) site 6 was only sampled in spring because it was deemed unnecessary due to its close proximity to other localities; and 2) site 25 was sampled only in fall because water levels were too high to sample in spring (Figure 2).

At each locality a 75 meter reach of the stream was sampled. Fish were collected using traditional kick-seining methods, using a 3.05 x 1.37 m wide, 0.32 cm mesh seine. To attempt equal sampling effort per locality, the number of kick-sets was determined by the average wetted stream width (Table 1; modified from Abernathy and Mattingly, 2011). Average wetted stream width was calculated from three transects taken at the upstream, mid, and downstream portions of the reach. Specific kick-set patches within the stream were chosen opportunistically with an attempt to sample all available habitat types proportionally to their occurrence. Kick-sets began at the downstream end and continued upstream to minimize disturbance of habitat.

Captured individuals of *E. pseudovulatum* were measured for total length (TL), sexed if possible, and then placed in a bucket with a battery-powered aerator. Individuals were sexed only during the breeding season due to the difficulty of determining sex in the field outside of the breeding season. Specimens of each species captured (including at least one *E. pseudovulatum* per locality) were collected as voucher specimens. Voucher specimens were euthanized using the anesthetic MS-222, preserved in 10% formalin for approximately two weeks, transferred to deionized water for approximately ten days, then converted to 70% ethanol for permanent storage in the Ichthyological Collection of the David H. Snyder Museum of Zoology at Austin Peay State University. All methods were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) before continuing with this study (IACUC Protocol #14.003). GPS coordinates were collected from each locality using a Garmin handheld GPS.

Presence/Absence Survey

All localities were examined for the presence or absence of *E. pseudovulatum* to determine the current distribution of the species. However, localities that were not measured for estimates of abundance and population size were referred to as “Presence” localities and followed no further methods as described in the “Field Collection” section. Only a single pass of the required number of kick-sets were completed for these localities.

Abundance and Population Size Estimation

Twelve localities were used to estimate abundance and population size (referred to as “Abundance” localities) with at least one locality in each tributary system (localities: 3, 5, 9, 11, 12, 13, 16, 17, 18, 19, 20, and 23; Figure 2). Estimates were completed using the Petersen mark-recapture method (Krebs, 1999). To fit the assumption of a closed system, the 75 m reach was enclosed using block-nets at the upstream and downstream ends of the reach. Two passes, each

of the designated number of kick-sets, were conducted. After the first pass was completed, all captured individuals of *E. pseudovulatum* were enumerated and marked by clipping the dorsal portion of the caudal fin while anesthetized. After clipping, individuals were allowed time to revive in an aerated bucket before being returned back to the stream at haphazardly selected locations within the reach. A minimum of thirty minutes was allowed for the fish to disperse before starting the second pass. During the second pass, individuals were noted as unmarked (newly captured) or marked (recaptured from first pass), measured for total length, and sexed if possible.

Abundance estimates were calculated using a modified version of the Petersen method for closed populations (Seber, 1982):

$$N = [(M + 1)(C + 1)/(R + 1)] - 1$$

where: N is the estimated abundance of the 75 m reach sampled; M is the number of individuals marked on the first pass; C is the total number of individuals captured on the second pass; and R is the number of individuals that were recaptured on the second pass. This model assumes that the individuals are effectively marked so that the marker is not lost or so that it does not alter the odds of survival or catchability of the individual. Because fin-clipping young-of-the-year individuals is difficult and risks their survival, young-of-the-year individuals captured during the fall were excluded from abundance estimates, which were identified by their small size (approximately less than 42 millimeters in total length). Additionally, young-of-the-year are small enough to fall through the seine or swim through block-nets, thereby decreasing the ability to capture them. From the abundance estimate, 95% confidence limits were calculated to estimate the upper and lower bounds of population size per 75 m reach. Due to the low number of recaptures, the Poisson distribution was used to obtain these estimates (Krebs, 1999).

Abundance and population size estimates were completed using Microsoft Excel (Microsoft, Redmond, WA). The “Matched Pairs” function in JMP Pro 10 (SAS Institute Inc., Cary, NC) was used to perform nonparametric tests for significant seasonal variation in abundance and population size estimates among sites.

To clarify, an abundance estimate is an estimate of the abundance of the species within the sampled 75 m reach based on the modified Peterson method. Population size estimates represent the minimum and maximum estimates of the population size within the sampled 75 m reach calculated by the 95% confidence interval of the abundance estimate. Relative abundance is the total number of individuals captured at a site per collection event.

Habitat

Reach-scale Parameters

Immediate land-use and anthropogenic effects observed at localities were documented via photographs and field notes. Assessment of reach-scale habitat quality was determined at each locality using standard habitat scoring methods from Barbour et al. (1999) for high-gradient streams (Appendix B). The “Habitat Assessment Data Sheet” consisted of 10 habitat parameter categories that are scored between 0 and 20: 1) epifaunal substrate/available cover, 2) embeddedness, 3) velocity/depth regime, 4) sediment deposition, 5) channel flow status, 6) channel alteration, 7) frequency of riffles, 8) bank stability, 9) vegetative protective, and 10) riparian vegetative zone width. The sum of these scores generates the overall habitat assessment score. Two evaluators each completed a data sheet, with the author consistently being one of the evaluators, and scores were averaged for each collection. Habitat assessments were completed during fall sampling. Specific conductance also was measured using a YSI meter; water temperature was measured using both the YSI meter and a mercury thermometer. In JMP Pro 10,

linear regression was used to analyze relationships between reach-scale variables and first pass relative abundance for all localities. Wetted stream width, specific conductance, and water temperature were analyzed for seasonal variation using the “Matched Pairs” function in JMP Pro 10. Quantile box plots of reach-scale variables were generated in IBM SPSS premium grad pack 22.0 (IBM Corp., Armonk, NY).

Microhabitat Parameters

Habitat use was measured at the 12 sites where abundance and population size estimation were conducted. During the first pass, before each kick-set was sampled, microhabitat was described by recording the presence or absence of certain habitat variables (see Table 2). Once a kick-set was completed, a labeled, flagged weight was dropped at that location so that the patch could be revisited after the pass was completed to measure averages of flow and depth. Average flow (m/sec) and depth (cm) was measured using a *Global Water* flow meter to take a transect of three measurements at each kick-set patch. General habitat characteristics of riffle, run, or pool were determined by flow and depth measurements.

Analyses of habitat use were assessed by season (spring and fall), pooled (“Total”), and by sex/age class (males, females, and juveniles). Analyses of males, females, and juveniles included only patches from spring samples due to the difficulty of sexing individuals in fall. Principal component analysis (PCA) is commonly used to analyze habitat use in stream fishes due to its ability to reduce dimensionality of complex, often correlated, multivariate data (Kwak and Peterson, 2007; Osier and Welsh, 2007; Midway et al., 2010; Compton and Taylor, 2013; Gibbs et al., 2014). Therefore, PCA was used to explore potential associations of habitat variables with *E. pseudovulatum* presence and to support further analyses of habitat use. PCA methods followed Compton and Taylor (2013) and were completed in SYSTAT 8.0 (Systat

Software, San Jose, CA). All data were $\log(X+1)$ transformed prior to analysis to improve linearity and better meet the assumptions of PCA (Compton and Taylor, 2013). The two principal components (PCs) with the greatest eigenvalues (values greater than 1.0) were selected as axes to plot factor scores within a scatterplot, and variables that best described observed variation within the data had component loadings greater than $|0.50|$. Kolmogorov-Smirnov two-sample tests on factor scores were used to determine whether the distribution of patches where *E. pseudovulatum* was present were significantly nonrandom with respect to the distribution of all available patches (Compton and Taylor, 2013).

Classification and regression trees (CART, developed by Breiman et al., 1984) are a leading method in modeling predictions of complex interactions between organisms and their environment, even proving to be better suited than generalized linear and additive models (De'ath, 2002). They have been utilized in numerous studies to form predictions of the presence of species within various suites of habitat variables (De'ath and Fabricius, 2000; Usio et al., 2006; Brewer et al., 2007; Osier and Welsh, 2007; Steen et al., 2008; Gibbs et al., 2014). CART explains a response variable, such as the presence or absence of a species, by creating a parsimonious hierarchy of explanatory variables, with the most influential variables first, and partitions data into mutually exclusive groups for each explanatory variable (De'ath and Fabricius, 2000). CART is nonparametric, robust to both categorical and continuous variables, has high predictive power, and can be easily interpreted for biological significance (Usio et al., 2006).

To determine which habitat variables best predicted the presence of *E. pseudovulatum*, CART was completed in IBM SPSS premium grad pack 22.0 using the Exhaustive CHAID algorithm (Brewer et al., 2007) and 10-fold cross-validation (Steen et al., 2008; Gibbs et al.,

2014). Tree size can vary based on the minimum number of cases for parent and child nodes. Therefore, multiple trees were formed using a gradient of minimum number of cases for parent and child nodes. A single tree was chosen if it was within one standard error of the lowest cross-validation risk estimate, had one of the highest overall correct classification rates, and made most sense biologically (Breiman et al., 1984; De'ath and Fabricius, 2000; De'ath, 2002; Gibbs et al., 2014). Cross-validation acts as a measure of the predictive ability of the model by producing multiple repetitions of the analysis on subsets of the given data and testing to see how often the model correctly explains the data subsets. The cross-validation risk estimate is the proportion of repetitions that incorrectly explain the data, while the correct classification rate is the proportion of repetitions that correctly explain the data (Breiman et al., 1984; De'ath and Fabricius, 2000). These parameters measure the performance of CART analyses.

Tests for univariate associations of variables with *E. pseudovulatum* presence were completed in JMP Pro 10. Equal samples sizes between number of patches where *E. pseudovulatum* was present vs. absent patches were achieved for univariate statistics by using a random number generator to randomly select a subset of absent patches. Wilcoxon signed-rank tests were conducted for continuous variables, and Fisher's exact tests were conducted for categorical variables.

Genetic Diversity and Phylogeography

Tissue samples were obtained from caudal fin clips taken from anesthetized fishes encountered during site surveys and abundance estimate collections. Tissues were preserved in 95% non-denatured ethanol in the field and replaced with fresh 95% non-denatured ethanol once in the lab. Whole genomic DNA was extracted from 59 individuals collected in spring from 14 localities representing each tributary system (localities: 1, 5, 7, 8, 9, 11, 12, 13, 16, 17, 18, 19,

20, and 23; Figure 2) using two methods: 1) GeneJET Genomic DNA Purification kit (Thermo-Scientific Inc.); and 2) 5% Chelex solution. GeneJET extractions were completed following the manufacturer's instructions except only 200 μ l of 95% ethanol and 90 μ l of elution buffer were used to yield a higher concentration of DNA. Extractions were quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo-Scientific Inc.).

A variety of genetic markers have been utilized for phylogeographic studies of darters and other upland-adapted fishes, and one of the most commonly used markers for these purposes is the mitochondrial gene NADH dehydrogenase subunit 2, known as ND2 (e.g., Avise, 2004; Freeland, 2006; George et al., 2006; Hollingsworth and Near, 2009; Fluker et al., 2011, 2014; Lang and Echelle, 2011). Mitochondrial genes, such as ND2, serve as reliable markers for assessing intraspecific gene flow because of their high mutational rate relative to nuclear markers (Vawter and Brown, 1986; Kocher et al., 1989; Kocher and Carleton, 1997). Also, mitochondrial genes undergo genetic drift and become fixed faster than nuclear genes because mtDNA is maternally-inherited and their effective population size is four times smaller than that of nuclear DNA (Birky et al., 1989; Palumbi et al., 2001). Additionally, mtDNA is relatively cheap and easy to work with because of the low cost of primers, the abundance of mtDNA within cells, and the ease of interpreting non-recombining lineages (Freeland, 2006).

ND2 was amplified using the primers GLN (5'-CTACCTGAAGAGATCAAAAC-3') and ASN (5'-CGCGTTTAGCTGTAAAC TAA-3') and polymerase chain reaction (PCR) with the following cycling protocol: 95 °C for 1 min; 32 cycles of 95 °C for 30 sec, 56 °C for 1 min, then 72 °C for 30 sec; followed by 72 °C for 7 min (Kocher et al., 1995). PCR products were gel-verified, then sent to the Interdisciplinary Center for Biotechnology Research at the University of

Florida for Sanger sequencing. Sequence data were edited and consensus sequences were made using CodonCode Aligner (CodonCode Corporation, Dedham, MA).

Haplotype and nucleotide diversity and average sequence divergence within and between clades were calculated using DnaSP (Librado and Rozas, 2009). TCS 1.21 was used to build a statistical parsimony haplotype network from resulting sequences with a 95% connection limit (Clement et al., 2000). Sequences were partitioned by codon position using Mesquite (Maddison and Maddison, 2014). Each partition was analyzed with jModelTest (Posada, 2008) and the Akaike Information Criterion (AIC) was used to determine the best model of nucleotide evolution for each codon position. These models were then incorporated into a partitioned, mix-model Bayesian analysis of the sequence data using MrBayes (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Two runs of 12,000,000 generations each were conducted, sampling every 100th tree, and the resulting trees from each run were examined for congruence in topology. A 50% majority-rule consensus tree was formed using Mesquite (Maddison and Maddison, 2014) excluding 2000 burn-in trees as determined by convergence in likelihood values. ND2 sequences of nine *Goneaperca* species (*E. chienense*, *E. corona*, *E. crossopterum*, *E. flabellare*, *E. forbesi*, *E. neopterum*, *E. nigripinne*, *E. oophylax*, and *E. virgatum*) and *E. nigrum* obtained from GenBank were included in the Bayesian analysis as outgroup species (GenBank accessions JQ088521, JQ088530, JQ088531, JQ088540, JQ088543, JQ088558, JQ088560, JQ088565, JQ088598, and JQ088561, respectively).

CHAPTER III

RESULTS

Status Survey

Etheostoma pseudovulatum was present at all 25 localities sampled in the spring and fall (Figure 2) and 795 individuals were collected in total. In the spring 62 males, 188 females, and 234 juveniles were captured, and in the fall 311 individuals were collected. The mean total length of all individuals captured was 48.79 mm (SD = 14.26), with a mean of 48.52 mm (SD = 13.91) for spring captures and 49.22 mm (SD = 14.80) for fall captures (no significant difference between seasons). Mean total length for males was 69.40 mm (SD = 7.96), 56.77 mm (SD = 6.18) for females, and 36.37 mm (SD = 5.90) for juveniles in the spring. It is important to note there were some individuals large enough to be adults, but were classified as juveniles due to the inability to identify their sex, seen as the outliers in the juveniles' frequency histogram (Figure 3). There were at least two age classes observed in the total length frequency histograms, with a possible third age class represented by individuals greater than approximately 65 mm (Figure 3).

No significant differences in estimates of abundance and population size among tributary systems were observed (Table 3). Abundance estimates ranged from 5 to 258 individuals per 75 m reach, and population size estimates ranged from 1 to 546 per 75 m reach. The highest estimates were primarily from localities within the largest system, Piney River, while the other five tributary systems generally had moderate-to-low estimates. Even though it appears that estimates were greater in the spring, there was no significant seasonal variation of estimates among sites ($p = 0.0640$ for abundance, $p = 0.0640$ and 0.1568 for lower and upper bounds of population size, respectively).

Although estimates of abundance and population size generated by this study are not directly comparable to those of previous studies due to sampling and data analysis differences, a general comparison of first-pass relative abundance results with that of the Ceas and Page (1995) status survey was conducted to better understand the historical context of these findings (Table 4). Collections from Ceas and Page were made on 3 April, 1995, by sampling for 15 minutes to 2 hours using seines and dip nets; data were reported as categories: abundant (greater than 20 individuals), common (10-20), present (less than 10), and absent. At most sites examined herein relative abundance was consistent with or higher than those reported previously (50% agreed with Ceas and Page, 37.5% were greater, and 12.5% were lower). For this study, the total number of individuals captured per collection attempt ranged from 1 to 92 individuals (Table 4).

In addition to *E. pseudovulatum*, 40 other co-occurring fish species were collected (Table 5). The species most commonly associated with *E. pseudovulatum*, collected from at least 16 of the 25 localities, in descending order were: *Clinostomus funduloides*, *Cottus carolinae*, *E. flabellare*, *Campostoma oligolepis*, *E. flavum*, *Semotilus atromaculatus*, *Chrosomus erythrogaster*, *E. bison*, *E. caeruleum*, and *Fundulus catenatus*.

Habitat

Reach-scale Parameters

Mean wetted stream width was 8.26 m in spring and 7.37 m in fall. Mean water temperature was 12.2°C in spring and 19.9°C in fall. Mean specific conductance was 0.1120 mS/cm^c in spring and 0.1709 mS/cm^c in fall. The mean total habitat assessment score for all sites was 147.2. Values of wetted stream width, water temperature, specific conductance, and total habitat assessment score per site for each season are reported in Table 7 and box plots for each are in Figure 5. Observations of land-use at localities are listed in Table 10.

Average wetted stream width was significantly greater in spring ($p = 0.0017^*$), and water temperature and specific conductance were significantly greater in fall ($p = 0.0001^*$ for both), therefore, linear regression was analyzed for these variables by season (Table 6). There were no significant relationships between any of the reach-scale variables and first pass relative abundance (Table 6). Scores of the habitat parameter categories from the “Habitat Assessment Data Sheet” can be interpreted as conditions that are: optimal (scores 15 to 20), suboptimal (10 to 15), marginal (5 to 10), and poor (0 to 5; Appendix B). Five of the ten habitat parameter categories had a median considered within optimal conditions (epifaunal substrate/available cover, embeddedness, channel alteration, frequency of riffles, and vegetative protective), and the other five were within suboptimal conditions (velocity/depth regime, sediment deposition, channel flow status, bank stability, and riparian vegetative zone width; Figure 4). The mean total habitat assessment score indicated, on average, sites exhibited suboptimal conditions (i.e., scores between 100-150).

Microhabitat Parameters

In total, 524 habitat patches were analyzed (153 patches where *E. pseudovulatum* was present, 371 absent patches), with 264 patches in the spring (60 present, 204 absent) and 260 in the fall (93 present, 167 absent). Results of PCA reveal that all three categories of individuals (i.e., spring, fall, and total) show similar patterns of habitat use (Figures 6, 7, and 8; Table 8 summarizes descriptive parameters for PCAs). For all patches in total (Figure 6) and spring patches (Figure 7) more individuals were present in areas with lower flow, greater depth, and the presence of undercut banks, root wads, and debris. In fall, *E. pseudovulatum* was typically found in similar conditions except that the presence of root wads and debris did not load heavily in the PCA. Results from Kolmogorov-Smirnov tests show that the distribution of present patches was

significantly nonrandom with respect to all available habitats for PC1 of all three categories and PC2 of “Total” which supports that these variables may drive the presence of *E. pseudovulatum*. Variation in habitat patch use observed for spring males, females, and juveniles overlapped considerably in the PCA, suggesting no major differences in habitat use between males, females, and juveniles in spring.

The CART analyses performed well in comparison to other studies, yielding correct classification rates ranging from 74.2-93.9%. Other studies have reported 72-76% for fish species distributions in Michigan (Steen et al., 2008), 80-85% for fish species distributions in Ontario (Olden and Jackson, 2002), and 84% for predicting an invasive crayfish distribution in Japan (Usio et al., 2006). Although none of the trees were identical, all of the CART results highlighted similar combinations of habitat variables used by *E. pseudovulatum* and were consistent with variables highlighted by the PCA (Figures 9-14; Table 9 lists descriptive parameters for CART analyses). For all habitat patches (correct classification rate = 79.2%; cross-validation risk estimate = 0.223), *E. pseudovulatum* was primarily observed in flows less than 0.10 m/sec, and within those flows individuals were typically in greater depths (16.66-113.33 cm) with either the occurrence of debris or in a patch adjacent to the bank. When found in intermediate flows (0.10-0.41 m/sec), individuals were associated with intermediate depths (21.0-37.66 cm). In the spring (correct classification rate = 79.5%; cross-validation risk estimate = 0.227), *E. pseudovulatum* was most often observed with the presence of undercut banks. If undercut banks were absent, individuals were typically found at patches with the occurrence of debris, root wads, or vegetation. In the fall (correct classification rate = 74.2%; cross-validation risk estimate = 0.296), individuals were primarily present in lower flows (0-0.07 m/sec), but if in intermediate flows (0.08-0.14 m/sec), they were typically found with undercut banks. The few

individuals in higher flows (0.15-0.83 m/sec) were found in vegetation, root wads, or debris. *Etheostoma pseudovulatum* males in the spring (correct classification rate = 93.9%; cross-validation risk estimate = 0.061) were most commonly found with undercut banks, however, in the absence of undercut banks they were either associated with the occurrence of vegetation, debris, or root wads. Female presence in the spring (correct classification rate = 89.4%; cross-validation risk estimate = 0.125) was primarily associated with the presence of debris and lower flows (0-0.11 m/sec); if debris was absent then individuals were typically found with undercut banks or root wads. Juvenile presence in the spring (correct classification rate = 91.3%; cross-validation risk estimate = 0.121) was most often associated with the occurrence of root wads and lower flows (0.02-0.19 m/sec). In the absence of root wads, juveniles were associated with the presence of vegetation and debris. However, juveniles in patches lacking vegetation were in undercut banks or sites adjacent to a stream bank.

Univariate tests revealed that the presence of *E. pseudovulatum*, whether male, female, or juvenile, or in the spring or fall, was significantly associated with lower flows and greater depths (Figures 15 and 16). The presence of the stream bank and undercut banks were significantly associated with *E. pseudovulatum* presence for all categories except for fall individuals (Figures 17 and 18). The presence of root wads and debris were significantly associated with *E. pseudovulatum* presence for all categories except for males (Figures 19 and 20). The presence of open bedrock was significantly associated with the absence of *E. pseudovulatum* only for the “Total” category (Figure 21). Vegetation (Figure 21) and backwater were not significantly associated with the presence of individuals in any category, likely due to low occurrence of these habitats.

Genetic Diversity and Phylogeography

There were 11 haplotypes recovered from the 59 individuals examined (Table 11, Figure 22). In total, haplotype diversity (H_d) was 0.624 and nucleotide diversity (π) was 0.0054 (Table 11). East Fork Wolf Creek and Only Creek each contained only one haplotype, resulting in no diversity. In ascending order of haplotype diversity, the remaining tributary system populations were: Beaverdam Creek ($H_d = 0.371$), Piney River (0.450), Little Piney Creek (0.533), and Happy Hollow Creek (0.600). In ascending order of nucleotide diversity, the remaining tributary system populations were: Beaverdam Creek ($\pi = 0.0004$), Happy Hollow Creek (0.0005), Piney River (0.0006), and Little Piney Creek (0.0046).

Results from the haplotype network (Figure 22) showed that one haplotype was shared among 34 individuals found in all tributary systems except Beaverdam Creek. Haplotypes were grouped into two clusters that differed by 13 or more nucleotide substitutions. One of the clusters consisted of individuals from Piney River, Little Piney Creek, Happy Hollow Creek, Only Creek, and East Fork Wolf Creek, while the other cluster, separated by 13 nucleotide substitutions, consisted of individuals from Beaverdam Creek and 2 of the 10 individuals examined from Little Piney Creek.

