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Comparison Of Spawning Habitat And Nest Density Between Buck Darter (etheostoma Nebra) And Striped Darter (etheostoma Virgatum) Populations In The Cumberland River Drainage, Kentucky

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COMPARISON OF SPAWNING HABITAT AND NEST DENSITY BETWEEN BUCK DARTER (ETHEOSTOMA NEBRA) AND STRIPED DARTER (ETHEOSTOMA VIRGATUM) POPULATIONS IN THE CUMBERLAND RIVER DRAINAGE, KENTUCKY

BY

JACOB MURPHY

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ABSTRACT

The Buck Darter (*Etheostoma nebra*) is an imperiled, small-stream species in the Cumberland River drainage in southern Kentucky. It was originally thought to be a population of the Striped Darter (*E. virgatum*) species and was prevalent throughout the Buck Creek system. It was separated from the Striped Darter and deemed a separate species in 2015; however, the population has declined dramatically. Presently, the species is found in three streams of the Flat Lick Creek system, two originally established populations in Big Spring Branch and Stewart Branch, and one newly introduced population, established in 2018, in an unnamed tributary. The decline in population makes it important to understand the Buck Darter spawning habits and nesting habitat. Thus, my study objectives were to compare populations of established and introduced Buck Darters and determine differences in spawning habitats, conductivity, and effects of temperature on timing of spawning and nest densities; as well as compare spawning habitats, conductivity, and effects of temperature on timing of spawning and nest densities between the Striped Darter (in Beaver Creek, Cumberland River drainage) and Buck Darter populations. Two 120-m reaches were observed for each stream throughout the spawning season. Slab rocks were checked for active nests and habitat measurements were taken at each nesting site. Buck Darters nested in shallow, slow-moving water located closer to the streambank than the center of the stream. Nests of established populations were found at greater stream widths with nests located farther from the streambank than nests of the introduced population, most likely a factor of stream size overall. Nesting sites also differed in flow velocity, with the unnamed

tributary having nests in slower moving water. At Beaver Creek, conductivity and dissolved oxygen were lower, and Striped Darters nested in areas with greater stream width, and nests were located farther from the nearest streambank than the established Buck Darter populations. Weekly nest densities were similar between the established and introduced populations and were lower in the Striped Darter population, possibly a factor of nest rock availability. The established and introduced Buck Darter populations also had a longer average nesting season (15.7 weeks) than the Striped Darter (6 weeks). Water temperature showed a significant effect on nest density in the unnamed tributary ($p = 0.020$) but not in any of the other streams. It did have an impact on timing of nesting, with all three groups starting the nesting season around 9-13°C and ending around 19-22°C. The Striped Darter population began nesting at a later start date and ended at an earlier date due to the colder start to the season and sharper increase of temperature throughout the season compared to the introduced and established Buck Darter streams. Comparing the Buck Darter's nest habitat and densities to the introduced population and the Striped Darter allows us to observe how they are surviving in the unnamed tributary and what differences there are between their declining population and the persisting Striped Darter species. This knowledge will aid in finding and restoring new streams in the Buck Creek watershed for the Buck Darter and hopefully help to slow the decline of their population.

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Chapter I: Introduction

Kentucky's freshwater streams and lakes provide habitat and resources to approximately 269 different fish species, ranking third nationally in freshwater fish diversity (Thomas 2021). Within this diverse group is the family *Percidae,* the most diverse fish family in Kentucky and home to the darter (*Etheostomatinae*) clade. Darters are small, benthic fishes found throughout North America and make up one of the most species-rich clades of freshwater fish on the continent (Near et al. 2011). Because of their intolerance to stream degradation, darters are often used as indicators of good water quality and stream conditions (Karr 1981, Thomas 2021). This intolerance to change has also contributed to declines of many darter species, leading to over a quarter of the species found in the southern United States being considered vulnerable, threatened, or endangered (Warren et al. 2000). With 73 species found in Kentucky, darters make up a large portion of the fish diversity of the state, but many are threatened by stream health changes (Thomas 2021).