The haplotypes also sorted into two well-supported clades in the Bayesian phylogenetic analysis (Figure 23). Clades were consistent with clusters recovered by the haplotype network. One clade contained all individuals from Beaverdam Creek and two individuals from Little Piney Creek (referred to as the Beaverdam Creek clade), while the other clade contained all individuals from Piney River, East Fork Wolf Creek, Only Creek, Happy Hollow Creek, and eight individuals from Little Piney Creek (referred to as the Piney River clade). Average

sequence divergence was 0.040% within the Beaverdam Creek clade, 0.041% within the Piney River clade, and 1.24% between the two clades.

CHAPTER IV

DISCUSSION

Conservation Status

Etheostoma pseudovulatum was present at all historical localities sampled which suggests that, relative to the last status survey (Ceas and Page, 1995) and historical information contained in museum records, there have been no changes in the overall distribution of the species.

Although some historical localities were not sampled and some localities were newly established, the general range of the species remains consistent with previous findings (Page et al., 1992; Ceas and Page, 1995). Likewise, because not all historical localities were sampled, it is possible that the species has been extirpated from those sites. However, this seems unlikely given that the species was present at sites upstream and/or downstream from non-sampled sites.

Additional sampling in adjacent tributary streams could reveal new localities outside the known range of the species. However, previous surveys of other localities have not yielded any *E. pseudovulatum* and authors have noted many of these adjacent localities lack suitable habitat for the species (Page et al., 1992; Ceas and Page, 1995; Fishnet2 Portal, 2013).

Overall, *E. pseudovulatum* appears to be relatively abundant throughout most of its distribution (Tables 3 and 4). The 795 individuals captured in this study were greater than numbers observed for other *Goneaperca* species with small native ranges, such as *E. striatulum* with a total of 102 individuals captured (Abernathy and Mattingly, 2011) and *E. forbesi* with a total of 75 individuals captured (Hansen et al., 2006). Estimates of abundance and population size for *E. pseudovulatum* were either greater or comparable to findings for other darters, such as *E. cragini*, *E. microperca* (79-546 and 129-1,341 individuals, respectively; Hargrave and Johnson, 2003), and *E. raneyi* (2,419 for all historical collection attempts; Sterling et al., 2013).

The authors concluded in these cases that the focal species were in need of monitoring but with no immediate need for federal protection. Although collection techniques in these studies vary and make it difficult to accurately compare results, they provide some context for understanding the relatively large number of *E. pseudovulatum* individuals captured in this study.

The pattern of estimated abundances and population sizes for tributary systems agrees with Ceas and Page (1995). The Piney River system had the majority of the highest estimates of abundance and population size, followed by Beaverdam Creek and the other four smaller tributary systems (Table 3). As concluded in Ceas and Page (1995), the Piney River system appears to harbor the most stable population of *E. pseudovulatum* overall. All but one of the localities in the Piney River system yielded an estimated abundance greater than 30 individuals per 75 m reach. The exception to this general pattern was observed for a mainstem Piney River site (locality #20), which had the largest wetted stream width of the abundance localities (average 22.4 m wide between spring and fall) but lacked ample suitable habitat for *E. pseudovulatum*. Ceas and Page (1995) noted that larger localities, such as mainstem Piney River or Beaverdam Creek, were too large to support persistent populations of *E. pseudovulatum*. Although few individuals were captured at this locality, it is possible that larger streams may simply have a lower density of *E. pseudovulatum*, therefore making it more difficult to capture individuals. The smaller estimates of abundance and population size in the five other tributary systems suggest that these systems may be more unstable and susceptible to local extirpation, deserving of regular monitoring.

When comparing our results of relative abundance with that of Ceas and Page (1995), most populations have remained stable over the past 20 years (Table 4). Of the localities that we could compare to their study, 87.5% of sites had values comparable to or higher than the 1995

status survey. Only two localities had lower relative abundances, Cow Hollow Creek (#7) and Coon Creek (#23), which are both small streams (less than 4 m wide) that may have changed in habitat characteristics since the 1995 survey, potentially by anthropogenic means. Although Ceas and Page (1995) did not detail the habitat quality of Cow Hollow Creek, this study found it had a narrow (approximately less than 10 m wide) riparian zone on each bank due to nearby farmland and residential properties, and suboptimal sedimentation levels likely resulting from these conditions (Table 10). For Coon Creek, the authors noted that although *E. pseudovulatum* was abundant, it was likely the population was limited to only the uppermost mile where they sampled due to a lack of suitable habitat elsewhere. This seemed to be the case in 2014, where the majority of the reach was a long riffle of hard-packed substrate, with a couple of pools and undercut banks. Also, there was a section of bank-stabilization rip-rap at the upstream end of the reach indicating additional impacts from surrounding land use.

Several of the historical localities sampled have some type of anthropogenic disturbance that could threaten the persistence of the species at these and other sites downstream (Table 10). The majority of these stressors observed were farmland and residential properties that reduced riparian zones. One locality in Beaverdam Creek (site #3) was altered between our spring and fall sampling. One of the neighboring properties clear-cut their yard about 100 m along the stream and laid gravel, which formed a new channel in the stream diverting water away from the original reach sampled. The reach was nearly half the average wetted stream width in fall (4.9 m) as it was in spring (9.4 m) and had significantly slower average flow in fall (0.03 m/sec) than in spring (0.32 m/sec; $p < 0.0001^*$ from ANOVA of flow at patches). It is likely that the property owners responsible for this did this with the intention of providing pasture for cattle. However, it is important to note the relative abundance and estimated abundance at site 3 was higher in the

fall (7 and 14, respectively) than in the spring (5 and 10, respectively). This could be due to the increase in overall slower flowing habitats stemming from the alteration to the site, which is associated with the presence of *E. pseudovulatum*. Although the modification seems to have temporarily increased abundance at the locality, long-term impacts are unknown, but should be monitored.

Habitat

Reach-scale Parameters

On average, localities of *E. pseudovulatum* were considered to have suboptimal habitat quality (Figure 4). Most noteworthy were the categories of bank stability, sediment deposition, and riparian vegetative zone width, which had medians that met criteria for suboptimal conditions (Figure 4). Because *E. pseudovulatum* is associated with features of eroded banks, such as undercut banks and exposed root wads, suboptimal conditions for bank stability is more likely to be favorable for this species. The suboptimal sediment deposition condition at sites is concerning due to its negative effects on benthic communities, *E. pseudovulatum* spawning habitat, and increased suspended solids which can reduce water clarity, decrease photosynthetic productivity, and even disrupt gill formation in juvenile fishes (Ceas and Page, 1995; Sutherland, 2005, 2007; Sutherland and Meyer, 2007; Helfman et al., 2009). Suboptimal riparian vegetative zone width is also concerning because riparian zone reduction has been associated with decreasing fish abundance, decreasing habitat diversity, increasing sedimentation, and changes in faunal and physical characteristics of streams (Jones et al., 1999). However, there were no significant relationships between any of the reach-scale variables and first pass relative abundance, which suggests these variables either do not affect the abundance of *E. pseudovulatum* or there is insufficient variation to detect any effects (Table 6).

Previous summaries of stream size for *E. pseudovulatum* stated the species was found in “headwaters” and “small creeks” (Page et al., 1992; Ceas and Page, 1995). Although there was no significant relationship between relative abundance and wetted stream width, three of the localities sampled were greater than 20 m wide, suggesting that this species may be more common in larger streams and rivers than previously thought.

Microhabitat Parameters

Overall, microhabitat use of *E. pseudovulatum* agrees with that of other *Stigmacerca* species (Page et al., 1992; Ceas and Page, 1995). Although none of the CART analyses were identical, they all showed that *E. pseudovulatum* was typically found in slow-flowing pools (defined by low flow and greater depth), often near banks with some type of cover such as undercuts, root wads, debris, or vegetation. If they were not found within slow-flowing pools then they were found with some type of cover as previously described (Figures 9-14). These results were further supported by PCA (Figures 6-8) as well as univariate tests (Figures 15-21), except for vegetation, which was not significantly associated with the species, likely due to the low sample size of the occurrence of this habitat. Additionally, the CART analyses performed well when comparing their correct classification rates and cross-validation risk estimates with other studies (Olden and Jackson, 2002; Usio et al., 2006; Steen et al., 2008).

Some seasonal variation in microhabitat use was noted. Unlike in spring, individuals in fall were not significantly associated with bank habitats based on univariate tests (Figures 17 and 18). This may mean they shift from using bank-adjacent patches during the breeding season to more general use of pool habitats outside of the breeding season. However, the CART analysis for fall patches showed that the presence of undercut banks is a predictor variable for *E. pseudovulatum* presence, but only in flows between 0.08 and 0.14 m/sec (Figure 11).

There were no major differences in microhabitat use between males, females, and juveniles during the breeding season. The results of PCA for the three sex/age classes showed no substantial separation, which further supports this conclusion. However, there was some minor variation worth noting. Unlike spring females and juveniles, males were not significantly associated with root wads or debris (Figures 19 and 20). However, both root wads and debris are predictors within the CART analysis for spring males (Figure 12). This contradiction may be explained by the effects of the low sample size of male-present patches on univariate tests, as CART analysis is better equipped for data with varying sample sizes (Breiman et al., 1984; De'ath and Fabricius, 2000; De'ath, 2002).

Genetic Diversity

Overall haplotype and nucleotide diversity observed for *E. pseudovulatum* ($H_d = 0.624$ and $\pi = 0.0054$, respectively) appears to be fairly low-to-moderate in comparison to other darters, such as *Percina burtoni* ($H_d = 0.891$ and $\pi = 0.0130$; George et al., 2006), and to other Nearctic and Palearctic freshwater fishes (nucleotide diversity ranging from 0.00007 in *Morone saxatilis* to 0.0774 in *Cyprinella lepida*; Bernatchez and Wilson, 1998). Populations from Happy Hollow Creek and Little Piney Creek have haplotype and nucleotide diversity comparable to the larger Piney River and Beaverdam Creek populations, despite having lower estimates of abundance and population size (Table 11). Yet, the equivalently small populations of East Fork Wolf Creek and Only Creek have no diversity. Low genetic diversity is correlated with low fitness and increased extinction risks (Frankel, 1974; Lande and Barrowclough, 1987; Spielman et al., 2004; Leimu et al., 2006; O'Grady et al., 2006). Therefore, the low measures of diversity observed in populations of *E. pseudovulatum* further justify the need for regular monitoring of the species. Additionally, because Beaverdam Creek, Little Piney Creek, Happy Hollow Creek,

and the Piney River contain unique haplotypes, each tributary system should be regularly monitored for species persistence and abundance. If any one of these populations were extirpated, then a portion of the already low overall genetic diversity would be lost. This is especially important for the Beaverdam Creek population because of its particularly unique haplotype assemblage.

It is important to note that unequal sample sizes between the tributary systems could have unintended effects on our results of genetic diversity (Table 11). Only 5 individuals each were examined from East Fork Wolf Creek, Only Creek, and Happy Hollow Creek, while 10 were examined from Little Piney Creek, 15 from Beaverdam Creek, and 19 from Piney River. Additional sampling could reveal more haplotypes within populations and could alter our interpretation of the genetic diversity within tributary systems, especially for the systems with a smaller sample size.

If one or more of the tributary systems require propagation or translocation of the species, the results from our phylogenetic analyses provide crucial context for carefully selecting a source population (George et al., 2009). It is critical to select a source population that is closely related to the target population so no unnatural genetic variation is introduced (George et al., 2009). For example, if the East Fork Wolf Creek population were to be extirpated, individuals from the Piney River would make a better source population than individuals from Beaverdam Creek because they share a haplotype with East Fork Wolf Creek.

Phylogeography

With respect to gene flow patterns of the species, the results support that the Duck River does not act as a hard barrier for *E. pseudovulatum*. Haplotype 1 was shared among all of the tributary systems except Beaverdam Creek, which supports that gene flow has likely been

maintained historically at some level between these five populations (Avise, 2004; George et al., 2006). Alternatively, the unique haplotype assemblage of Beaverdam Creek and relatively high divergence from the other populations suggest that Beaverdam Creek has likely experienced long-standing isolation or reduced genetic exchange with other tributaries except possibly Little Piney Creek (Near et al., 2001; Avise, 2004; Powers et al., 2004; George et al., 2006; Ray et al., 2006; Lang and Echelle, 2011). Bayesian analysis provided additional evidence for this in that haplotypes sorted into two well-supported clades: 1) Beaverdam Creek and two individuals from Little Piney Creek; and 2) the other five populations, including eight individuals from Little Piney Creek (Figure 23). Additionally, average sequence divergence was higher between the two clades than within each clade.

Although mitochondrial markers prove useful for phylogeographic studies, because mtDNA is maternally inherited, patterns detected reflect only the female lineage. Therefore, if males and females exhibit different dispersal behaviors, then it is possible that the true dynamics of gene flow in the species may differ from what is interpreted from mtDNA data analysis (Avise, 2004; Freeland, 2006). This is particularly important in cases where genetic differentiation is observed, but merely reflects female philopatry (where females show no to little dispersal), while males disperse longer distances and maintain gene flow between populations (Hoelzer, 1997). This point concerns the interpretation of the genetic isolation of the Beaverdam Creek population. However, it's unlikely that female philopatry explains the observed differentiation in Beaverdam Creek when there are no signs of sex-biased gene flow in the other five tributary systems. Additionally, it's important to note that mtDNA does not measure contemporary gene flow (Avise, 2004). Nuclear markers are sexually unbiased and would provide useful information to better assess the effects of female philopatry and measure

contemporary gene flow in *E. pseudovulatum* (Hoelzer, 1997; Avise, 2004; Freeland, 2006; Hollingsworth and Near, 2009).

The use of haplotype networks and phylogenetic analyses have played a critical role in understanding phylogeographic patterns of various freshwater fishes, including darters (Echelle et al., 1975; Near et al., 2001; Powers et al., 2004; Near and Keck, 2005; George et al., 2006; Ray et al., 2006; Hollingsworth and Near, 2009; Lang and Echelle, 2011; Bossu et al., 2013; Echelle et al., 2015), suckers (Berendzen et al., 2003), catfishes (Turner and Robison, 2006; Blanton et al., 2013), and other taxa (Bernatchez and Wilson, 1998). For example, George et al. (2006) concluded genetic isolation between populations of *Percina burtoni* based on the concordance of their haplotype network and phylogenetic tree results. As demonstrated in numerous studies, the use of these tools can provide reliable information for the development of our understanding of the genetic diversity in context of the geographic range of a species.

The fact that *E. pseudovulatum* showed little genetic differentiation across its range, excluding Beaverdam Creek, was unexpected. Numerous fishes considered to be adapted to small, headwater streams, such as *E. pseudovulatum*, exhibit considerable genetic isolation and differentiation across their distribution (Echelle et al., 1975; Starnes and Etnier, 1986; Bernatchez and Wilson, 1998; Turner and Trexler, 1998; Near et al., 2001; Berendzen et al., 2003; Powers et al., 2004; George et al., 2006; Ray et al., 2006; Turner and Robison, 2006; Hollingsworth and Near, 2009; Sterling et al., 2012; Blanton et al., 2013; Bossu et al., 2013; Fluker et al., 2014; Echelle et al., 2015). One example is the microendemism and intraspecific divergence of barcheek darters (*Oopareia*, a subclade of *Goneaperca*; Near et al., 2011; Hollingsworth and Near, 2009). Hollingsworth and Near (2009) found that four of the seven species of barcheek darters they examined showed intraspecific genetic divergence, with *E.*

basilare showing the greatest structure with five divergent clades restricted to five tributaries of the Caney Fork system (Cumberland River drainage) that had a most recent common ancestor as old as 8.0 million years ago.

One hypothesis for such genetic differentiation is the effect of life history strategy on the dispersal ability of a species (Turner and Trexler, 1998; Turner and Robison, 2006; Hollingsworth and Near, 2009; Fluker et al., 2014). Life history traits, such as spawning habitat, egg size, and fecundity, have been shown to influence dispersal and gene flow patterns in darters (Turner and Trexler, 1998; Hollingsworth and Near, 2009; Fluker et al., 2014). Turner and Trexler (1998) found that female size, fecundity, and egg size were associated with gene flow for 15 darter species. They found that darters that exhibited small female size, low fecundity, and large egg size (such as *E. flabellare*) had reduced gene flow when compared to darters of opposing life history traits (such as *Percina caprodes*). They hypothesized that larger individuals can travel further because of greater energy reserves, and that larger eggs result in larger larvae which drift less and become benthic sooner than species with smaller larvae that experience farther dispersal rates (Simon and Wallus, 2005). Fluker et al. (2014) discuss how species with strict breeding habitats may be limited to headwater streams, thus reducing dispersal via larger rivers, which lack such habitat requirements.

Despite sharing life history traits associated with reduced gene flow, *E. pseudovulatum* shows little limitation of gene flow between populations, except for Beaverdam Creek. Another species that has defied the evidence of upland-adapted fishes being restricted by large rivers is *E. proeliare*. Lang and Echelle (2011) recovered clades that included individuals from populations on both sides of the lower Mississippi River, including one clade that had individuals from the Lake Pontchartrain drainage and individuals west of the Mississippi River, suggesting that the

species has maintained gene flow at some level across the Mississippi River. The findings of *E. proeliare* and *E. pseudovulatum* do not support common hypotheses of smaller, headwater adapted fishes being restricted by large river barriers and may suggest additional research could reveal this pattern in other fishes of similar life history traits.

Given the overall pattern of maintained gene flow among populations of *E. pseudovulatum*, the largely unique haplotype assemblage observed in Beaverdam Creek is interesting. There are no known physical barriers, such as a dam or a waterfall, present between Beaverdam Creek and the Duck River, which could possibly explain this pattern. One of the most common explanations for phylogeographic breaks in freshwater fishes is vicariance resulting from past geologic events. Historical patterns of eastern North American fluvial geomorphology are commonly used to explain the distribution of fishes, and vice versa (Starnes and Etnier, 1986; Mayden, 1988; Bernatchez and Wilson, 1998; Near et al., 2001; Berendzen et al., 2003; Near and Keck, 2005; George et al., 2006; Ray et al., 2006; Lang and Echelle, 2011; Blanton et al., 2013; Bossu et al., 2013). For example, Near et al. (2001) examined the phylogeography of *Percina evides* using the mitochondrial gene cytochrome *b* to test competing hypotheses of the geological processes that explain Central Highlands biogeography. The same approaches can be used to examine the influence of historical vicariant events on fish distributions and diversity on localized scales, such as cases of stream capture events (Kuehne and Bailey, 1961; Branson and Batch, 1971; Buth and Mayden, 1981; Hocutt and Wiley, 1986; Lang and Echelle, 2011).

Therefore, examining the underlying geology of the range of *E. pseudovulatum* (Figure 24) can provide insights into the historical geomorphology of the area, giving us ideas of how past geologic events have shaped current patterns of genetic diversity. Bedrock substrata, such as

limestone, are formed by long-term erosion caused by the persistently-strong flow of a stream, thereby reflecting the position a stream's channel, while fine substrata, such as sand, are deposited by areas in a stream with less flow, such as the inside of a bend in the stream (Charlton, 2007). Something unique to Beaverdam Creek is a large section of sand that goes 4 km upstream from the stream's confluence with the Duck River (Figure 24). The other tributary systems primarily exhibit limestone at their mouth with no or only a small segment of sand. Also, there is a trail of limestone that goes around the southern border of the large section of sand.

From what is known about reconstructing fluvial geomorphology using current geology, the pattern seen at the confluence of Beaverdam Creek may suggest that the channel of the Duck River once traveled through the position of the limestone that borders the large sand deposit and at some point was captured at the northern tips of the bend, resulting in a shift in the Duck River 4 km north of its former position. If true, this event could have effectively formed a barrier for fish dispersal, either by unsuitable habitat or by physical disconnection from the Duck River, thereby isolating *E. pseudovulatum* long enough for the Beaverdam Creek population to accumulate 13 mutational differences from the other tributary systems. Subsequently, the remaining long-stretch of mostly sand deposits in the lower reaches of Beaverdam Creek may continue to limit movement into Beaverdam Creek. However, given that Beaverdam Creek haplotypes are found in Little Piney Creek, it is not an absolute barrier to dispersal.

Numerous studies have provided evidence of stream capture and other forms of changes in fluvial patterns to explain the unique distributions of fishes (Kuehne and Bailey, 1961; Branson and Batch, 1971; Buth and Mayden, 1981; Hocutt and Wiley, 1986; Lang and Echelle, 2011). For example, stream capture between the Middle Tennessee River and Mobile Basin has

been proposed based on shared taxa. These systems share species, such as *Lythrurus lirus* (Snelson Jr., 1980; Starnes and Etnier, 1986), and *L. bellus*, *Nocomis leptocephalus*, and *Noturus funebris*, which are widespread within the Mobile Basin and elsewhere but limited to only Bear and Yellow creeks in the Tennessee River drainage, suggesting Bear and Yellow creeks were once part of the Mobile System but were captured by the Tennessee River (Boschung and Mayden, 2004). Although shifts in the fluvial geomorphology of the Duck River may have occurred, our hypothesis is based solely on the phylogeographic results of *E. pseudovulatum* and the geologic map of the area. There is no additional evidence to support this hypothesis and further research would be required, such as investigating the phylogeography of fishes ecologically similar to *E. pseudovulatum* within its range. Haplotypes unique to Beaverdam Creek populations of other species could potentially exist and would provide supplemental support for this hypothesis. Additional information about the geological history of the area could also provide better context for the explanation of the pattern we see in *E. pseudovulatum*.

The implications of the phylogeographic results on the conservation status of the species could be promising. If individuals disperse through the Duck River to other tributary systems, then the risk of local extirpation of smaller tributary system populations is lower due to the ability of the species to recolonize (Fagan, 2002). Additionally, gene flow among the different populations should increase the likelihood of maintaining genetic diversity, which is important to the health of the species (Frankel, 1974; Avise, 2004). However, it is important to note this study does not consider the rate of genetic exchange between populations, which would more accurately determine the probability of recolonization and the ability of the species to maintain moderate levels of genetic diversity (Avise, 2004). Also, the fact that Beaverdam Creek is likely isolated from the other populations could mean the Beaverdam Creek population and its unique

haplotypes could be at greater risk of local extirpation and that efforts should be made to monitor that population, specifically as a separate unit relative to others.

Considerations for Federal Listing

The criteria that the US Fish and Wildlife Service uses to determine the listing of a species are: 1) the present or threatened destruction, modification, or curtailment of its habitat or range; 2) overutilization for commercial, recreational, scientific, or educational purposes; 3) disease or predation; 4) the inadequacy of existing regulatory mechanisms; and 5) other natural or manmade factors affecting its survival (Endangered Species Act, Section 4). When considering these criteria, it is recommended that *E. pseudovulatum* does not currently warrant federal protection. Despite the finding that localities of *E. pseudovulatum* were, on average, of suboptimal conditions based on habitat assessments, there were no major threats observed to the habitat or range of the species. When comparing results to those of Ceas and Page (1995), the abundance and distribution of the species have remained relatively stable over the past 20 years. Also, there was no evidence of *E. pseudovulatum* being overutilized for commercial, recreational, scientific, or educational purposes, and there were no indications observed of disease or predation that could threaten the persistence of the species.

The last two criteria for listing are more concerning. It appears *E. pseudovulatum* is not regularly monitored. And although there are no manmade factors affecting its survival, there are natural factors that could potentially affect the persistence of the species, including the lower abundance, population size, and genetic diversity in the smaller tributary systems. However, *E. pseudovulatum* appears to be relatively abundant throughout its range. And the potential for recolonization of smaller tributary systems helps to buffer the threat of local extirpation, as well as contribute to the possibility of maintaining gene flow among and between populations,

thereby reducing the risks of low genetic diversity. Nevertheless, the species should be regularly monitored with special consideration given to the smaller tributary systems (Happy Hollow Creek, Little Piney Creek, Only Creek, and East Fork Wolf Creek) and the genetically distinct Beaverdam Creek population.

Table 1. Number of kick-sets performed at a site determined by the average wetted stream width (modified from Abernathy and Mattingly, 2011).

Average wetted stream width (W)	Number of kick-sets
$W < 6 \text{ m}$	20
$6 \text{ m} \leq W < 11 \text{ m}$	40
$11 \text{ m} \leq W < 16 \text{ m}$	60
$16 \text{ m} \leq W$	80

Table 2. Categories used to define habitat characteristics of kick-set patches. General habitat characteristics of riffle, run, or pool were determined by three transect-measurements of flow and depth.

Category	Definition
Bank	Area of stream adjacent to a stream bank.
Undercut Bank	A bank where the current has eroded the edge so that some of the bank overhangs the water surface.
Root wad	Roots from woody plants that are exposed and in the water.
Debris	Any type of organic material that can provide cover, such as leaves, sticks, or logs, in the stream.
Backwater	Pool that is outside of the main channel of the stream and has no flow.
Vegetation	Living vegetation that is in the water, such as grasses.
Bedrock	Open areas where exposed, flat bedrock makes up the substrate.

Table 3. Estimates of abundance (N) and lower and upper bounds of population size per 75 meter reach per season using a modified Petersen method equation (Seber, 1982). Locality numbers refer to those provided in Figure 2. Appendix A provides detailed locality information.

Locality	Tributary System	Spring			Fall		
		N	Lower	Upper	N	Lower	Upper
3	Beaverdam Ck.	7	0.9	7	14	3.7	27.5
5	Beaverdam Ck.	149	34	149	17	4.5	32.3
9	E. Fk. Wolf Ck.	35	10.4	67.5	27	4.5	27
11	Little Piney Ck.	47	14.1	90.3	5	1	10.4
12	Happy Hollow Ck.	13	3.4	25.6	62	13.7	62
13	Only Ck.	32	6.7	32	15	4.1	29.4
16	Piney R.	164	92.3	345.6	125	48.2	278
17	Piney R.	62	18.9	118.9	67	28.9	148.6
18	Piney R.	258	121.2	546.3	89	27.5	170.3
19	Piney R.	144	67.4	305.4	53	26.5	360.6
20	Piney R.	7	0.9	7	8	1.8	16.1
23	Piney R.	59	18	113.2	34	7.2	34

Table 4. Comparison of relative abundances from Ceas and Page (1995) and the current study. Collections from Ceas and Page were made on 3 April, 1995, by sampling for 15 minutes to 2 hours using seines and dip nets; data were reported as categories: abundant (greater than 20 individuals), common (10-20), present (less than 10), and absent. Abundance values shown for the current study reflect relative abundance of the first pass for each site sampled by season. Numbers in parentheses represent the total number of individuals captured, including second pass captures and young-of-the-year. Dashes represent sites for which abundance data were not available. Locality numbers refer to those provided in Figure 2. Appendix A provides detailed locality information.

Locality	Tributary System	Relative Abundance		
		1995	2014 Spring	2014 Fall
1	Beaverdam Ck.	10-20	11	7
2	Beaverdam Ck.	<10	10	2
3	Beaverdam Ck.	-	3 (5)	9 (10)
4	Beaverdam Ck.	<10	20	5
5	Beaverdam Ck.	-	14 (23)	6 (9)
6	Beaverdam Ck.	-	33	-
7	Beaverdam Ck.	>20	8	4
8	Beaverdam Ck.	<10	10	3
9	E. Fk. Wolf Ck.	-	7 (14)	3 (9)
10	E. Fk. Wolf Ck.	<10	5	2
11	Little Piney Ck.	0	5 (19)	2 (4)
12	Happy Hollow Ck.	<10	6 (8)	6 (14)
13	Only Ck.	<10	10 (12)	7 (9)
14	Piney R.	-	13	3
15	Piney R.	-	8	1
16	Piney R.	>20	44 (68)	20 (92)
17	Piney R.	<10	17 (22)	15 (41)
18	Piney R.	<10	36 (66)	11 (35)
19	Piney R.	>20	24 (48)	18 (31)
20	Piney R.	-	1 (4)	2 (7)
21	Piney R.	-	1	1
22	Piney R.	0	7	3
23	Piney R.	>20	11 (19)	6 (13)
24	Piney R.	10-20	50	5
25	Piney R.	-	-	1

Table 5. Associated species collected at least once during the study. Taxa are ranked in descending order of the frequency of occurrence at the 25 localities sampled. Occupied localities are listed and locality numbers refer to those provided in Figure 2. Appendix A provides detailed locality information.

Taxa	Frequency	Localities
<i>Clinostomus funduloides</i>	25	1-25
<i>Cottus carolinae</i>	25	1-25
<i>Etheostoma flabellare</i>	24	1-12, 14-25
<i>Campostoma oligolepis</i>	23	1-12, 14-24
<i>Etheostoma flavum</i>	23	1-12, 14-22, 24-25
<i>Semotilus atromaculatus</i>	19	1, 4-5, 7-19, 22-24
<i>Chrosomus erythrogaster</i>	17	1-2, 4, 7, 9-15, 17-20, 23-24
<i>Etheostoma bison</i>	16	1-10, 14-17, 23-24
<i>Etheostoma caeruleum</i>	16	4-8, 10-11, 15-18, 20-22, 24-25
<i>Fundulus catenatus</i>	16	1, 2, 4-8, 15-18, 20-22, 24-25
<i>Rhinichthys obtusus</i>	11	7, 9-11, 13-17, 19, 23
<i>Hypentelium nigricans</i>	10	3, 5-6, 8, 16, 20-22, 24-25
<i>Lythrurus fasciolaris</i>	10	2-3, 6, 8, 15-18, 21, 24
<i>Lepomis cyanellus</i>	9	1, 4, 8, 16, 18-19, 20, 22, 24
<i>Lampetra aepyptera</i>	8	1-2, 4, 6, 16, 18, 22, 24
<i>Notropis telescopus</i>	8	5-6, 8, 18, 20-22, 25
<i>Noturus fasciatus</i>	7	1-7
<i>Pimephales notatus</i>	7	2, 3, 15-17, 21
<i>Ambloplites rupestris</i>	6	2, 16, 20-22, 24

Table 5. Continued.

<i>Etheostoma blennioides</i>	6	6, 8, 18, 20, 22, 25
<i>Etheostoma rufilineatum</i>	6	8, 15, 20-22, 25
Lamprey ammocoete	6	3, 5-6, 11, 20-21
<i>Notropis leuciodus</i>	6	6, 8, 15, 18, 20, 25
<i>Etheostoma planasaxatile</i>	5	6, 8, 18, 20, 25
<i>Lepomis macrochirus</i>	5	3, 16-18, 24
<i>Luxilus chrysocephalus</i>	5	2, 8, 17, 20-21
<i>Nocomis effusus</i>	5	1, 3, 5-6, 8
<i>Etheostoma blenni</i>	4	4, 8, 15, 20
<i>Fundulus olivaceus</i>	3	2, 4, 6
<i>Gambusia affinis</i>	2	8, 21
<i>Hybopsis amblops</i>	2	17, 24
<i>Micropterus dolomieu</i>	2	21-22
<i>Cottus bairdii</i>	1	4
<i>Cyprinella galactura</i>	1	21
<i>Esox americanus</i>	1	8
<i>Etheostoma obama</i>	1	20
<i>Etheostoma zonale</i>	1	25
<i>Micropterus punctulatus</i>	1	3
<i>Percina evides</i>	1	8

Table 6. Reach-scale variables with p-values of linear regression testing relationship between first pass relative abundance and each variable. Habitat assessment categories are presented by their ID number and title. Wetted stream width, water temperature, and specific conductance are presented by season due to significant seasonal variation. Refer to Figure 4 for scores of habitat assessment categories.

Variable	P-value
1-Epifaunal Substrate/Available Cover	0.895
2-Embeddedness	0.568
3-Velocity/Depth Regime	0.726
4-Sediment Deposition	0.852
5-Channel Flow Status	0.858
6-Channel Alteration	0.481
7-Frequency of Riffles	0.406
8-Bank Stability	0.126
9-Vegetative Protection	0.880
10-Riparian Vegetative Zone Width	0.642
Total Score	0.808
Wetted stream width (spring)	0.537
Wetted stream width (fall)	0.186
Water temperature (spring)	0.875
Water temperature (fall)	0.117
Specific conductance (spring)	0.412
Specific conductance (fall)	0.453

Table 7. Values of total habitat assessment score per locality, and wetted stream width (m), water temperature ($^{\circ}\text{C}$), and specific conductance ($\text{mS}/\text{cm}^{\circ}$) per locality for each season. For total habitat assessment scores, letters in parentheses reflect the following conditions: O – optimal (150-200); S – suboptimal (100-150); M – marginal (50-100); and P – poor (0-50). Locality numbers refer to those provided in Figure 2. Appendix A provides detailed locality information.

Locality	Habitat Score	Width		Temperature		Conductance	
		Spring	Fall	Spring	Fall	Spring	Fall
1	145 (S)	4.97	3.26	10.2	23.1	0.043	0.056
2	102.5 (S)	6.2	4.1	11.5	24.3	0.052	0.063
3	148.5 (S)	9.43	4.9	10.9	21.5	0.058	0.077
4	152 (O)	14.67	9.26	12.8	22.0	0.068	0.085
5	144.5 (S)	8.8	5.53	11.5	20.3	0.08	0.106
6	110 (S)	23.46	-	10.7	-	0.079	-
7	146.5 (S)	3.2	1.67	13.9	24.0	0.079	0.087
8	161 (O)	9	9.56	14.6	22.3	0.072	0.096
9	143.5 (S)	5.83	4.1	10.2	20.7	0.059	0.081
10	158 (O)	6.57	4.46	10.1	20.5	0.073	0.108
11	155 (O)	5.1	4.1	14.8	17.8	0.098	0.188
12	183.5 (O)	3.67	2.53	11.3	18.6	0.146	0.223
13	155 (O)	1.5	2.13	13.6	16.6	0.129	0.175
14	140 (S)	4.83	3.83	8.6	19.2	0.139	0.195
15	166.5 (O)	11.37	12.56	11.0	-	0.166	-
16	138.5 (S)	10.27	7.72	11.9	20.7	0.135	0.128
17	133 (S)	3.9	5.93	14.8	11.7	0.15	0.176
18	167.5 (O)	8.3	8.76	13.2	16.2	0.197	0.292
19	149 (S)	4.6	2.51	16.2	16.5	0.224	0.354
20	163.5 (O)	23.16	21.68	15.9	10.5	0.204	-
21	148 (S)	9.43	8.67	10.2	24.4	0.25	0.343
22	140 (S)	9.3	8.2	12.8	22.9	0.171	0.296
23	153.5 (O)	3.9	2.4	11.5	21.2	0.034	0.155
24	140 (S)	6.8	7.23	11.8	23.3	0.173	0.304
25	135 (S)	-	31.87	-	-	-	-

Table 8. Descriptive parameters of principal component analyses for "Total," spring, and fall. Includes number of patches (n), component loadings (PC values), and the eigenvalue, percent of total variance explained, and p-value of Kolmogorov-Smirnov two-sample test per principal component (asterisks indicate statistical significance; $p < 0.05$). The variable backwater was not included in fall because there were too few occurrences of backwater habitat.

Component Loadings	Total (n=524)		Spring (n=264)		Fall (n=260)	
	PC1	PC2	PC1	PC2	PC1	PC2
Flow	-0.546	0.251	-0.6	0.09	-0.621	0.388
Depth	0.31	-0.604	0.099	-0.594	0.678	-0.426
Bank	0.635	0.386	0.748	0.133	0.225	0.729
Undercut	0.679	0.317	0.711	0.231	0.515	0.521
Roots	0.597	0.3	0.599	0.182	0.428	0.485
Debris	0.328	-0.665	0.246	-0.659	0.473	-0.408
Backwater	0.066	-0.207	0.135	-0.327	-	-
Vegetation	0.048	-0.181	0.119	-0.319	-0.036	-0.026
Bedrock	-0.264	0.282	-0.029	0.53	-0.438	-0.189
Eigenvalue	1.798	1.365	1.888	1.388	1.762	1.573
Percent of total variance explained	19.98	15.164	20.974	15.428	22.021	19.659
KS test p-value	<0.01*	0.01*	<0.01*	0.056	<0.01*	0.32

Table 9. Descriptive parameters of classification and regression trees seen in Figures 9 through 14. Shown are: the figure number, minimum number of cases for parent and child nodes, cross-validation risk estimate and its standard error (the proportion of cases that are incorrectly classified), and the overall percent correct (the correct classification rate, i.e., the proportion of cases that are correctly classified).

Tree	Figure	Parent	Child	Risk	SE	% Correct
Total	9	25	25	0.223	0.018	79.2
Spring	10	75	5	0.227	0.026	79.5
Fall	11	5	1	0.296	0.028	74.2
Males	12	75	1	0.061	0.015	93.9
Females	13	10	10	0.125	0.020	89.4
Juveniles	14	5	1	0.121	0.020	91.3

Table 10. Observations of land-use at localities collected. Left and right bank determined while facing downstream. Locality numbers refer to those provided in Figure 2. Appendix A provides detailed locality information. Total habitat assessment scores of localities are presented in Table 7. Criteria for habitat assessments can be seen in “Habitat Assessment Data Sheet” in Appendix B.

Locality	Land-use notes
1	Large open field on right bank, possibly used as a hayfield, with riparian zone width less than 6 m. Left bank riparian zone width 10 to greater than 18 m wide on a hillside.
2	Church property (field and parking lot) on left bank, which leaves a riparian zone width less than 7 m. Right bank riparian zone 3-10 m wide but has a farm on the other side of it. One collector noted that the locality has changed much since first visiting the site for another study in 2013: it has accumulated more sediment over the years which has caused the channel to shift positions and gravel bars to increase.
3	Between the spring and fall sampling of this site, about 15 m upstream of where reach starts the land-owners clear-cut a large area and laid a large amount of gravel which formed a new channel forking off from the original channel of the stream, diverting water away from the reach. Although water was still flowing through the original channel, it became much slower and more pools were present than observed in the spring. This was possibly done to form land for cattle farming.
4	Left bank riparian zone 3-12 m wide due to row crop field. Right bank riparian zone 16 to greater than 18 m wide with row crop field on other side of riparian zone.
5	Riparian zone 5-15 m wide on both banks with the left bank riparian zone bordered by road and the right bank bordered by row crop field. One neighboring property owner mentioned it often floods heavily and scours banks.
6	Left bank riparian zone 2-5 m wide adjacent to a residential property and an open field. Right bank riparian zone 5-10 m wide. About 0.5 mi upstream of locality, the stream runs alongside the road where it appears to be heavily eroding the hillside underneath the road.

Table 10. Continued.

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| 7 | Riparian zone 5-8 m wide on both banks, with a large row crop field adjacent to left bank riparian zone and residential properties adjacent to right bank riparian zone. |
| 8 | Riparian zone 15 to greater than 18 m wide on both banks. No particular land-use observed in nearby area. |
| 9 | Left bank riparian zone 5-15 m wide. Right bank riparian zone 8-10 m wide due to adjacent dirt road and row crop field. |
| 10 | Left bank riparian zone 12-18 m wide with adjacent small horse field.
Right bank riparian zone 5-12 m wide with adjacent small cabin. |
| 11 | Riparian zone greater than 18 m on both banks. Signs that are posted indicate the land is leased out, likely for hunting. The dirt/gravel road runs close alongside the stream and the road runs through the stream about four or five times. |
| 12 | Riparian zone greater than 18 m on both banks. Appears to be very natural area. Property was owned by nearby state correctional facility and was transferred to TWRA to be formed into a WMA in 2014. |
| 13 | Left bank riparian zone 10-18 m wide. Right bank riparian zone 1-10 m wide due to adjacent road and open field. Area appears to be mostly residential, with no riparian zone just downstream of reach due to stream flowing through mowed lawns. |
| 14 | Left bank riparian zone 8-16 m wide. Right bank riparian zone 5-10 m wide with adjacent dirt road. No farms seen in immediate area; sparsely dispersed residential properties. A clear-cut area occurs about 50 m along stream where powerlines cross. |
| 15 | Right bank riparian zone 10 to greater than 18 m wide. Left bank riparian zone 5-10 m wide due to residential property adjacent to stream. |
| 16 | Right bank riparian zone 10 to greater than 18 m wide. Left bank riparian zone 5-10 m wide due to adjacent dirt road and residential properties. |
| 17 | Left bank riparian zone 15 to greater than 18 m wide. Right bank riparian zone 1-5 m wide due to residential property. A paved road runs close alongside stream. Less than a mile downstream of reach, the stream runs through a cattle farm property where they've clear-cut the area for about 100 m length of stream. |
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Table 10. Continued.

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| 18 | Right bank riparian zone 15 to greater than 18 m wide. Left bank riparian zone 10-15 m wide with adjacent dirt road and a small horse field with residential property. |
| 19 | Right bank riparian zone greater than 18 m wide. Left bank riparian zone 0-5 m due to adjacent paved road and open field. Just upstream of reach is the John Noel State Natural Area at Bon Aqua which is a primarily forested area that provides a thick riparian zone. |
| 20 | Riparian zone 11 to greater than 18 m wide on both banks. Several open and row crop fields were observed while driving to site. The paved road fords directly through the stream with a low bridge structure that likely is flooded during high-water events. |
| 21 | Left bank riparian zone 10-20 m wide with adjacent residential property. Right bank riparian zone 1-8 m wide due to adjacent row crop field. |
| 22 | Left bank riparian zone 1-5 m wide with a mowed lawn of residential property adjacent to stream. Right bank riparian zone 1-7 m wide with adjacent row crop field. Bank stabilization (rip-rap) located on left bank for a 10-15 m length of the stream. |
| 23 | Left bank riparian zone 8-15 m wide with adjacent paved road. Right bank riparian zone 11-18 m wide with adjacent residential property driveway. Bank stabilization (rip-rap) located on left bank for a 10 m length of stream. |
| 24 | Left bank riparian zone 4-10 m wide with adjacent mowed lawn of church property. Right bank riparian zone 7-10 m wide with adjacent row crop field. There appears to be more row crop fields along the stream in the area of this site. |
| 25 | Left bank riparian zone 10-15 m wide. Right bank riparian zone 7-10 m wide with adjacent open field, possibly a cattle farm due to potential signs of cattle accessing stream. |
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Table 11. Summary statistics of variation in the mitochondrial ND2 gene. The top 11 rows list the frequency of individuals examined with haplotypes (H1-11) for each tributary system population and frequency of total individuals examined. The bottom three rows include number of individuals examined (n), haplotype diversity (Hd), and nucleotide diversity (π) by tributary system and total individuals.

Haplotype	Piney R.	Happy Hollow Ck.	Only Ck.	E. Fk. Wolf Ck.	Little Piney Ck.	Beaverdam Ck.	Total
H1	14	3	5	5	7	-	34
H2	1	-	-	-	-	-	1
H3	1	-	-	-	-	-	1
H4	-	2	-	-	-	-	2
H5	-	-	-	-	1	-	1
H6	3	-	-	-	-	-	3
H7	-	-	-	-	1	12	13
H8	-	-	-	-	-	1	1
H9	-	-	-	-	-	1	1
H10	-	-	-	-	-	1	1
H11	-	-	-	-	1	-	1
n	19	5	5	5	10	15	59
Hd	0.450	0.600	0	0	0.533	0.371	0.624
π	0.0006	0.0005	0	0	0.0046	0.0004	0.0054

Figure 1. Identified historical localities for *Etheostoma pseudovulatum*. Numbers are site identifiers used in Figures 2, 22, and 23, Tables 3, 4, 5, 7, 10, and 11, and Appendix A, which provides specific locality information for each. Letters refer to tributary systems of the Duck River: P = Piney River; O = Only Creek; H = Happy Hollow Creek; L = Little Piney Creek; W = East Fork Wolf Creek; and B = Beaverdam Creek.

Tennessee

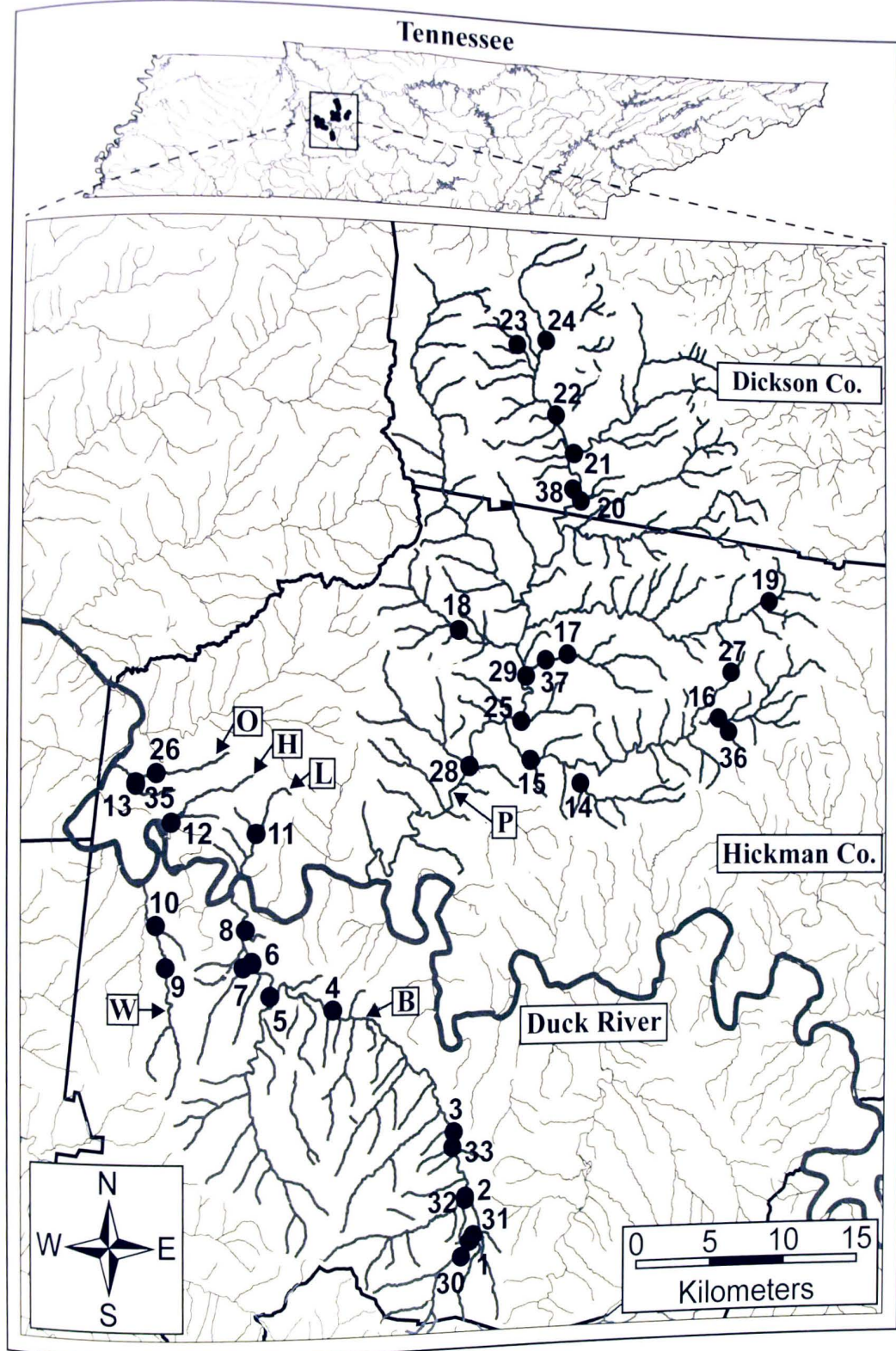


Figure 2. Localities sampled in this study. Numbers are site identifiers used in Figures 22, and 3, Tables 3, 4, 5, 7, 10, and 11, and Appendix A, which provides specific locality information for each. White circles represent localities where presence, abundance, and population size were estimated. Solid black circles represent localities where only the presence of *Etheostoma pseudovulatum* was measured. Letters refer to tributary systems of the Duck River: P = Piney River; O = Only Creek; H = Happy Hollow Creek; L = Little Piney Creek; W = East Fork Wolf Creek; and B = Beaverdam Creek.