The Buck Darter (*Etheostoma nebra)* is a species found in few streams in southern Kentucky. It belongs to the clade known as barcheek darters, *Oopereia,* and is classified in the subgenus *Catonotus* based on its nesting habits (Near & Thomas 2015). The Buck Darter was considered an independent population of the Striped Darter (*E. virgatum)* since it was first recorded in 1955, up until 2015 when Near and Thomas (2015) deemed it a separate species through morphological distinctions and DNA sequencing.

The Buck Darter was originally observed throughout the Buck Creek system of the Cumberland River drainage in Kentucky, with records from the mainstem Buck Creek as well as seven tributaries in the system, including Flat Lick Creek (Cicerello & Butler 1985). Since then, its population range has declined to only Flat Lick Creek (Thomas & Brandt 2013) and, more recently, only in Stewart Branch and Big Spring Branch, first-order tributaries to Flat Lick Creek (Black 2018). In addition to these two tributaries, a different, unnamed tributary has recently become the location of a newly introduced Buck Darter population. This stream, a separate first-order tributary to Flat Lick Creek, is close in proximity to Stewart Branch and Big Spring Branch, and has similar habitat, leading personnel from Conservation Fisheries, Inc. (Knoxville, TN) and the U.S. Fish and Wildlife Service (USFWS) to choose it as the location for the introduced Buck Darter population. Every year since 2018, several hundred individual Buck Darters (with elastomer dye markings) have been released into the unnamed tributary during spring (pers. comm., Dr. Michael Floyd, USFWS). These individuals were excess hatchings from a successfully propagated "Ark" population at Conservation Fisheries, Inc. (Knoxville, TN), which was first established from eggs collected from nests at Stewart Branch. Biologists have monitored the stream and recaptured several individuals, indicating survivability within the new population.

The Striped Darter has been observed in small streams across much of the Upper and Lower Cumberland Drainage in southern Kentucky (Porter et al. 2002), with its population in Beaver Creek being the closest in stream size and location to the streams of the Buck Darter populations. Beaver Creek is a third-order tributary draining directly into the Cumberland River in McCreary County, Kentucky. Museum collection records indicate the prevalence of Striped Darters in two locations throughout Beaver Creek (Near & Thomas 2015) and the species' populations have shown to coincide with other *Catonotus* species (Porter et al. 2002). This differs from the Buck Darter populations which are the only Darter species located in Big Spring Branch, Stewart Branch, and the unnamed tributary (Near & Thomas 2015).

Nesting habitat choice and success are important factors for understanding fish reproduction and conservation of dwindling populations. Within the genus *Etheostoma*, the subgenus *Catonotus* (Bailey & Gosline 1955) characterized a group of five species of darters based on similar spawning habits (Page 1975). This group has since expanded and presently includes *E. nebra* and *E. virgatum* (Near & Thomas, 2015). Lake (1936) described *E. flabellare's* (Fantail Darter) spawning habits as having a territorial male who creates an opening under a slab rock. The male guards this opening and attracts females who invert themselves under the rock to lay their eggs, which the male later fertilizes. Lake (1936) also found that the incubation period for the darter eggs is around 21 days, with nests staying active longer than three weeks due to multiple deposits of eggs from different females at separate times. Kornman (1980) noted that for successful Striped Darter males, more than one female enters the nest and deposits her eggs on the underside of the rock. The male then sits at the opening of the nest, guarding an area of 15 – 20 cm around the nest from predators (Kornman 1980). All *Catonotus* darter species show these same spawning habits.

To attract more females and have increased reproductive success, males choose nests in preferred locations with superior habitat features. The Fantail Darter prefers shallower waters with decreased flow and increased water temperatures (Lake 1936). The Striped Darter has also been observed migrating upstream during spawning seasons and nesting in shallow waters to avoid predation and high-water levels during the rainy season (Kornman 1980). They mostly nest in depressions in the stream where the current is lowest, as well as in areas that have stream-washed substrates (Kornman 1980). *Catonotus* darter species' spawning seasons can range from early March through late May (Flynn & Hoyt 1979) or early April through late June (Kornman 1980). *Catonotus* spawning season length is related to water flow, day length, and water chemistry, but is mostly related to water temperature, starting at approximately 12-15°C and peaking between 14 and 19°C (Kornman 1980, Page & Burr 1976, Page 1974). Striped Darter spawning was shown to last through late June when the water temperature reached approximately 22°C (Kornman 1980). These temperatures are similar to those observed in Stewart Branch and Big Spring Branch; however, water temperature did not reach 22°C until July and stayed around that temperature through the entire month, which potentially led to the Buck Darter's spawning season being extended through the summer (Black 2018). These lower, more stable temperatures are presumed to be because of multiple springs that feed into Stewart Branch and Big Spring Branch (Black 2018).