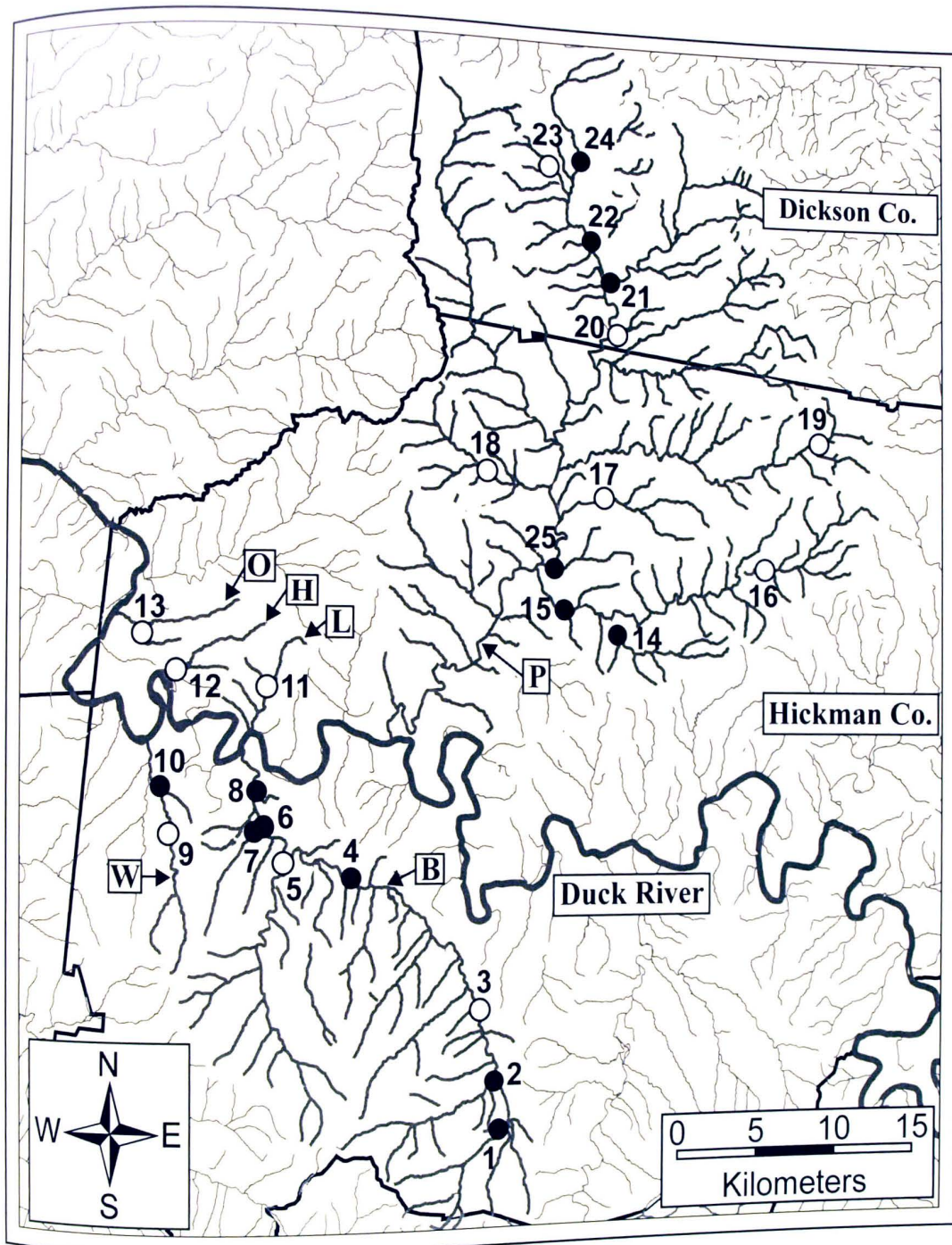
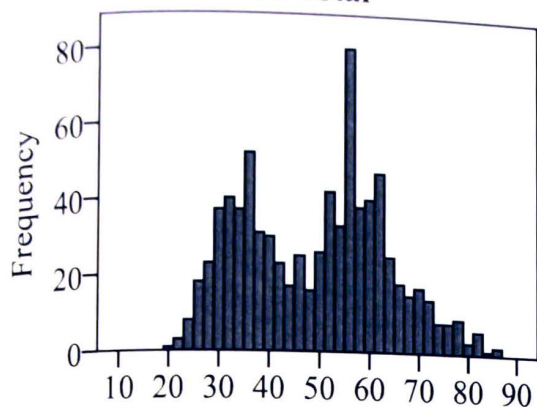
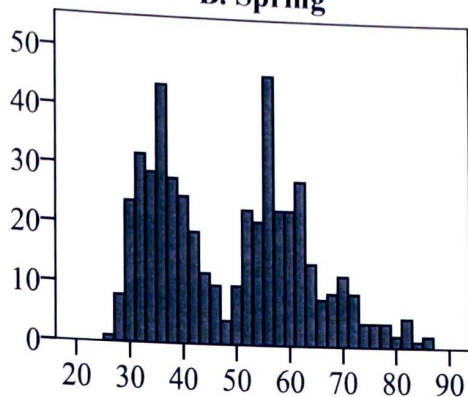


Figure 3. Frequency histograms of total length (mm) for: A) all individuals collected; B) all individuals collected in the spring; C) all individuals collected in the fall; D) males collected in the spring; E) females collected in the spring; and F) juveniles collected in the spring. The "juveniles" category includes fishes too small to confidently sex, with the exception of a few larger individuals where the sex was undetermined.

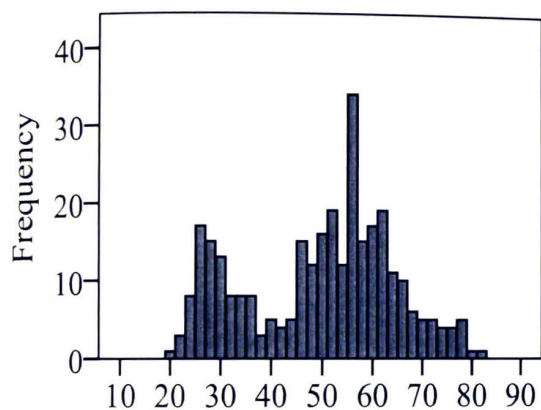
A. Total



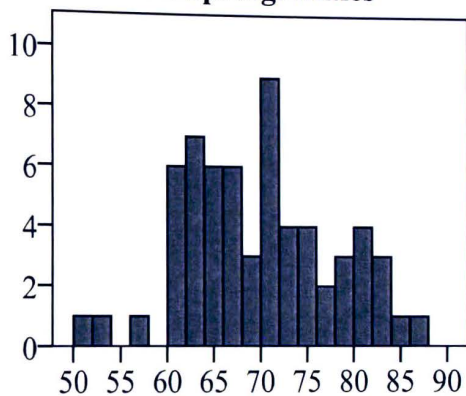
B. Spring



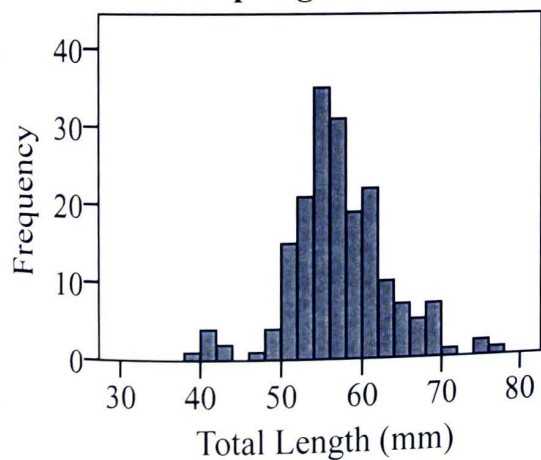
C. Fall



D. Spring: Males



E. Spring: Females



F. Spring: Juveniles

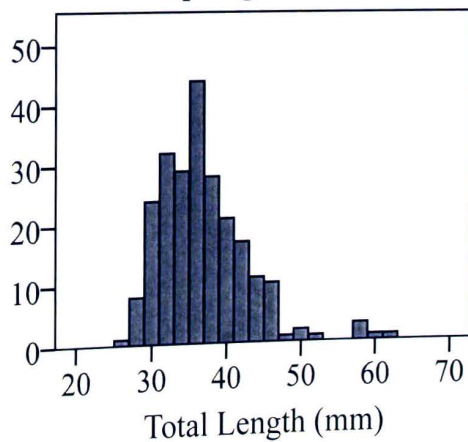
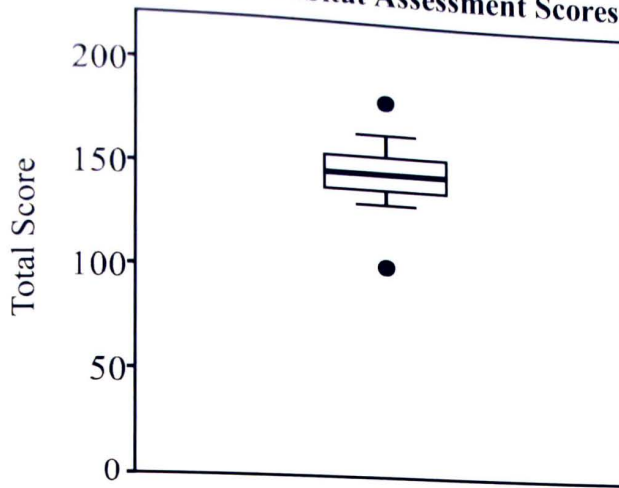


Figure 4. Box plots of: A) total habitat assessment scores; and B) scores of the ten habitat parameter categories included in the assessment sheet. The habitat parameter categories are: 1) epifaunal substrate/available cover, 2) embeddedness, 3) velocity/depth regime, 4) sediment deposition, 5) channel flow status, 6) channel alteration, 7) frequency of riffles, 8) bank stability, 9) vegetative protective, and 10) riparian vegetative zone width (Appendix B). Upper and lower edges of boxes represent upper and lower quartiles, black lines inside boxes represent median values, lines extending from boxes represent non-extreme values outside of interquartile range, and outliers are represented as black dots.

A. Total Habitat Assessment Scores



B. Habitat Parameter Category Scores

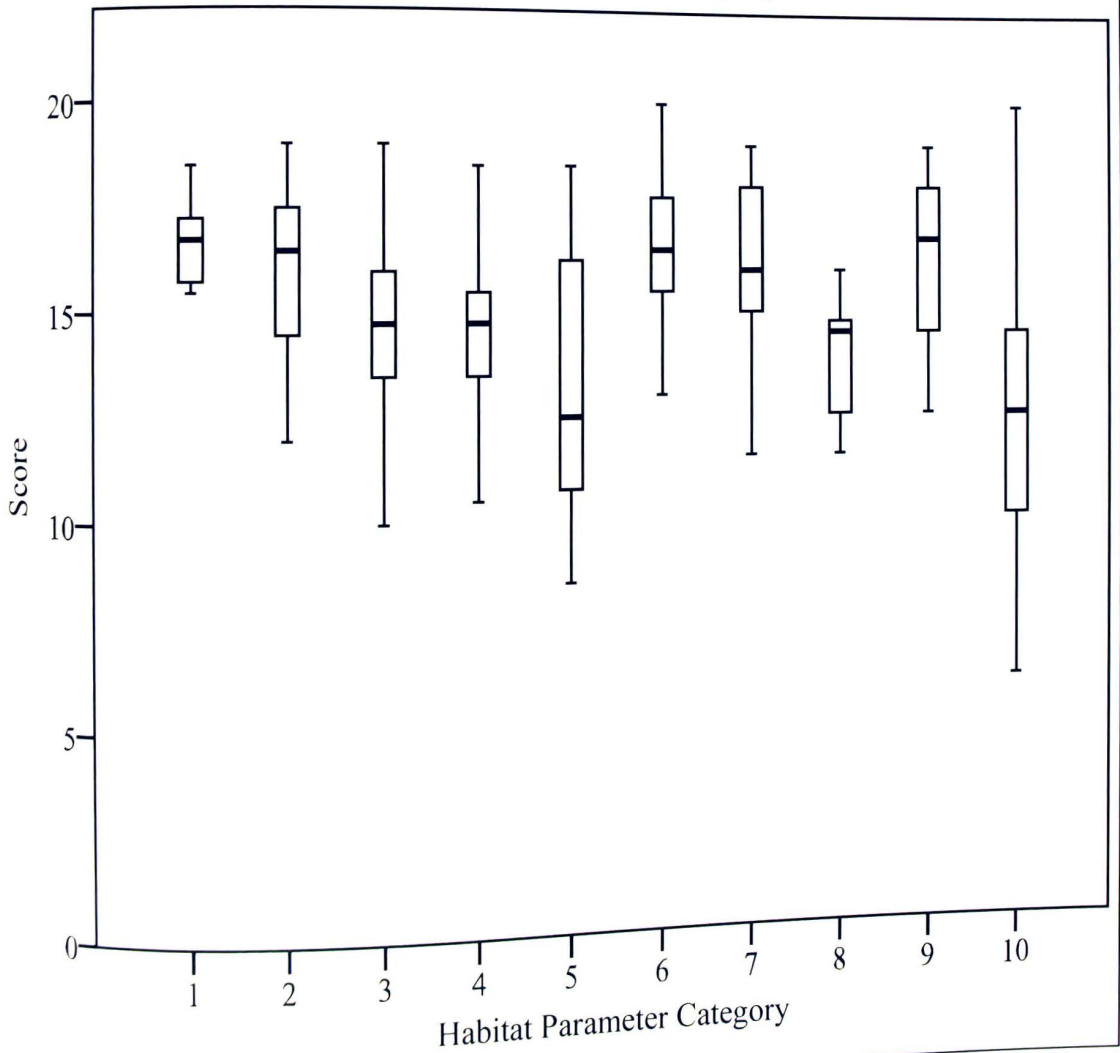


Figure 5. Box plots of: A) wetted stream width; B) water temperature; and C) specific conductance. White boxes represent spring values. Gray boxes represent fall values. Upper and lower edges of boxes represent the upper and lower quartiles, black lines inside boxes represent median values, lines extending from boxes represent non-extreme values outside of interquartile range, and outliers are represented as black dots. Asterisks reflect a significant difference between spring and fall measurements.

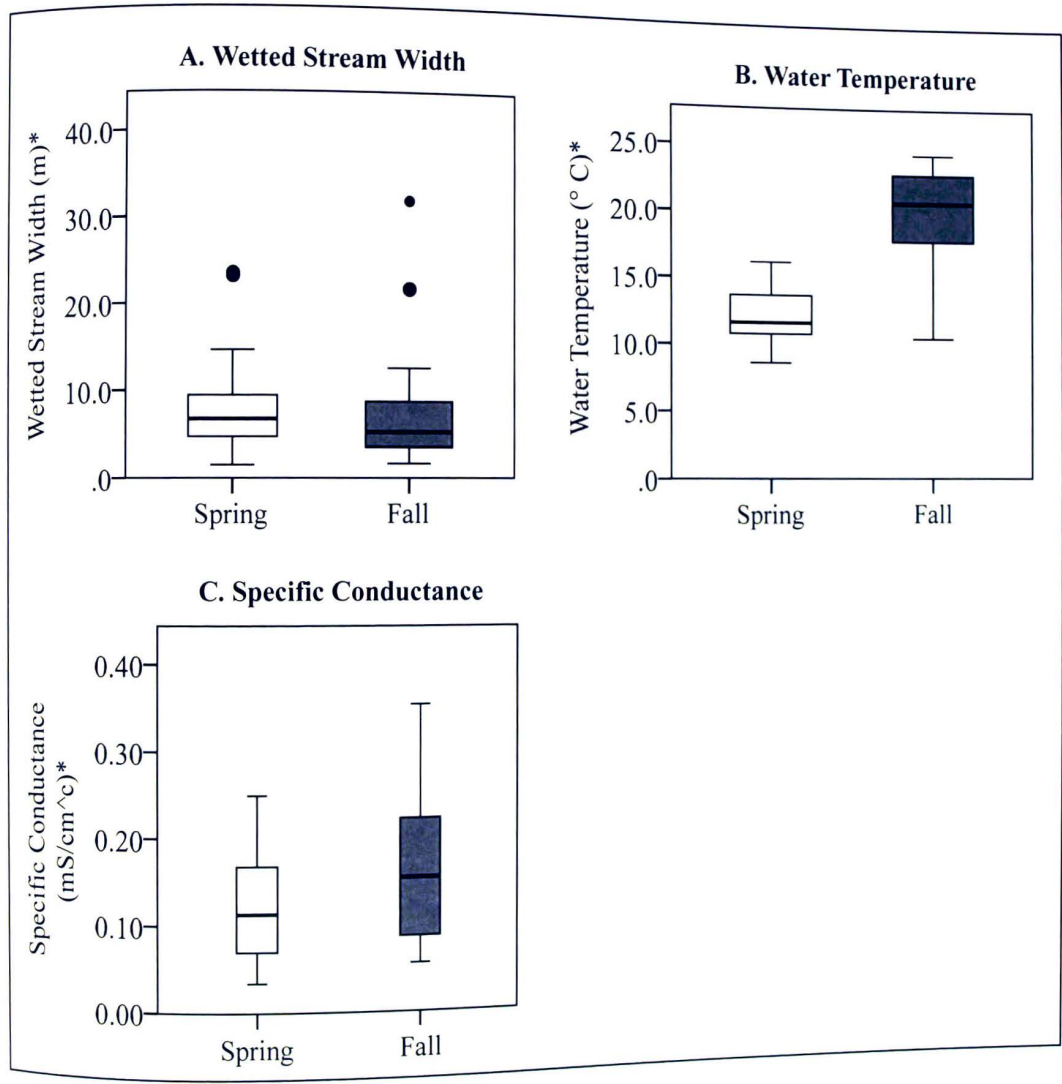


Figure 6. Scatterplot of factor scores resulting from the principal component analysis (PCA) of "Total" (spring and fall samples pooled) habitat data. Circles represent habitat patches sampled where *Etheostoma pseudovulatum* was present; gray X's represent patches where the species was absent (524 patches were included in total). Values next to PC titles reflect the percent of total variance explained by each component. Listed on each axis are the variables with the highest component loadings (Table 8). Direction arrows indicate the pattern of increase or decrease of variables.

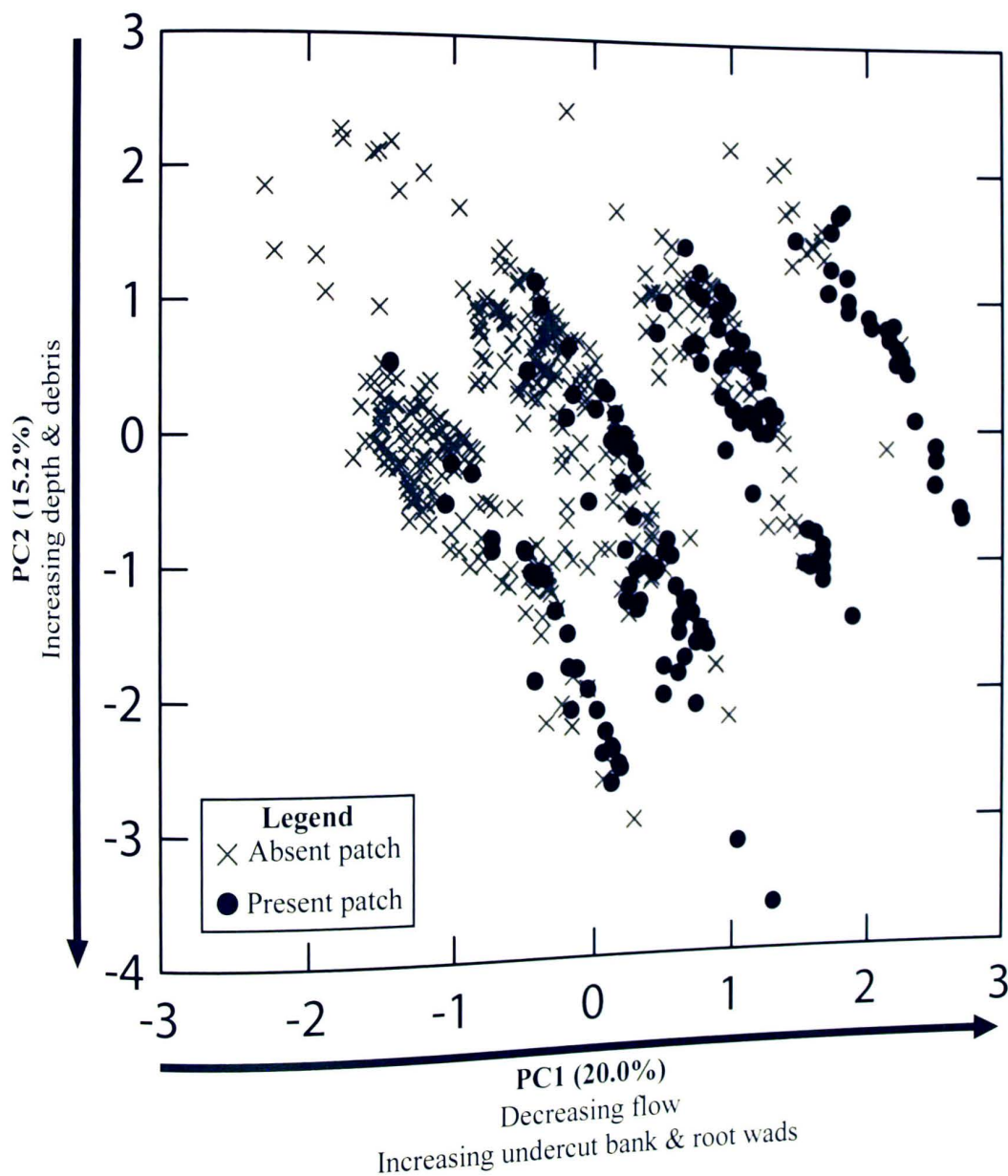


Figure 7. Scatterplot of factor scores resulting from the principal component analysis (PCA) of spring habitat data. Circles represent habitat patches sampled where *Etheostoma pseudovulatum* was present; gray X's represent patches where the species was absent (264 patches were included in total). Values next to PC titles reflect the percent of total variance explained by each component. Listed on each axis are the variables with the highest component loadings (Table 8). Direction arrows indicate the pattern of increase or decrease of variables.