These habitat variables, along with many others, help researchers understand which streams provide adequate spawning sites for darter populations. Spawning habitat and nesting success; however, have not been observed closely for *E. nebra*. An understanding of Buck Darter nesting habits and how they compare to the well-studied Striped Darter would provide insight into adequate stream habitat and potential locations for new populations and help guide future management decisions. The objective of my research was to answer the following questions:

1. Are the introduced Buck Darters spawning in the unnamed tributary? If so, are the spawning habitats (i.e., water depth, flow velocity, substrate), physicochemical parameters, and timing of spawning events similar to those of the established populations in Stewart and Big Spring Branch?

2. Is there a difference in nest densities between the introduced and established Buck Darter populations?

3. Are spawning habitats, physicochemical parameters, and timing of spawning similar between the closely related Striped and Buck Darters?

4. Are there differences in nest densities between Striped and Buck Darters?

Chapter II: Methods

Study Area

The study was conducted at four streams, three in the Buck Creek drainage and one that is a direct tributary to the Cumberland River. The three streams in the Buck Creek drainage are Stewart Branch, Big Spring Branch, and an unnamed stream, which are first order tributaries of Flat Lick Creek in Pulaski County, Kentucky. Flat Lick Creek runs between two ecoregions, with the upstream portion, including Big Spring Branch and the unnamed tributary, located in the Eastern Highland Rim of the Interior Plateau ecoregion (Woods et al. 2002). The downstream portion of Flat Lick Creek runs through the Plateau Escarpment of the Southwestern Appalachians ecoregion before opening into Buck Creek, including Stewart Branch which starts in the Plateau Escarpment and ends in the Eastern Highland Rim. The fourth study stream was Beaver Creek, a direct tributary of the Cumberland River in McCreary County, Kentucky, and located in the Plateau Escarpment of the Southwestern Appalachians ecoregion. The Plateau Escarpment is a sandstone and coal region known for its cliffs and gorges. Streams in this ecoregion are some of the highest quality in Kentucky, with boulder or bedrock substrates and a higher stream gradient than the Eastern Highland Rim. The Eastern Highland Rim is a karst region of hills and plains with a high stream density. Streams in this limestone-rich area are high in nutrients and moderate in gradient, underlined with a cobble, gravel, or bedrock substrate.

Big Spring Branch and Stewart Branch are both clear, spring-fed streams that drain to the north (Figure 1) (Near & Thomas 2015). They both consist of shallow pools and runs, lined with sand, gravel, and cobble, along with many slab rocks that are perfect for darter nests. The unnamed tributary is similar in location and size to Big Spring Branch and Stewart Branch, chosen as the home for the new Buck Darter population because of its similarities in spring-fed water and substrate (pers. comm., Dr. Michael Floyd, USFWS). All three streams are also similar in their surrounding land being agricultural fields and forests (Cicerello & Butler 1985, Black 2018). Beaver Creek has a larger, rockier channel that flows northeast, draining into the Cumberland River and showing a high elevation change from headwater to drainage (Kirsch 1891, Englund & Teaford 1981). It is surrounded by limestone rich land in a forested Wildlife Management Area in the Daniel Boone National Forest, with fewer springs than the Flat Lick Creek watershed (Figure 2). Two 120-m long reaches were chosen in Big Spring Branch, Stewart Branch, and Beaver Creek, and one 120-m long reach was chosen in the unnamed tributary for our study sites, based on previous nesting observations and museum collections (Figure 3 (data retrieved from KyGovMaps open data portal), Figure 4 (data retrieved from KyGovMaps open data portal), Appendix A) (Near & Thomas 2015, Black 2018).