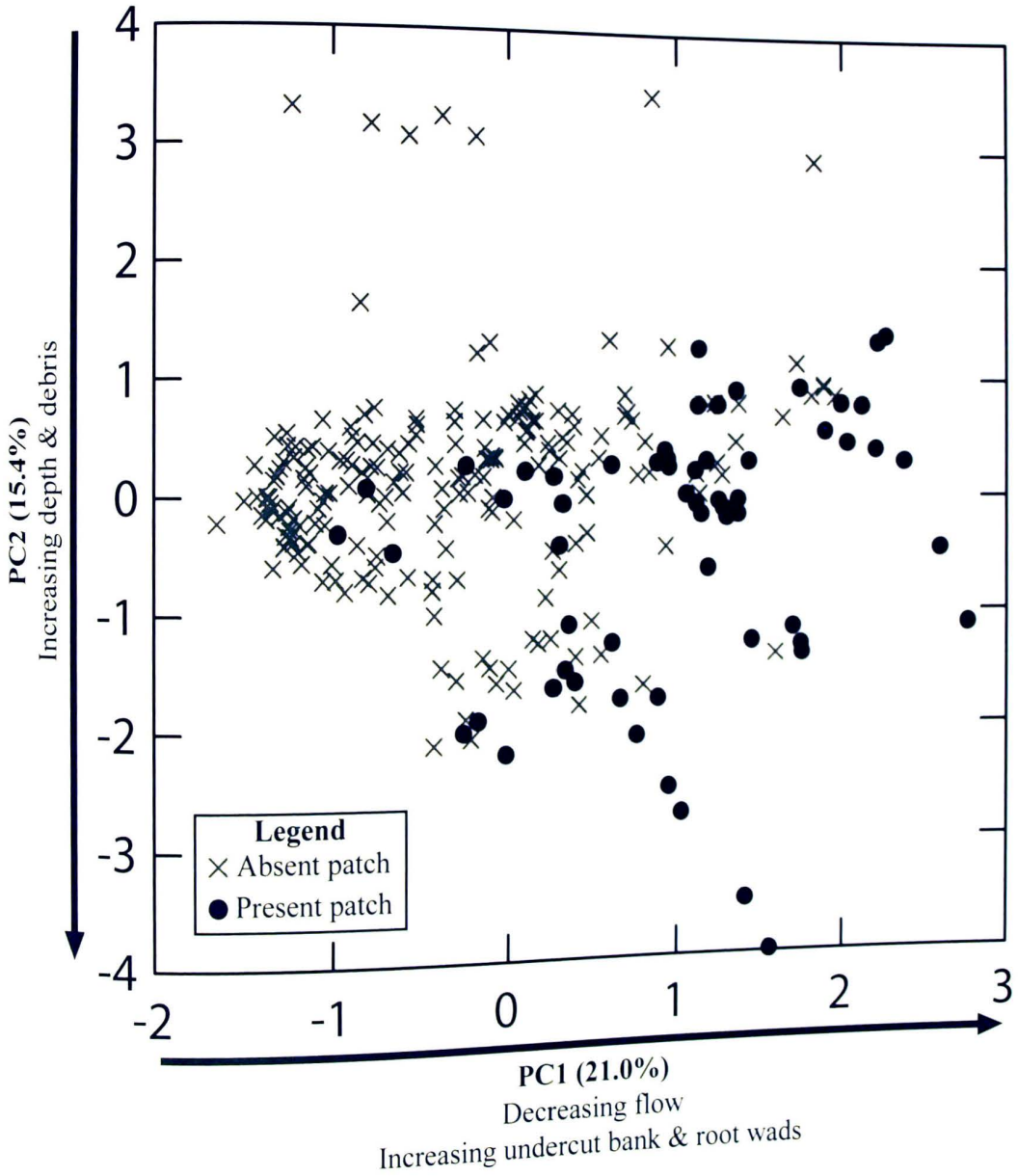


Figure 8. Scatterplot of factors scores resulting from the principal component analysis (PCA) of fall habitat data. Circles represent habitat patches sampled where *Etheostoma pseudovulatum* was present; gray X's represent patches where the species was absent (260 patches were included in total). Values next to PC titles reflect the percent of total variance explained by each component. Listed on each axis are the variables with the highest component loadings (Table 8). Direction arrows indicate the pattern of increase or decrease of variables.

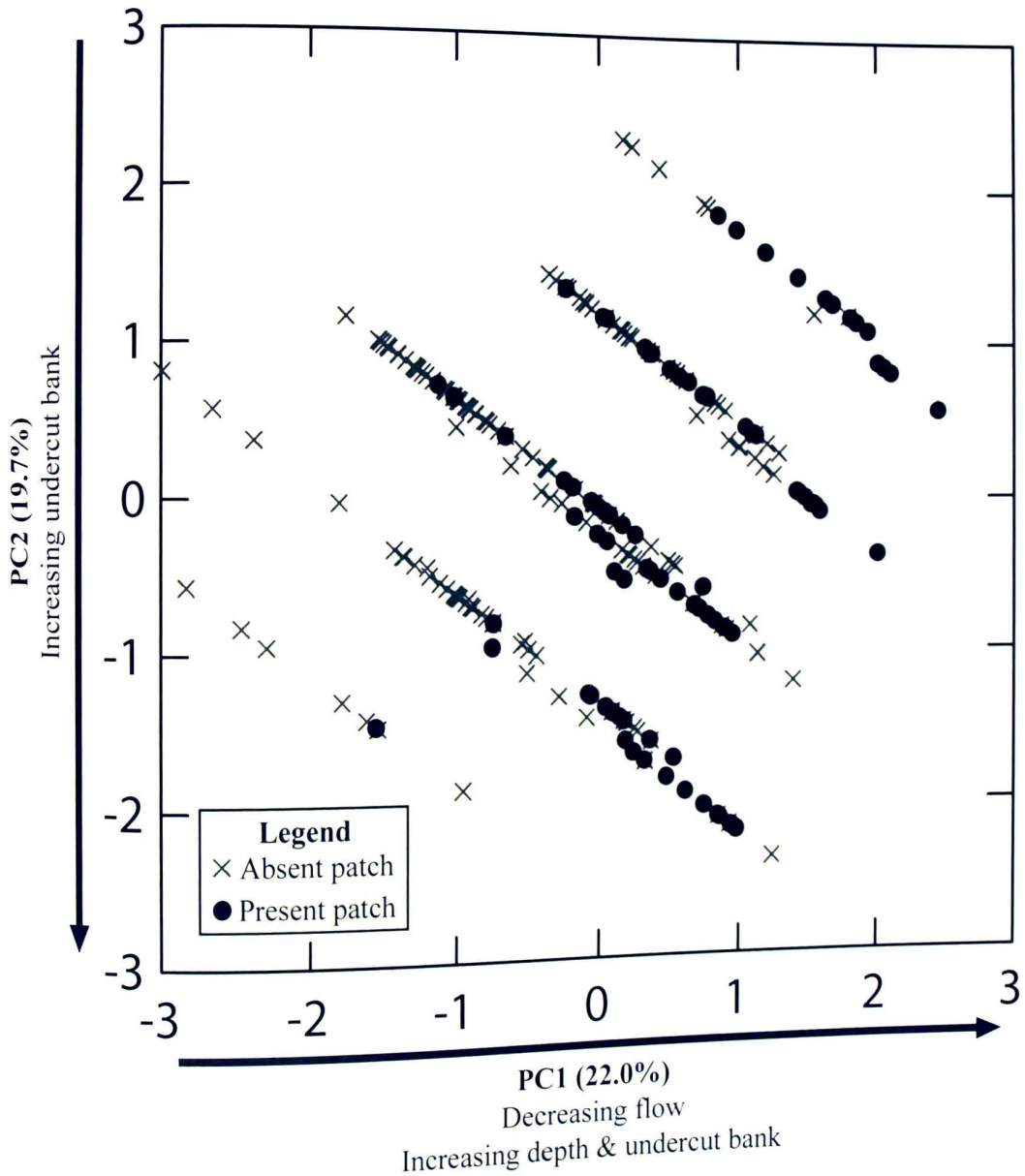


Figure 9. Classification and regression tree (CART) analysis of habitat data with patches of *Etheostoma pseudovulatum* present and absent for "Total" (spring and fall patches pooled together) habitat data. Branches represent splitting of data based on explanatory variables listed by values or categories (i.e., present/absent). Bold numbers in boxes are the predicted probability of *E. pseudovulatum* presence and the number of present patches over the total number of patches sampled for that particular suite of habitat characteristics is provided in parentheses. The correct classification rate of this model was 79.2% with a cross-validation risk estimate of 0.223. Values of other descriptive parameters can be seen in Table 9.

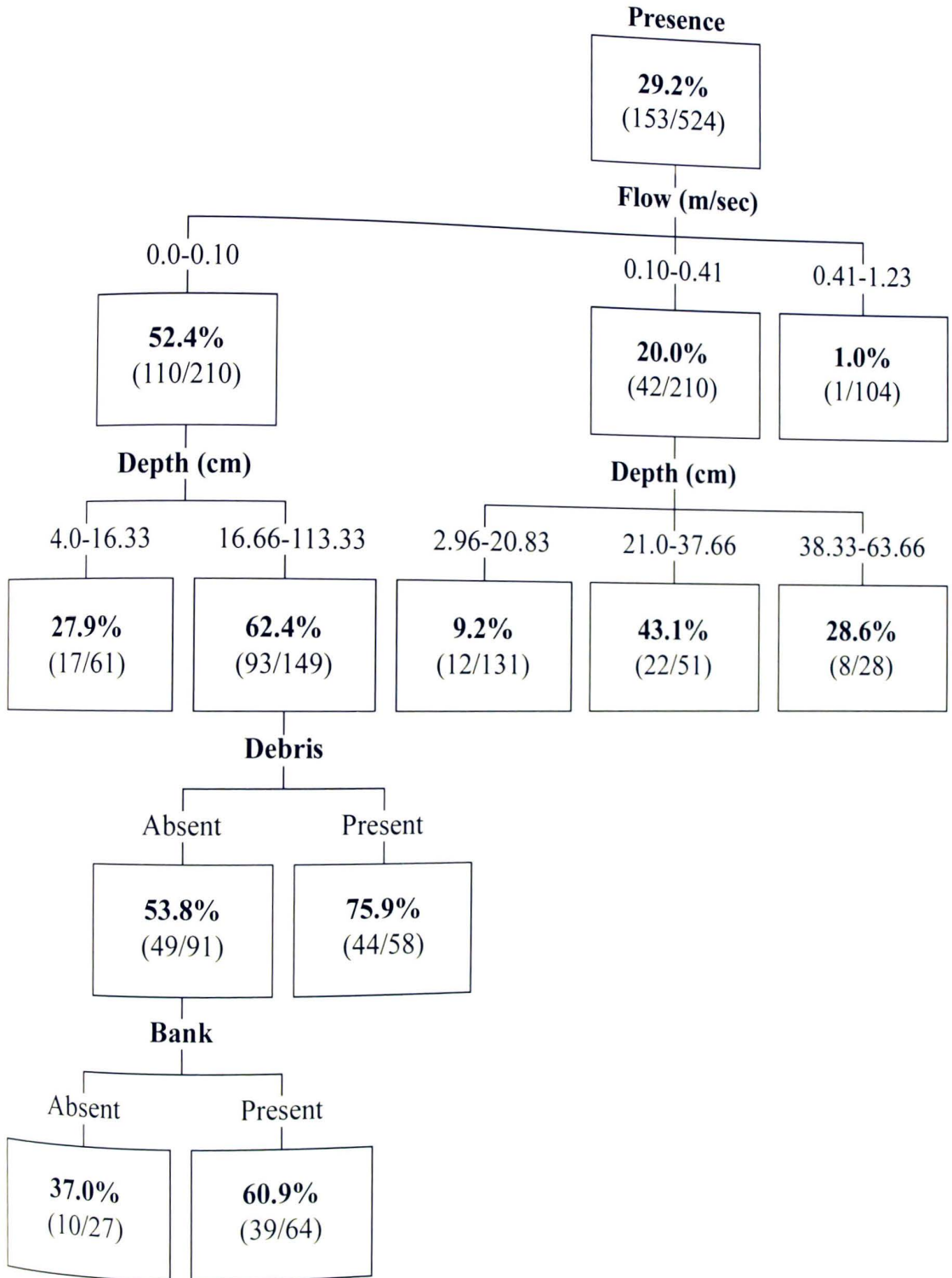


Figure 10. Classification and regression tree (CART) analysis of habitat data with patches of *Etheostoma pseudovulatum* present and absent for spring samples only. Branches represent splitting of data based on explanatory variables listed by values or categories (i.e., present/absent). Bold numbers in boxes are the predicted probability of *E. pseudovulatum* presence and the number of present patches over the total number of patches sampled for that particular suite of habitat characteristics is provided in parentheses. The correct classification rate of this model was 79.5% with a cross-validation risk estimate of 0.227. Values of other descriptive parameters can be seen in Table 9.

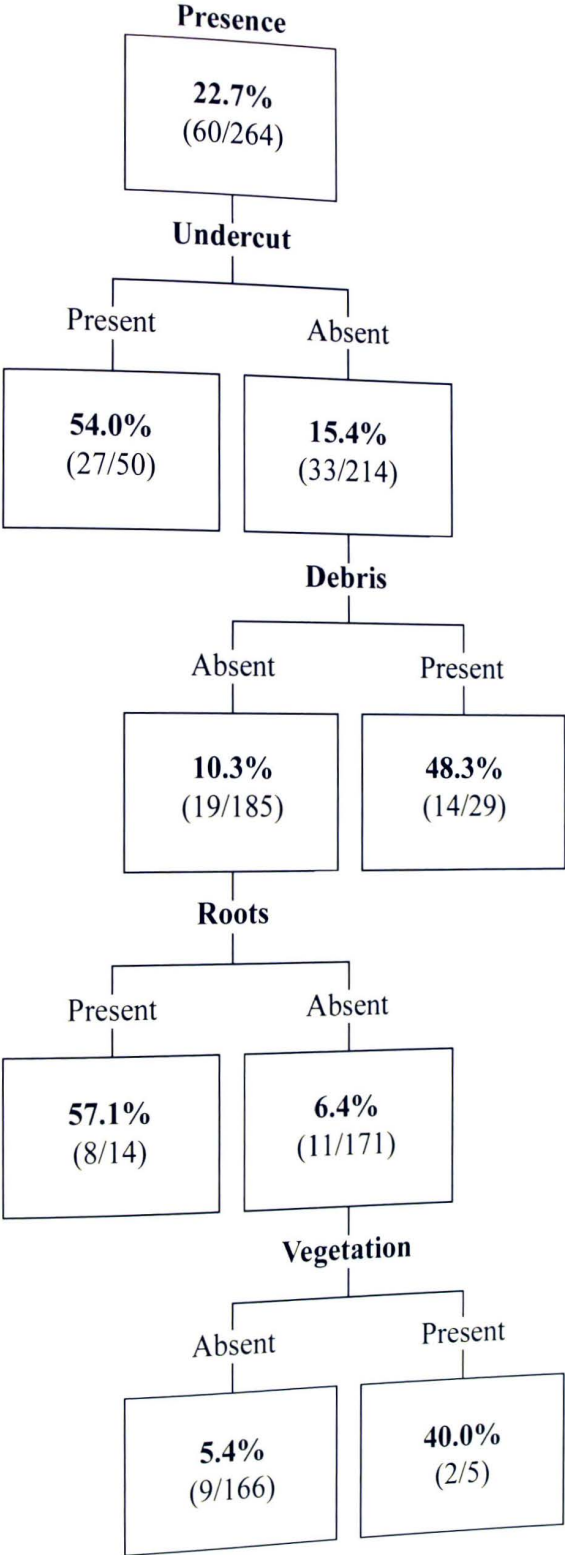


Figure 11. Classification and regression tree (CART) analysis of habitat data with patches of *Etheostoma pseudovulatum* present and absent for fall samples only. Branches represent splitting of data based on explanatory variables listed by values or categories (i.e., present/absent). Bold numbers in boxes are the predicted probability of *E. pseudovulatum* presence and the number of present patches over the total number of patches sampled for that particular suite of habitat characteristics is provided in parentheses. The correct classification rate of this model was 74.2% with a cross-validation risk estimate of 0.296. Values of other descriptive parameters can be seen in Table 9.

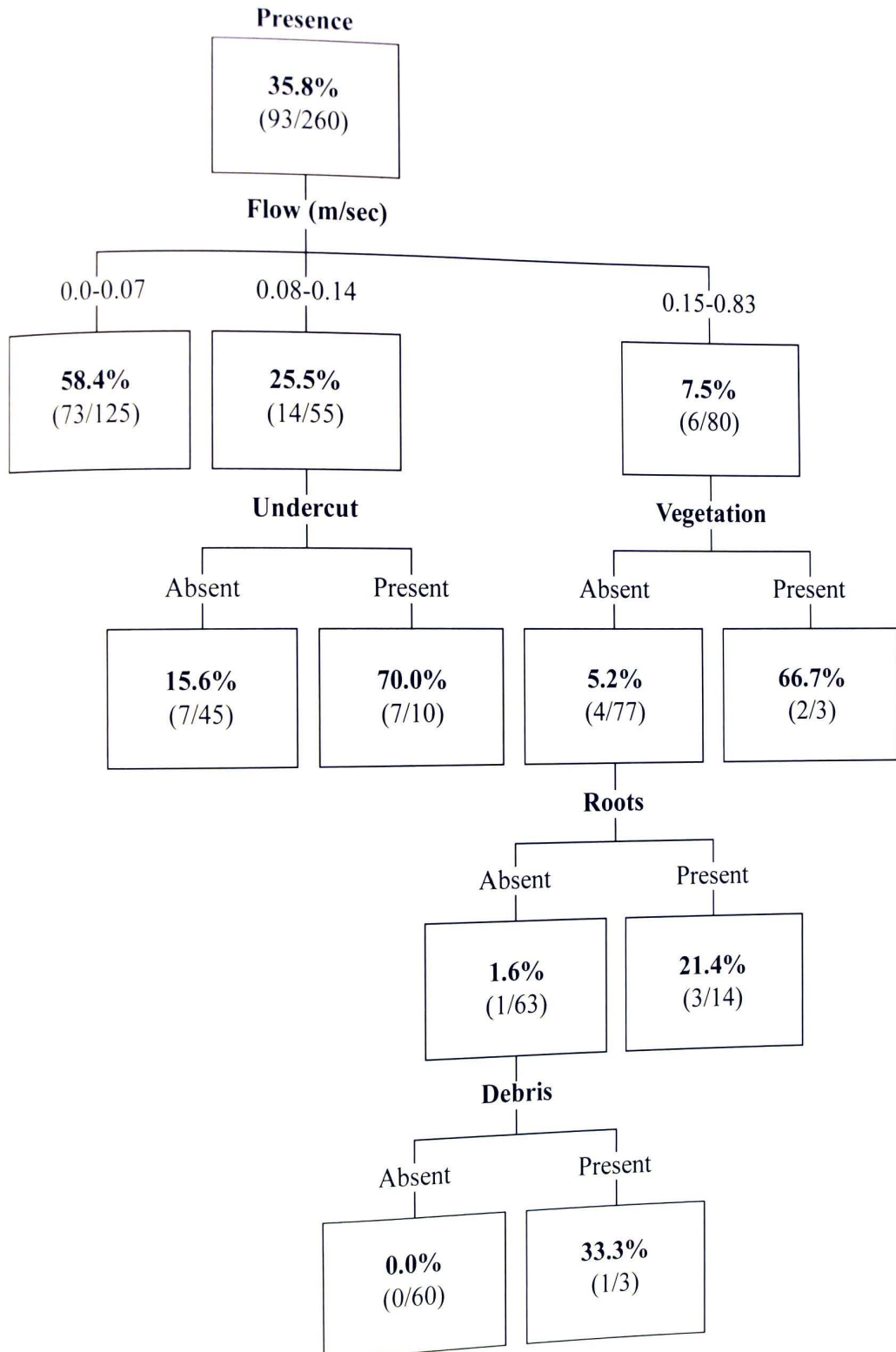


Figure 12. Classification and regression tree (CART) analysis of habitat data with patches of *Etheostoma pseudovulatum* present and absent for males in spring. Branches represent splitting of data based on explanatory variables listed by values or categories (i.e., present/absent). Bold numbers in boxes are the predicted probability of *E. pseudovulatum* presence and the number of present patches over the total number of patches sampled for that particular suite of habitat characteristics is provided in parentheses. The correct classification rate of this model was 93.9% with a cross-validation risk estimate of 0.061. Values of other descriptive parameters can be seen in Table 9.

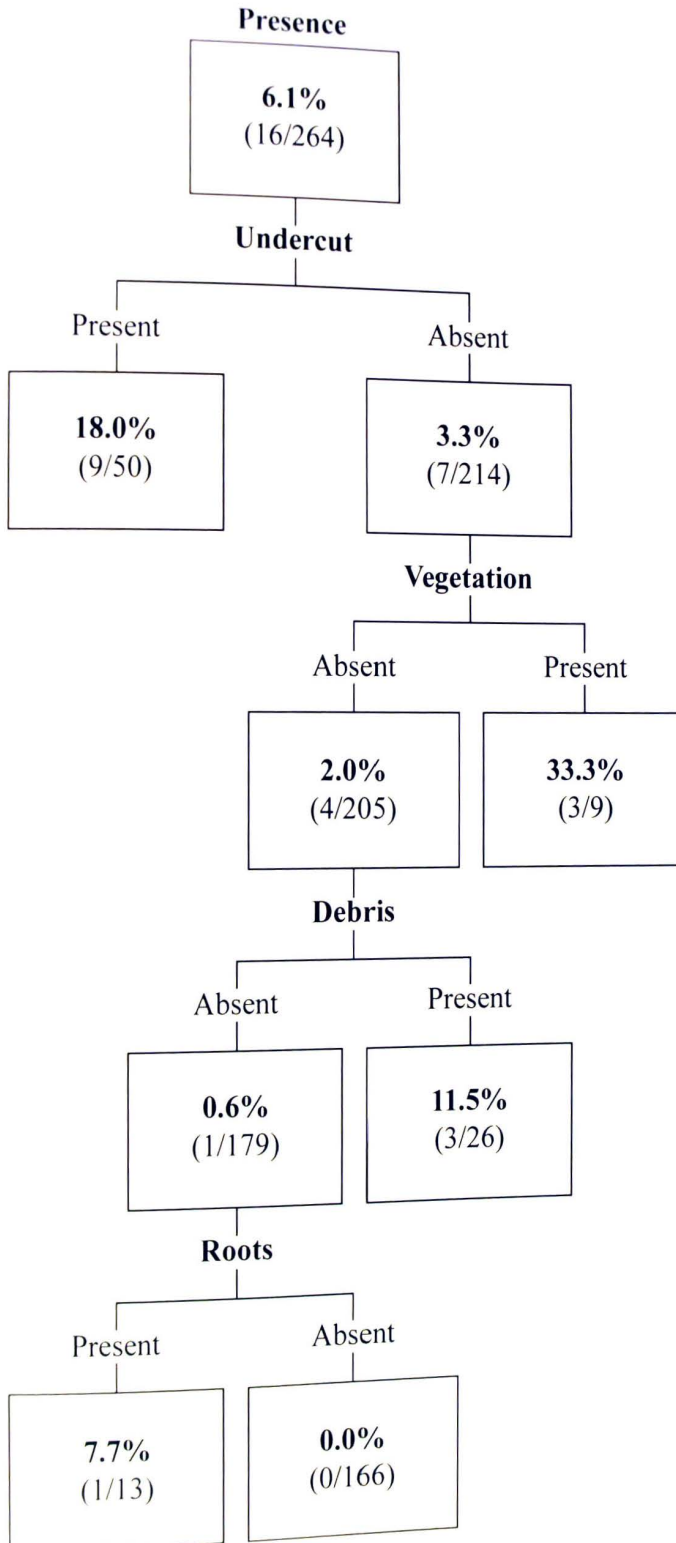


Figure 13. Classification and regression tree (CART) analysis of habitat data with patches of *Etheostoma pseudovulatum* present and absent for females in spring. Branches represent splitting of data based on explanatory variables listed by values or categories (i.e., present/absent). Bold numbers in boxes are the predicted probability of *E. pseudovulatum* presence and the number of present patches over the total number of patches sampled for that particular suite of habitat characteristics is provided in parentheses. The correct classification rate of this model was 89.4% with a cross-validation risk estimate of 0.125. Values of other descriptive parameters can be seen in Table 9.

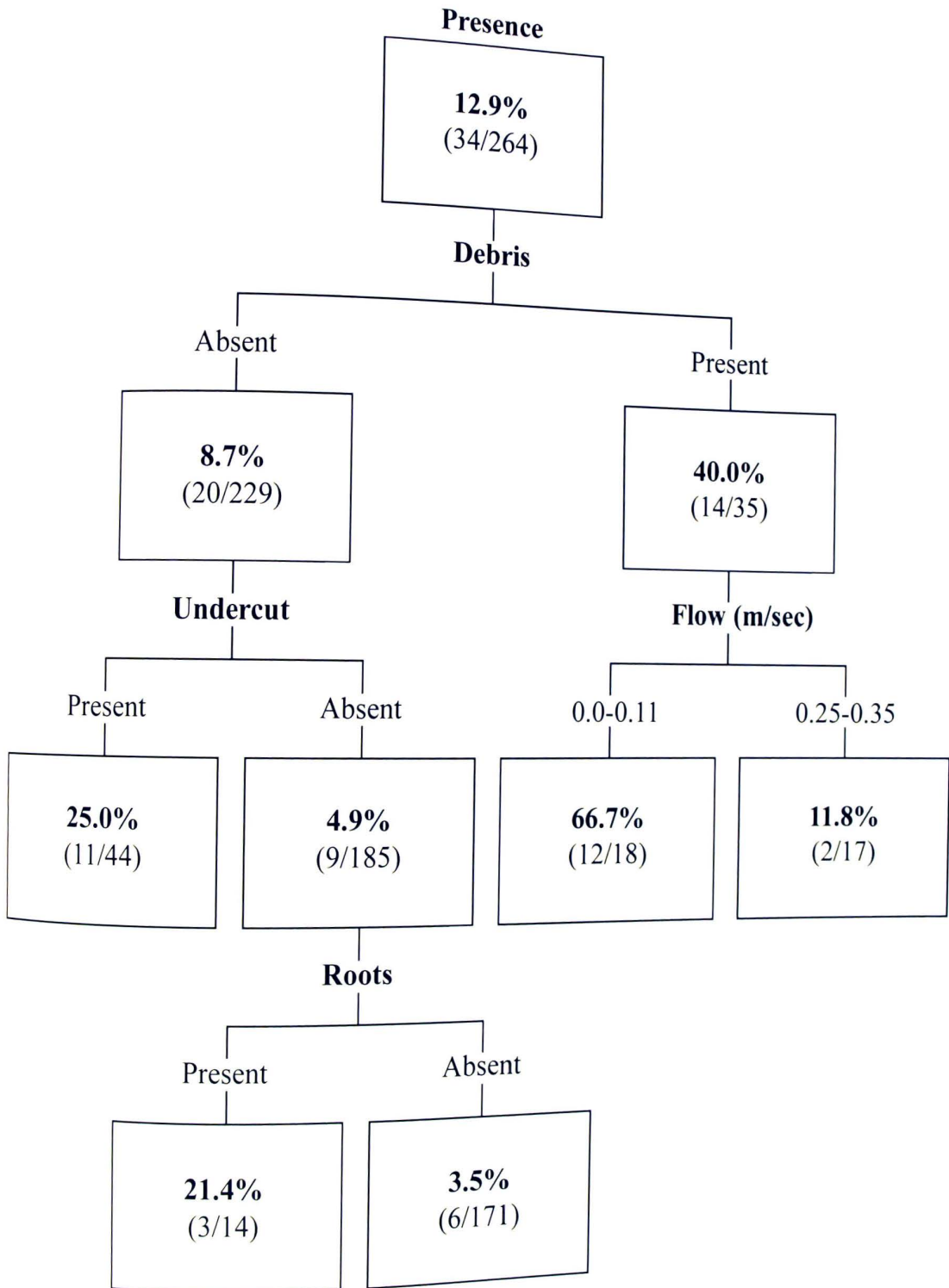


Figure 14. Classification and regression tree (CART) analysis of habitat data with patches of *Etheostoma pseudovulatum* present and absent for juveniles in spring. Branches represent splitting of data based on explanatory variables listed by values or categories (i.e., present/absent). Bold numbers in boxes are the predicted probability of *E. pseudovulatum* presence and the number of present patches over the total number of patches sampled for that particular suite of habitat characteristics is provided in parentheses. The correct classification rate of this model was 91.3% with a cross-validation risk estimate of 0.121. Values of other descriptive parameters can be seen in Table 9.

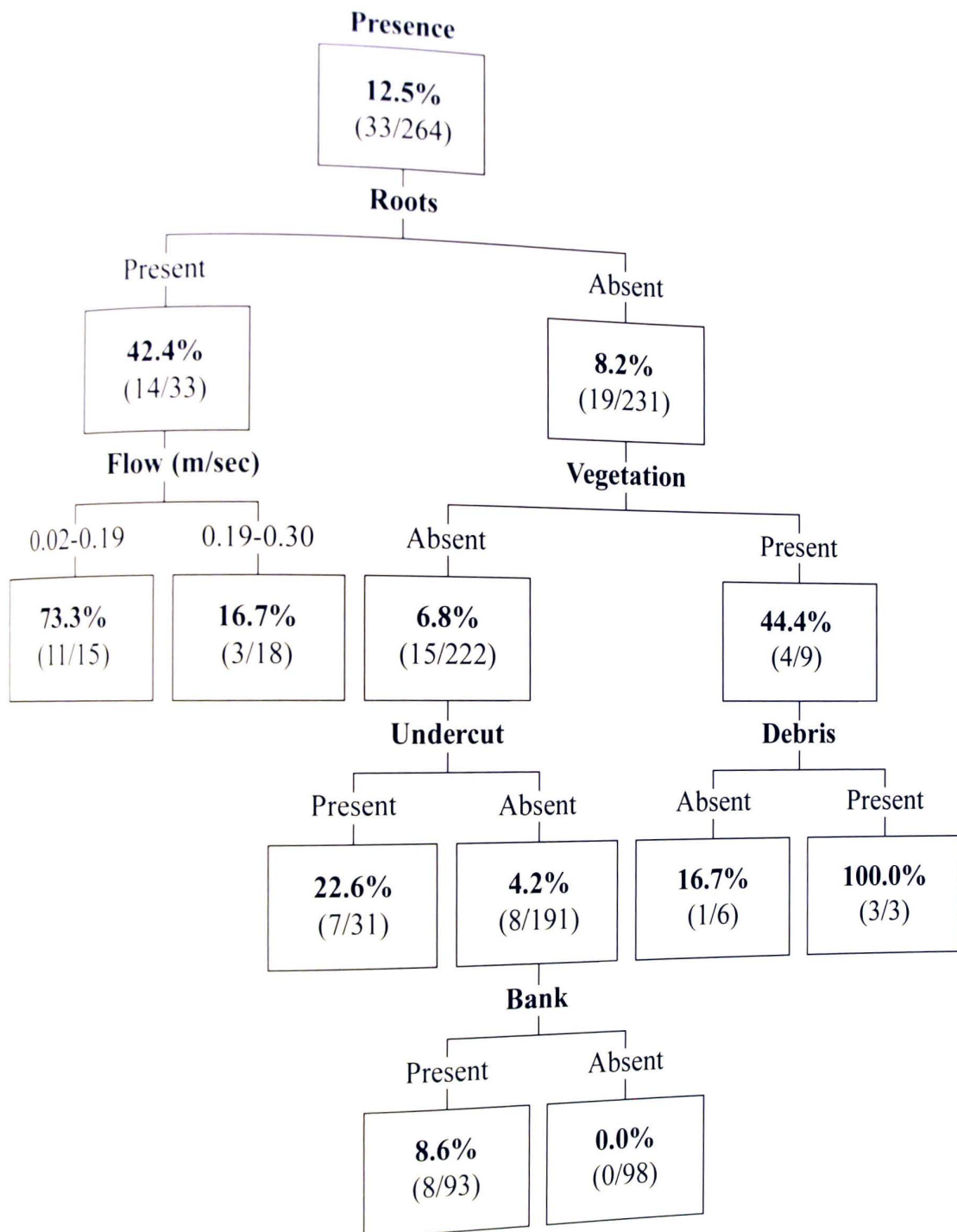
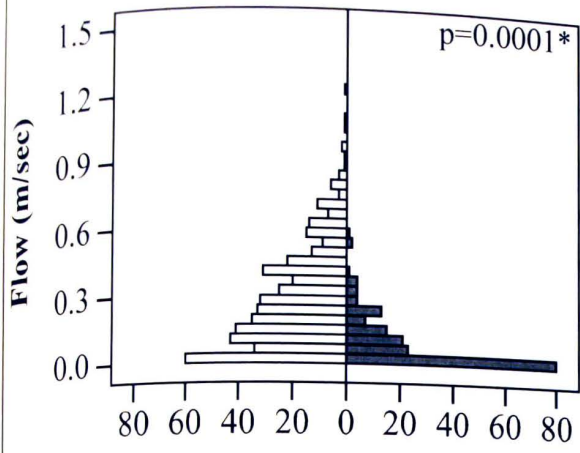
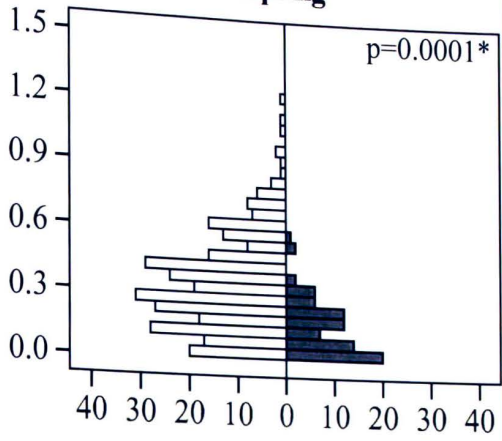


Figure 15. Frequency histograms of flow at patches where *Etheostoma pseudovulatum* was absent (white bars) and present (gray bars) for: A) all individuals collected throughout study; B) all individuals collected in the spring; C) all individuals collected in the fall; D) males collected in the spring; E) females collected in the spring; and F) juveniles collected in the spring. P-values for univariate tests with equal sample sizes for present and absent habitat patches are in the upper right-hand corners. An asterisk indicates variables that were significant ($p < 0.05$).

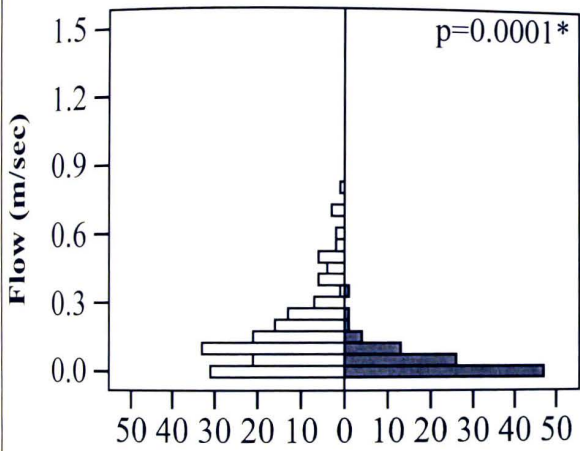
Total



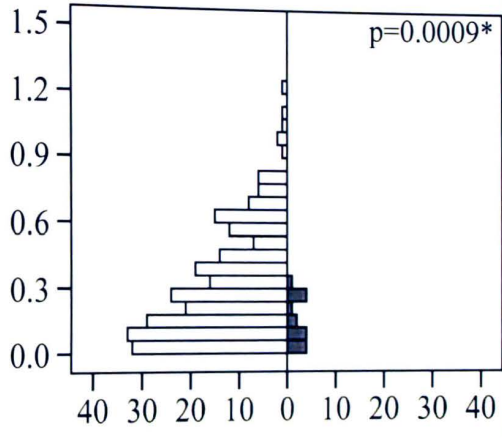
Spring



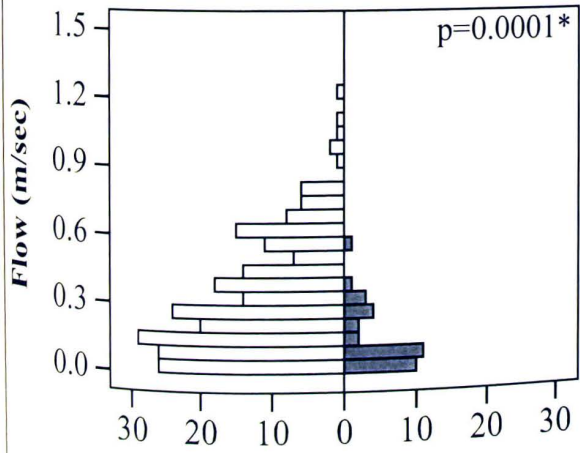
Fall



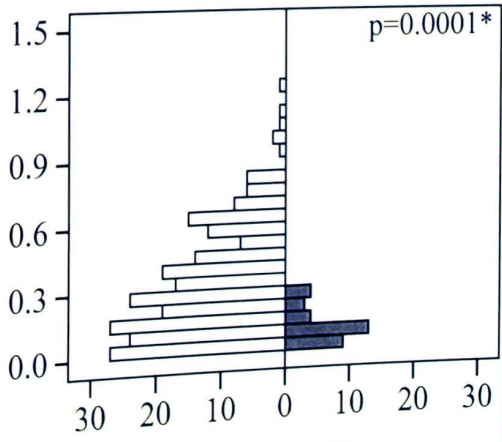
Males



Females



Juveniles



Frequency

Frequency

Figure 16. Frequency histograms of depth at patches where *Etheostoma pseudovulatum* was absent (white bars) and present (gray bars) for: A) all individuals collected throughout study; B) all individuals collected in the spring; C) all individuals collected in the fall; D) males collected in the spring; E) females collected in the spring; and F) juveniles collected in the spring. P-values for univariate tests with equal sample sizes for present and absent habitat patches are in the upper right-hand corners. An asterisk indicates variables that were significant ($p < 0.05$).

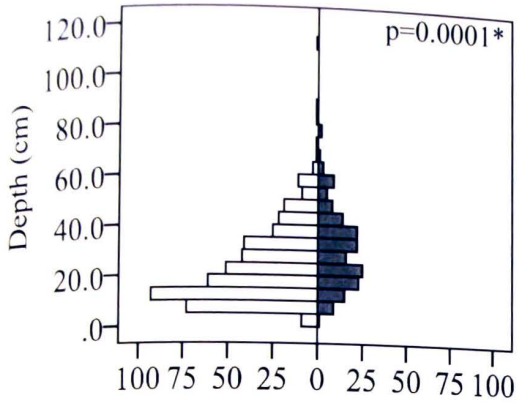
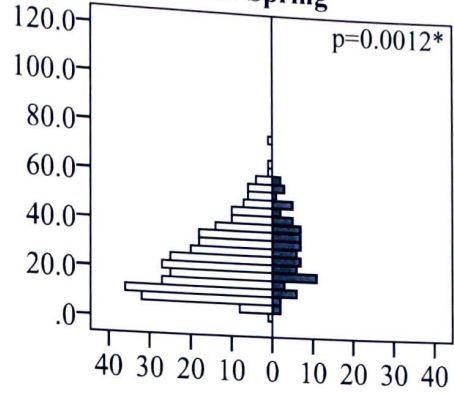
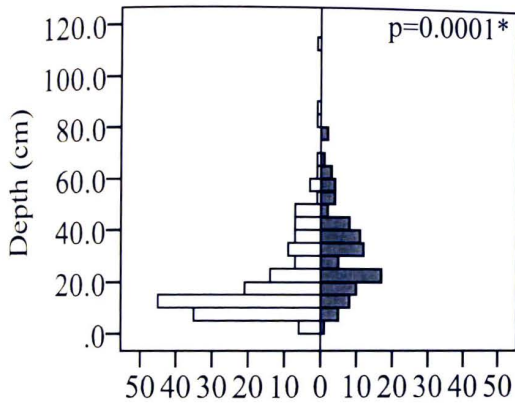
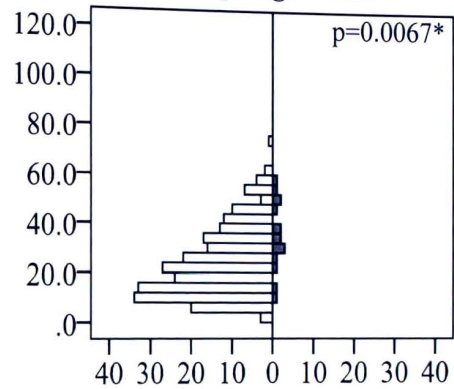
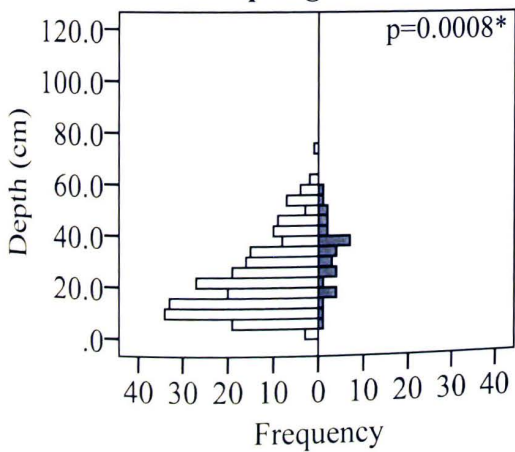
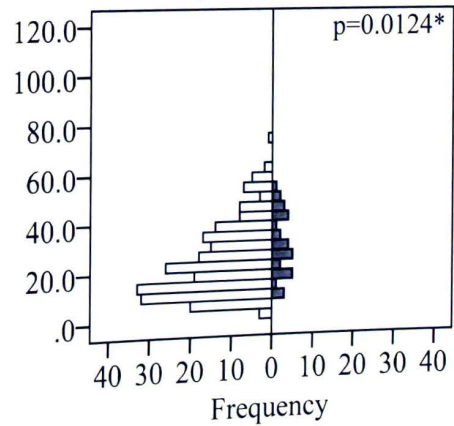
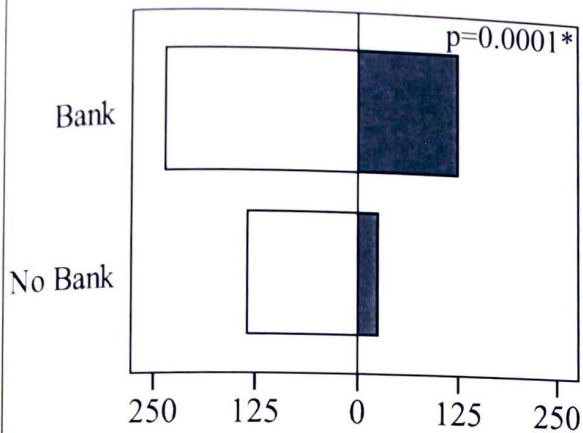
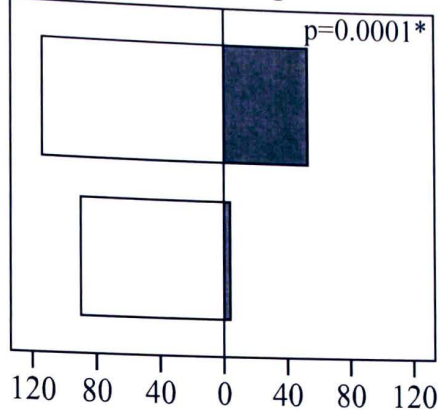
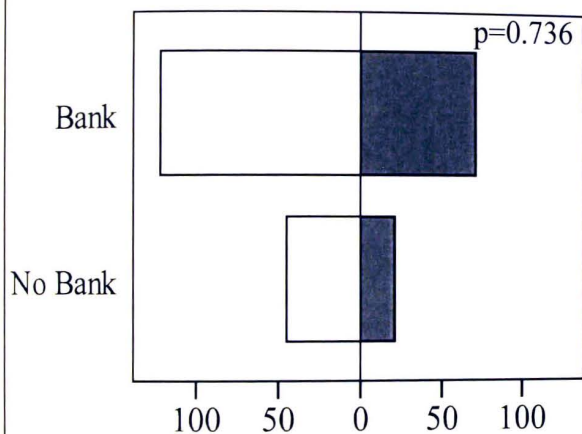
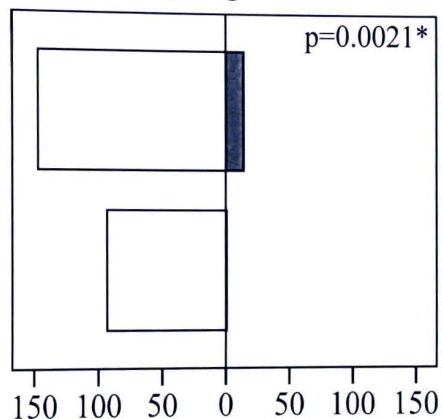
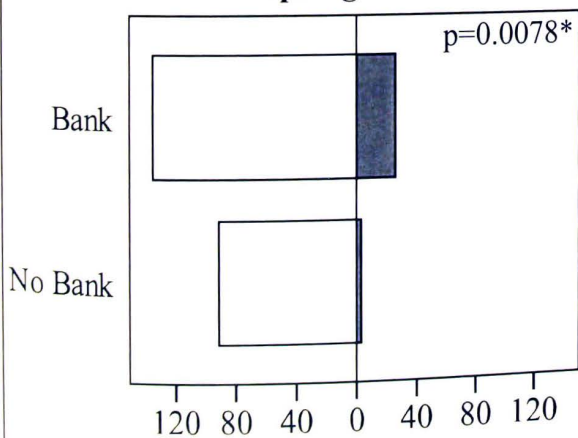
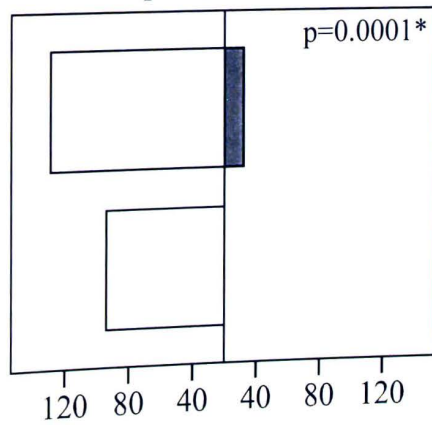
A. Total**B. Spring****C. Fall****D. Spring: Males****E. Spring: Females****F. Spring: Juveniles**

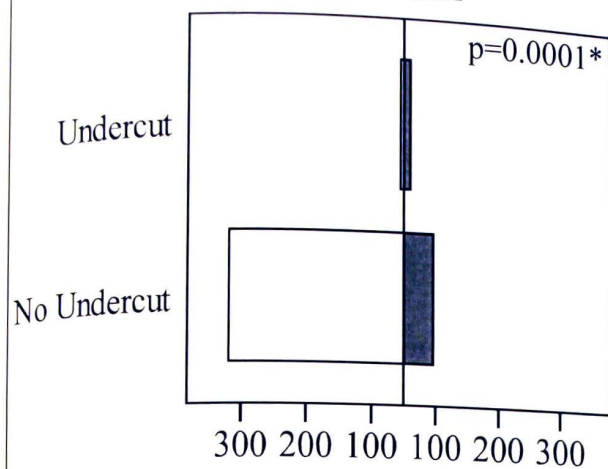
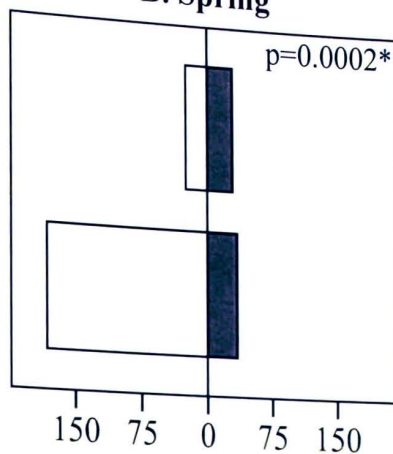
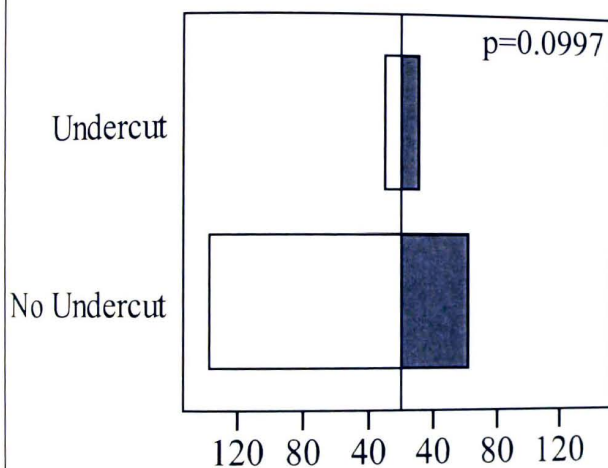
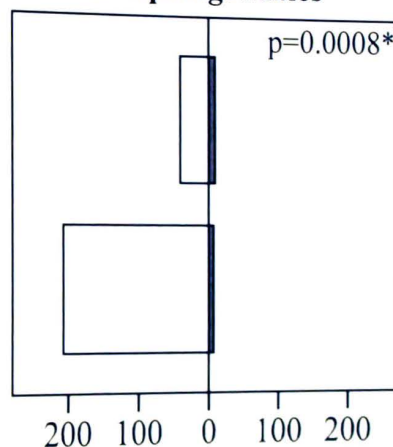
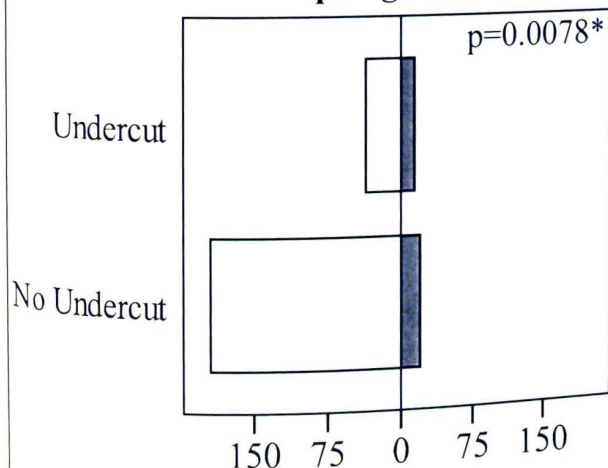
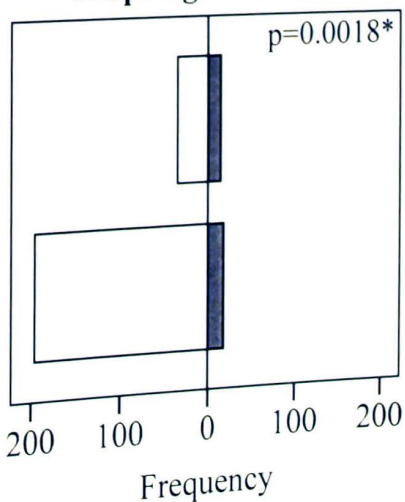
Figure 17. Frequency histograms of presence of stream bank in patches where *Etheostoma pseudovulatum* was absent (white bars) and present (gray bars) for: A) all individuals collected throughout study; B) all individuals collected in the spring; C) all individuals collected in the fall; D) males collected in the spring; E) females collected in the spring; and F) juveniles collected in the spring. P-values for univariate tests with equal sample sizes for present and absent habitat patches are in the upper right-hand corners. An asterisk indicates variables that were significant ($p < 0.05$).

A. Total**B. Spring****C. Fall****D. Spring: Males****E. Spring: Females****F. Spring: Juveniles**

Frequency

Frequency

Figure 18. Frequency histograms of presence of undercut banks in patches where *Etheostoma pseudovulatum* was absent (white bars) and present (gray bars) for: A) all individuals collected throughout study; B) all individuals collected in the spring; C) all individuals collected in the fall; D) males collected in the spring; E) females collected in the spring; and F) juveniles collected in the spring. P-values for univariate tests with equal sample sizes for present and absent habitat patches are in the upper right-hand corners. An asterisk indicates variables that were significant ($p < 0.05$).

A. Total**B. Spring****C. Fall****D. Spring: Males****E. Spring: Females****F. Spring: Juveniles**

Frequency

Frequency

Figure 19. Frequency histograms of presence of root wads in patches where *Etheostoma* *pseudovulatum* was absent (white bars) and present (gray bars) for: A) all individuals collected throughout study; B) all individuals collected in the spring; C) all individuals collected in the fall; D) males collected in the spring; E) females collected in the spring; and F) juveniles collected in the spring. P-values for univariate tests with equal sample sizes for present and absent habitat patches are in the upper right-hand corners. An asterisk indicates variables that were significant ($p < 0.05$).

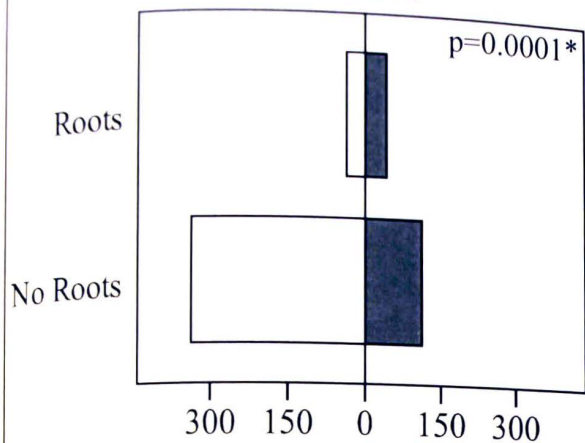
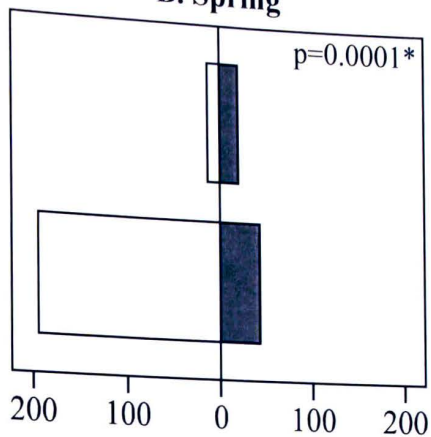
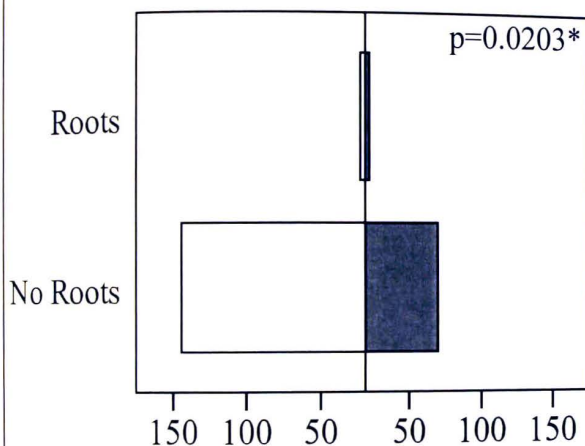
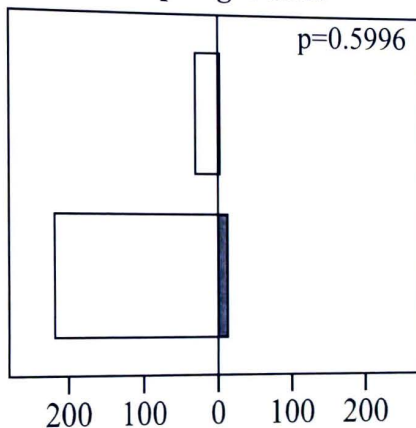
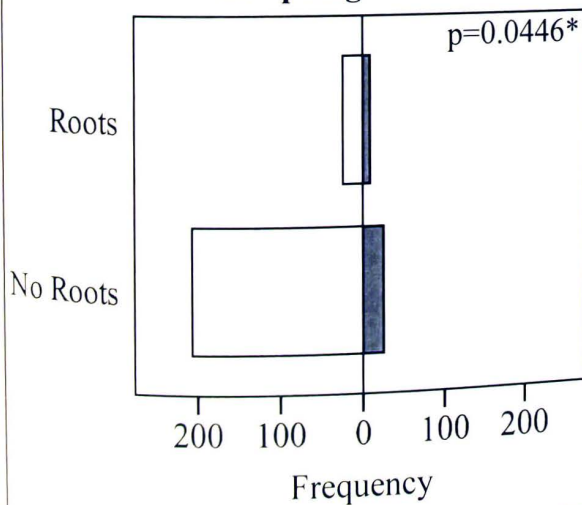
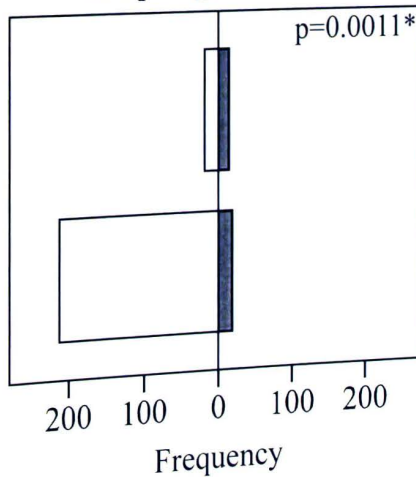
A. Total**B. Spring****C. Fall****D. Spring: Males****E. Spring: Females****F. Spring: Juveniles**

Figure 20. Frequency histograms of presence of debris in patches where *Etheostoma* *audouvilatum* was absent (white bars) and present (gray bars) for: A) all individuals collected throughout study; B) all individuals collected in the spring; C) all individuals collected in the fall; D) males collected in the spring; E) females collected in the spring; and F) juveniles collected in the spring. P-values for univariate tests with equal sample sizes for present and absent habitat patches are in the upper right-hand corners. An asterisk indicates variables that were significant ($p < 0.05$).

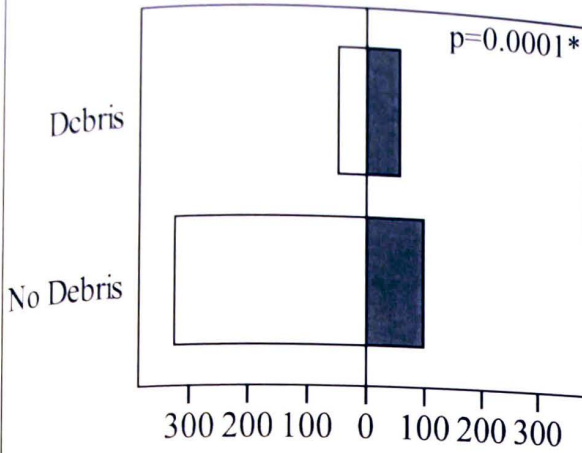
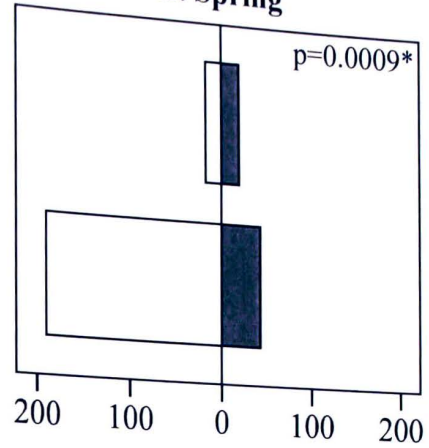
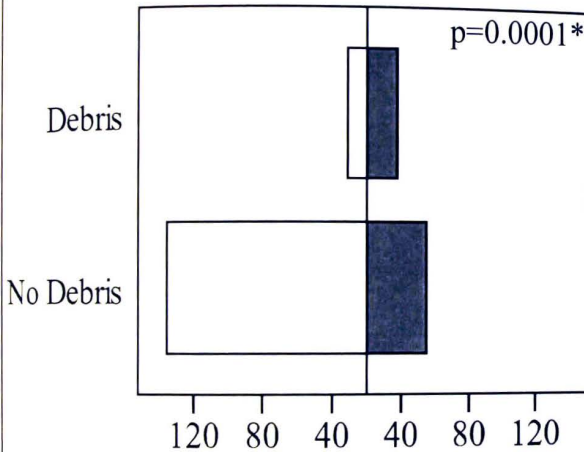
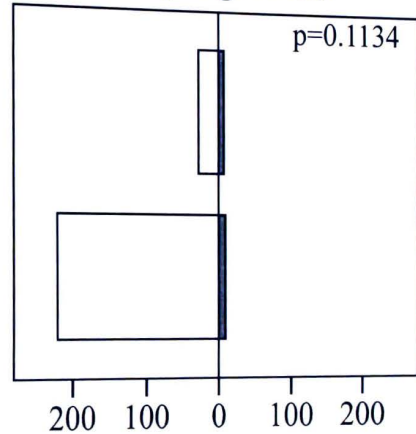
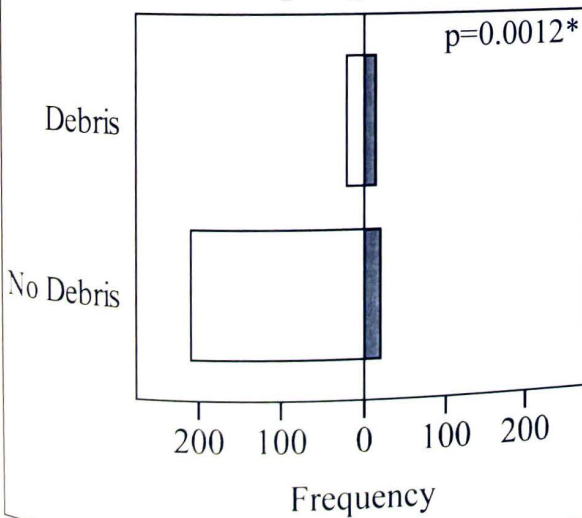
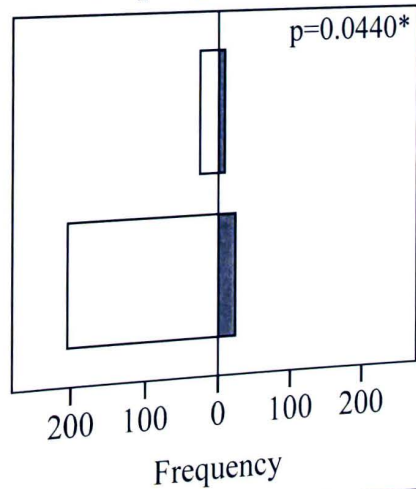
A. Total**B. Spring****C. Fall****D. Spring: Males****E. Spring: Females****F. Spring: Juveniles**

Figure 21. Frequency histograms of presence of A) vegetation and B) open bedrock in patches where *Etheostoma pseudovulatum* was absent (white bars) and present (gray bars) for all individuals collected throughout study. P-values for univariate tests with equal sample sizes for present and absent habitat patches are in the upper right-hand corners. An asterisk indicates variables that were significant ($p < 0.05$).

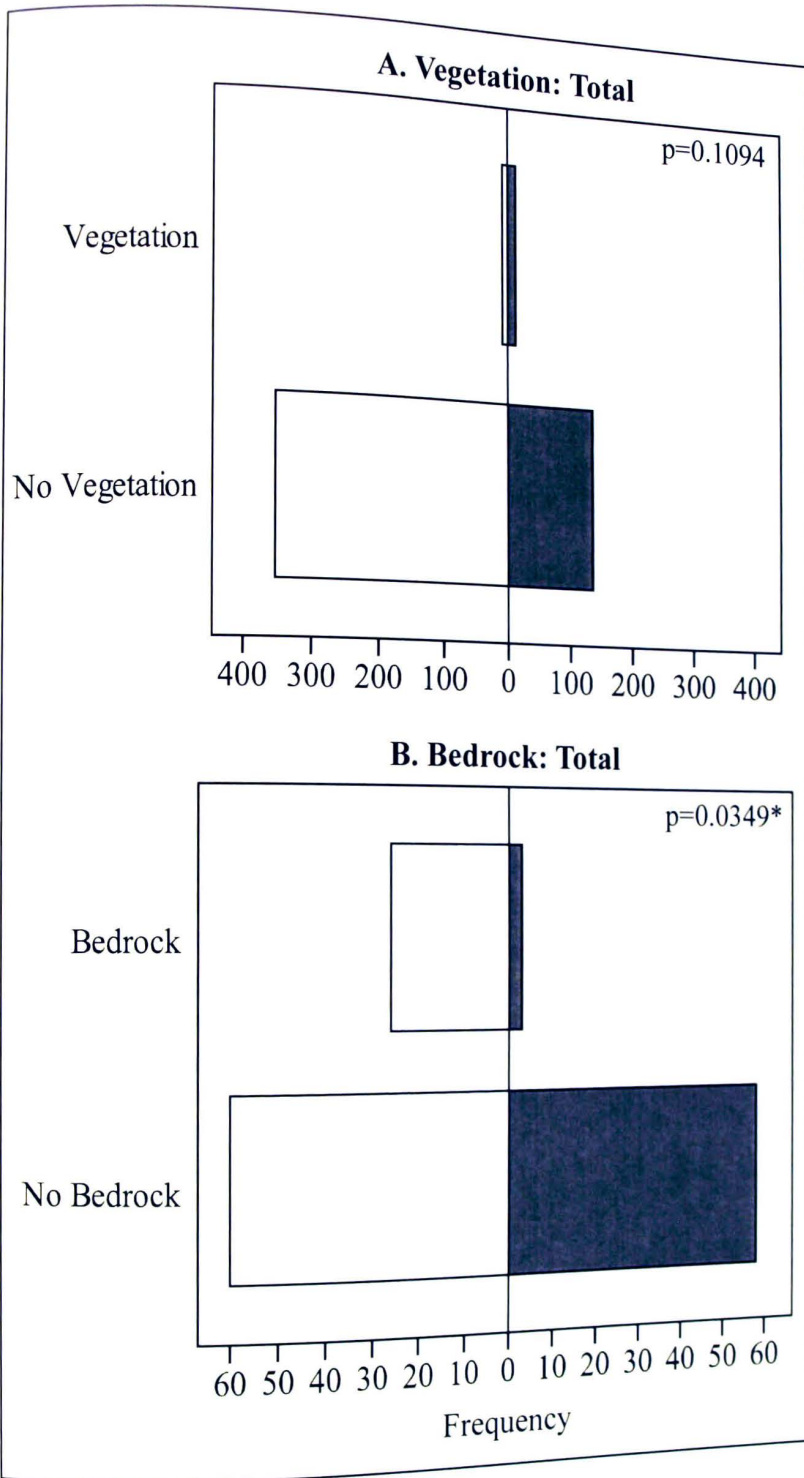


Figure 22. Ninety-five percent statistical parsimony haplotype network for *Etheostoma pseudovulatum* representing 11 ND2 haplotypes from 59 individuals from 14 localities. Haplotypes are represented by pie charts and labeled in bold. Pie size reflects frequency of individuals with that haplotype. Pie slices represent frequency of individuals from a tributary system with that haplotype (see legend.) Haplotype 1 (H1) had the highest outgroup probability, and thus, was estimated to be the most ancestral haplotype. Inset is a map of the range of the species with tributary systems color-coded to match the two clusters exhibited in the haplotype network: light gray represents Piney River, Only Creek, East Fork Wolf Creek, and Happy Hollow Creek (top cluster; H1, H2, H3, H4, and H6); black represents Beaverdam Creek (bottom cluster; H7, H8, H9, and H10); and moderate gray represents Little Piney Creek which had individuals in both clusters (top: H1 and H5; bottom: H7 and H11). Each line separated by a black circle represents one nucleotide difference between haplotypes.

Legend

- ☐ Piney R.
☒ Beaverdam Ck.
☐ Little Piney Ck.
☒ Happy Hollow Ck.
☐ E. Fk. Wolf Ck.
☒ Only Ck.

Figure 23. Fifty percent majority-rule phylogenetic tree resulting from Bayesian analysis of *Etheostoma pseudovulatum* from 59 individuals from 14 localities. Posterior probabilities are located at nodes. Outgroup not shown. Branches are labeled by tributary system with the haplotype ID. Number of individuals included in each haplotype for each tributary system can be seen in Table 11.

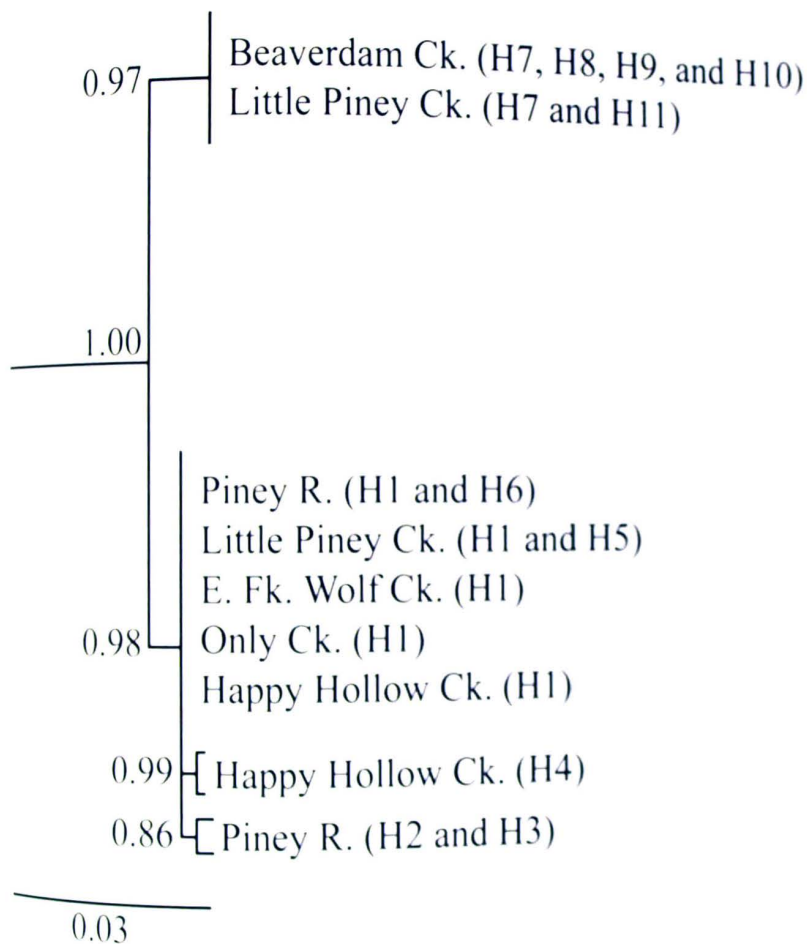
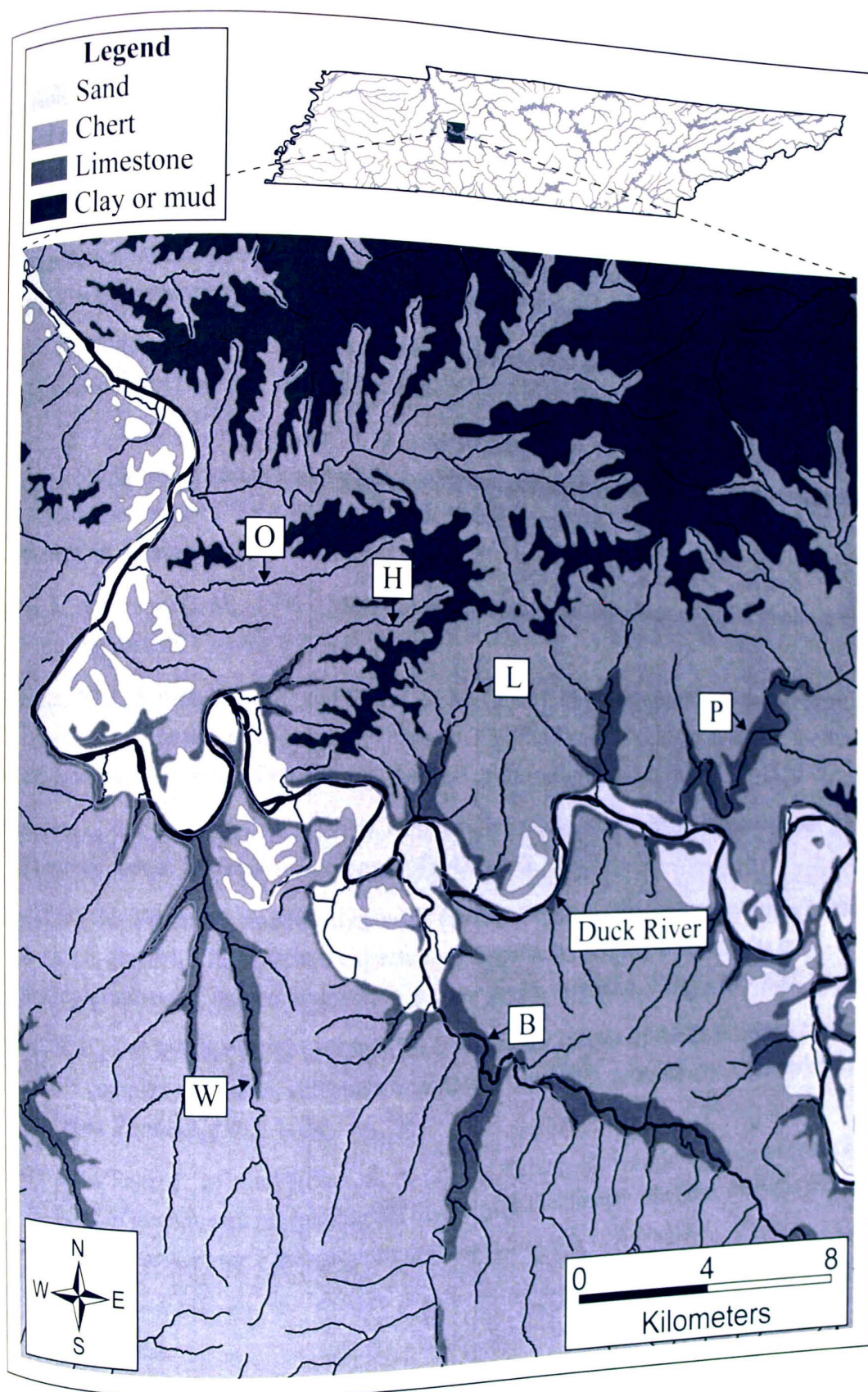


Figure 24. Underlying geology within the range of *Etheostoma pseudovulatum*, highlighting the confluence of Beaverdam Creek with the Duck River system. The Duck River is shown in its current channel position. The relatively unique haplotype assemblage of Beaverdam Creek may be explained by past geological events. Note the large expanse of sand in the lower reaches of the tributary; a feature that is lacking to this extent in all other tributaries where haplotypes are shared among populations of *E. pseudovulatum*. Letters refer to tributary systems of the Duck River: P = Piney River; O = Only Creek; H = Happy Hollow Creek; L = Little Piney Creek; W = East Fork Wolf Creek; and B = Beaverdam Creek. Tennessee geologic layer downloaded from the US Geological Survey website <http://mrdata.usgs.gov/geology/state/state.php?state=TN>.



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VIII. APPENDIX A

Materials Examined

Specific locality information for all historical localities identified through literature and museum database searches. Localities sampled in this study are presented first followed by others not sampled. Locality IDs correspond to numbers in Figures 1 and 2.

Locality information reads as follows:

Locality ID. State: county: locality string (Drainage); latitude and longitude +/- accuracy; field collection numbers (museum collection records).

Historical localities examined:

1. TN: Hickman Co.: Piney Fork Creek near TN-48, 0.5 mi NE of Aetna (Beaverdam Ck.-Duck R.-Tennessee R.-Ohio R.); Lat: 35.66204 Long: -87.49987 +/-14 ft.; ZLW-06-2014, ZLW-27-2014 (USNM 231310.509).
2. TN: Hickman Co.: Brushy Fork Creek at TN-48 of Aetna (Beaverdam Ck.-Duck R.-Tennessee R.-Ohio R.); Lat: 35.68102 Long: -87.50237 +/-9 ft.; ZLW-03-2014, ZLW-28-2014 (USNM 230491.5089).
3. TN: Hickman Co.: Beaverdam Creek 4 mi N of Aetna off East Beaverdam Creek Road, 0.5 mi from TN-48 (Duck R.-Tennessee R.-Ohio R.); Lat: 35.70666 Long: -87.50934 +/-9 ft.; ZLW-02-2014, ZLW-31-2014 (INHS 77593).
4. TN: Hickman Co.: Beaverdam Creek at bridge of Backside Beaverdam Creek Road, 3 mi E of Coble (Duck R.-Tennessee R.-Ohio R.); Lat: 35.76482 Long: -87.57976 +/-9 ft.; ZLW-05-2014, ZLW-29-2014 (INHS 77817).

5. TN: Hickman Co.: Sulphur Fork Creek at Mitchell bridge on Backside Beaverdam Creek Road and Sulphur Fork Creek Road (Beaverdam Ck.-Duck R.-Tennessee R.-Ohio R.); Lat: 35.77103 Long: -87.61524 +/-9 ft.; ZLW-04-2014, ZLW-38-2014 (UT 91.5796).
6. TN: Hickman Co.: Beaverdam Creek, 1 mi E of Coble at bridge of TN-438 (Duck R.-Tennessee R.-Ohio R.); Lat: 35.78693 Long: -87.62722 +/-9 ft.; ZLW-07-2014 (INHS 82756).
7. TN: Hickman Co.: Cow Hollow Creek at Coble on TN-438 near crossing of Briar Pond Road (Beaverdam Ck.-Duck R.-Tennessee R.-Ohio R.); Lat: 35.78390 Long: -87.63020 +/-9 ft.; ZLW-19-2014, ZLW-30-2014 (INHS 58414).
8. TN: Hickman Co.: Beaverdam Creek at TN-50, 1 mi N of Coble (Duck R.-Tennessee R.-Ohio R.); Lat: 35.80000 Long: -87.62932 +/-9 ft.; ZLW-22-2014, ZLW-32-2014 (UT 91.1629).
9. TN: Hickman Co.: East Fork Wolf Creek, 3 mi W of Coble off Wolf Creek Road (Wolf Ck.-Duck R.-Tennessee R.-Ohio R.); Lat: 35.77654 Long: -87.67401 +/-9 ft.; ZLW-08-2014, ZLW-36-2014 (INHS 91989).
10. TN: Hickman Co.: East Fork Wolf Creek on Mathis Loop Road, 3 mi NE of Coble (Wolf Ck.-Duck R.-Tennessee R.-Ohio R.); Lat: 35.80285 Long: -87.68281 +/-9 ft.; ZLW-09-2014, ZLW-37-2014 (INHS 33682).
11. TN: Hickman Co.: Little Piney Creek off Little Piney Road, 3.5 mi SW of Spot (Duck R.-Tennessee R.-Ohio R.); Lat: 35.83049 Long: -87.62912 +/-10 ft.; ZLW-24-2014, ZLW-39-2014 (INHS 91948).

12. TN: Hickman Co.: Happy Hollow Creek 1.5 mi SE of Only near TN-50 bridge via TWRA WMA (Duck R.-Tennessee R.-Ohio R.); Lat: 35.85014 Long: -87.66586 +/-11 ft.; ZLW-13-2014, ZLW-46-2014 (INHS 36030).
13. TN: Hickman Co.: Only Creek tributary at intersection of Only Road and Dyer Road (Only Ck.-Duck R.-Tennessee R.-Ohio R.); Lat: 35.86308 Long: -87.69202 +/-9 ft.; ZLW-17-2014, ZLW-35-2014 (INHS 91987).
14. TN: Hickman Co.: Bell Branch, 6.5 mi NNE of Centerville, at powerline clearing of Bell Branch Trace (Mill Ck.-Piney R.-Duck R.-Tennessee R.-Ohio R.); Lat: 35.86449 Long: -87.43401 +/-9 ft.; ZLW-10-2014, ZLW-34-2014 (INHS 91987).
15. TN: Hickman Co.: Mill Creek at TN-48, 1 mi N of Nunnely, 0.5 mi upstream of bridge (Piney R.-Duck R.-Tennessee R.-Ohio R.); Lat: 35.87312 Long: -87.46343; ZLW-11-2014, ZLW-55-2014 (UT 91.5796).
16. TN: Hickman Co.: Mill Creek, 1.6 km S of Wrigley at TN-100 bridge (Piney R.-Duck R.-Tennessee R.-Ohio R.); Lat: 35.89307 Long: -87.35056 +/-9 ft.; ZLW-01-2014, ZLW-40-2014 (INHS 58630).
17. TN: Hickman Co.: Little Spring Creek, 2 mi NE of Pinewood at Pinewood Road (Piney R.-Duck R.-Tennessee R.-Ohio R.); Lat: 35.92043 Long: -87.44244 +/-10 ft.; ZLW-20-2014, ZLW-54-2014 (INHS 36024).
18. TN: Hickman Co.: Beaver Creek, 2.2 mi W of TN-48 off Old Beaver Creek Road (Piney R.-Duck R.-Tennessee R.-Ohio R.); Lat: 35.93054 Long: -87.50612 +/-10 ft.; ZLW-14-2014, ZLW-48-2014 (INHS 62771).

19. TN: Hickman Co.: Big Spring Creek at Missionary Ridge Road, 0.75 mi SE of Bon Aqua, Bon Aqua Springs (Piney R.-Duck R.-Tennessee R.-Ohio R.); Lat: 35.94586 Long: -87.32037 +/-13 ft.; ZLW-23-2014, ZLW-33-2014 (INHS 36029).
20. TN: Dickson Co.: Piney River at Double Branch Road 0.5 mi from I-40, 2 mi SW of Mount Sinai (Duck R.-Tennessee R.-Ohio R.); Lat: 35.98930 Long: -87.43493 +/-9 ft.; ZLW-21-2014, ZLW-56-2014 (UMMZ 104881).
21. TN: Dickson Co.: East Piney River at North Mount Sinai Road (Piney R.-Duck R.-Tennessee R.-Ohio R.); Lat: 36.01035 Long: -87.43823 +/-9 ft.; ZLW-16-2014, ZLW-41-2014 (UT 91.5797).
22. TN: Dickson Co.: West Fork Piney River at TN-48 0.2 km downstream of bridge, private access (Piney R.-Duck R.-Tennessee R.-Ohio R.); Lat: 36.02695 Long: -87.44931 +/-9 ft.; ZLW-12-2014, ZLW-43-2014 (UT 91.7629).
23. TN: Dickson Co.: Coon Creek at Coon Creek Road, 3 mi SE of Tennessee City (Piney R.-Duck R.-Tennessee R.-Ohio R.); Lat: 36.05801 Long: -87.47345 +/-9 ft.; ZLW-18-2014, ZLW-42-2014 (INHS 35923).
24. TN: Dickson Co.: West Fork Piney River at Eno Road, 0.1 mi W of Eno (Piney R.-Duck R.-Tennessee R.-Ohio R.); Lat: 36.06058 Long: -87.45564 +/-13 ft.; ZLW-15-2014, ZLW-44-2014 (INHS 62760).

New localities examined:

25. TN: Hickman Co.: Piney River at TN-48, 2 mi N of Nunnely (Duck R.-Tennessee R.-Ohio R.); Lat: 35.89066 Long: -87.47062; ZLW-57-2014.

Historical localities not examined (dry, too deep, or inaccessible:

26. TN: Hickman Co.: Only Creek tributary along Nunnely-Only Road (Only Ck.-Duck R.-Tennessee R.-Ohio R.); Lat: 35.867269 Long: -87.680087; (INHS 35980).
27. TN: Hickman Co.: Mill Creek, 1 mi N of Wrigley (Piney R.-Duck R.-Tennessee R.-Ohio R.); Lat: 35.913848 Long: -87.343404; (INHS 63484).
28. TN: Hickman Co.: Little Piney Cr. at co. rd. 6173 (Piney R.-Duck R.-Tennessee R.-Ohio R.); Lat: 35.87083 Long: -87.50118; (UT 91.2580).
29. TN: Hickman Co.: Piney River at Pinewood on Co. Rd., ~1 mi from jct. with Rt. 48, 9 air mi N of Centerville (Duck R.-Tennessee R.-Ohio R.); Lat: 35.910719 Long: -87.467779; (CU 52472).

Historical localities not examined because adjacent to other localities:

30. TN: Hickman Co.: Beaverdam Creek, Aetna (Duck R.-Tennessee R.-Ohio R.); Lat: 35.65514 Long: -87.5048; (INHS 61767).
31. TN: Hickman Co.: Piney Fork Creek at TN-48 (Beaverdam Ck.-Duck R.-Tennessee R.-Ohio R.); Lat: 35.66455 Long: -87.4977; (USNM 231310.509).
32. TN: Hickman Co.: Tributary to Beaverdam Creek at TN-48, about 1.0 miles S junction with TN-100 (Duck R.-Tennessee R.-Ohio R.); Lat: 35.6817 Long: -87.5025; (NCSM 28827).
33. TN: Hickman Co.: Beaverdam Creek; at TN-48 and 100 intersection, ~10 km SW Centerville; RCH 10-08 (Duck R.-Tennessee R.-Ohio R.); Lat: 35.70384 Long: -87.5102; (YPM ICH 023883).
34. TN: Hickman Co.: Beaverdam Creek at Beaverdam Creek Road (Duck R.-Tennessee R.-Ohio R.); Lat: 35.78564 Long: -87.6258; (UT 91.5796).

35. TN: Hickman Co.: trib. Duck River, Only, Rt. 229 (Duck R.-Tennessee R.-Ohio R.); Lat: 35.86331 Long: -87.6918; (INHS 33651).
36. TN: Hickman Co.: Mill Creek, 1 mi. S Wrigley (Piney R.-Duck R.-Tennessee R.-Ohio R.); Lat: 35.887 Long: -87.345; (KU 14399, KU 16216).
37. TN: Hickman Co.: Little Spring Creek along Pinewood Road ~3 air km E Pinewood; TJN08-19 (Piney R.-Duck R.-Tennessee R.-Ohio R.); Lat: 35.91797 Long: -87.456; (YPM ICH 018560).
38. TN: Hickman Co.: Piney River at I-40 (Duck R.-Tennessee R.-Ohio R.); Lat: 35.99455 Long: -87.4396; (UT 91.5797).

IX. APPENDIX B

Habitat Assessment Data Sheet

HABITAT ASSESSMENT DATA SHEET FOR HIGH-GRADIENT STREAMS (FRONT)

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STREAM NAME		LOCATION	
STATION #	RIVER MILE	STREAM CLASS	
LAT	LONG	RIVER BASIN	
STORET #		AGENCY	
INVESTIGATORS			
FORM COMPLETED BY		DATE	REASON FOR SURVEY
		TIME	AM PM

Habitat Parameter	Condition Category																			
	Optimal					Suboptimal					Marginal					Poor				
1. Epifaunal Substrate/Available Cover	Greater than 70% of substrate favorable for epifaunal colonization and fish cover; mix of snags, submerged logs undercut banks, cobble or other stable habitat and at stage to allow full colonization potential (i.e., logs/snags that are not new-fall and not transient)					40-70% mix of stable habitat; well-suited for full colonization potential; adequate habitat for maintenance of populations; presence of additional substrate in the form of new-fall, but not yet prepared for colonization (may rate at high end of scale)					20-40% mix of stable habitat; availability less than desirable; substrate frequently disturbed or removed					Less than 20% stable habitat; lack of habitat is obvious; substrate unstable or lacking				
SCORE	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
2. Embeddedness	Gravel, cobble, and boulder particles are 0-25% surrounded by fine sediment. Layering of cobble provides diversity of niche space.					Gravel, cobble and boulder particles are 25-50% surrounded by fine sediment.					Gravel, cobble, and boulder particles are 50-75% surrounded by fine sediment.					Gravel, cobble, and boulder particles are more than 76% surrounded by fine sediment.				
SCORE	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
3. Velocity/Depth Regime	All four velocity/depth regimes present (slow-deep, slow-shallow, fast-deep, fast-shallow) (Slow is <0.3m/s deep is >0.5m)					Only 3 of the 4 regimes present (if fast-shallow is missing score lower than regimes).					Only 2 of the 4 habitat regimes present (if fast-shallow or slow-shallow are missing, score low)					Dominated by 1 velocity/depth regime (usually slow-deep)				
SCORE	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
4. Sediment Deposition	Little or no enlargement of islands or point bars and less than 5% (<20% for low-gradient streams) of the bottom affected by sediment deposition					Some new increase in bar formation, mostly from gravel, sand or fine sediment; 5-30% (20-50% for low-gradient) of the bottom affected; slight deposition in pools					Moderate deposition of new gravel, sand or fine sediment on old and new bars; 30-50% (50-80% for low-gradient) of the bottom affected; sediment deposits at obstructions, constrictions, and bends; moderate deposition of pools prevalent.					Heavy deposits of fine material, increased bar development; more than 50% (80% for low-gradient) of the bottom changing frequently; pools almost absent due to substantial sediment deposition				
SCORE	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
5. Channel Flow Status	Water reaches base of both lower banks, and minimal amount of channel substrate is exposed.					Water fills > 75% of the available channel; or 25 % of channel substrate is exposed.					Water fills 25-75 % of the available channel, and/or riffle substrates are mostly exposed.					Very little water in channel and mostly present as standing pools.				
SCORE	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1

HABITAT ASSESSMENT DATA SHEET FOR HIGH-GRADIENT STREAMS (BACK)

Habitat Parameter	Condition Category																			
	Optimal					Suboptimal					Marginal					Poor				
6. Channel Alteration	Channelization or dredging absent or minimal; stream with normal pattern.					Some channelization present, usually in areas of bridge abutments; evidence of past channelization, i.e., dredging, (greater than past 20 yr) may be present, but recent channelization is not present					Channelization may be extensive; embankments or shoring structures, present on both banks; and 40 to 80% of stream reach channelized and disrupted.					Banks shored with gabion or cement; over 80% of the stream reach channelized and disrupted. Instream habitat greatly altered or removed entirely.				
SCORE	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
7. Frequency of Rif es (or bends)	Occurrence of rif es relatively frequent; ratio of distance between rif es divided by width of the stream <7:1 (generally 5-7); variety of habitat is key. In streams where rif es are continuous, placement of boulders or other large, natural obstruction is important.					Occurrence of rif es infrequent; distance between rif es divided by the width of the stream is between 7 to 15.					Occasional rif e or bend; bottom contours provide some habitat; distance between rif es divided by the width of the stream is between 15 to 25.					Generally all flat at water or shallow rif es; poor habitat; distance between rif es divided by the width of the stream is a ratio of >35.				
SCORE	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
8. Bank Stability (score each bank) Note: determine left or right side by facing downstream.	Banks stable; evidence of erosion or bank failure absent or minimal; little potential for future problems <5% of bank affected.					Moderately stable; infrequent, small areas of erosion mostly healed over. 5-30% of bank in reach has areas of erosion.					Moderately unstable; 30-60 % of bank in reach has areas of erosion; high erosion potential during floods					Unstable; many eroded areas; "raw" areas frequent along straight sections and bends; obvious bank sloughing; 60-100% of bank has erosional scars				
SCORE (LB)	Left Bank	10	9			8	7	6			5	4	3	2		1	0			
SCORE (RB)	Right Bank	10	9			8	7	6			5	4	3			2	1	0		
9. Vegetative Protective (score each bank) Note: determine left or right side by facing downstream	More than 90% of the streambank surfaces and immediate riparian zone covered by native vegetation, including trees, understory shrubs, or nonwoody macrophytes; vegetative disruption through grazing or mowing minimal or not evident; almost all plants allowed to grow naturally.					70-90% of the streambank surfaces covered by native vegetation, but one class of plants is not well-represented; disruption evident but not affecting full plant growth potential to any great extent more than one-half of the potential plant stubble height remaining.					50-70% of the streambank surfaces covered by vegetation; disruption obvious; patches of bare soil or closely cropped vegetation common; less than one-half of the potential plant stubble height remaining					Less than 50% of the streambank surfaces covered by vegetation; disruption of streambank vegetation is very high; vegetation has been removed to 5 centimeters or less in average stubble height				
SCORE (LB)	Left Bank	10	9			8	7	6			5	4	3			2	1	0		
SCORE (RB)	Right Bank	10	9			8	7	6			5	4	3			2	1	0		
10. Riparian Vegetative Zone Width (score each bank riparian zone)	Width of riparian zone > 18 meters; human activities (i.e. parking lots, roadbeds, clear-cuts, lawns or crops) have not impacted zone					Width of riparian zone 12-18 meters; human activities have impacted zone only minimally					Width of riparian zone 6-12 meters; human activities have impacted zone a great deal.					Width of riparian zone <6 meters; little or no riparian vegetation due to human activities.				
SCORE (LB)	Left Bank	10	9			8	7	6			5	4	3			2	1	0		
SCORE (RB)	Right Bank	10	9			8	7	6			5	4	3			2	1	0		