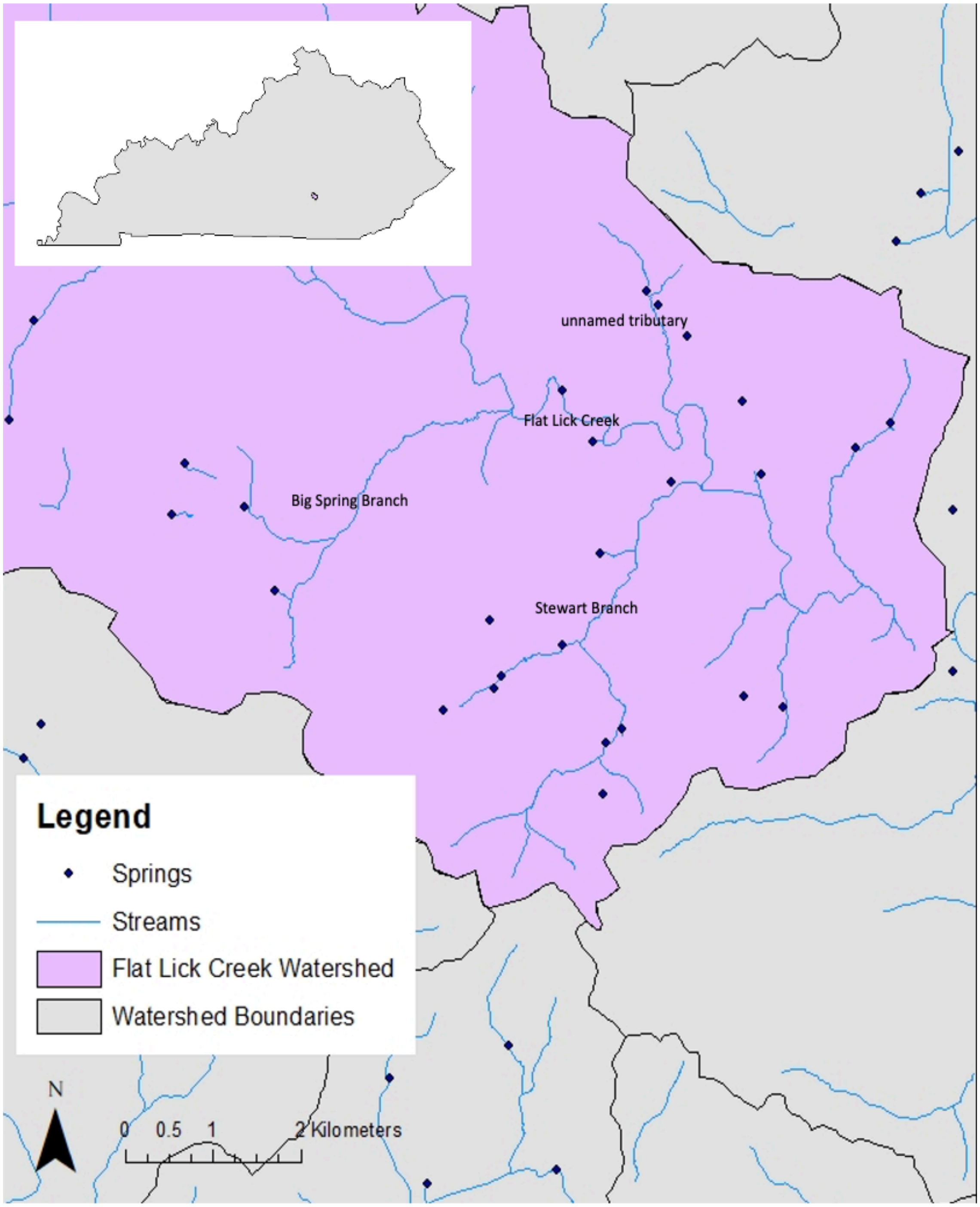

Figure 1. Map of Flat Lick Creek watershed in Pulaski County, Kentucky, showing spring locations and watershed boundaries.

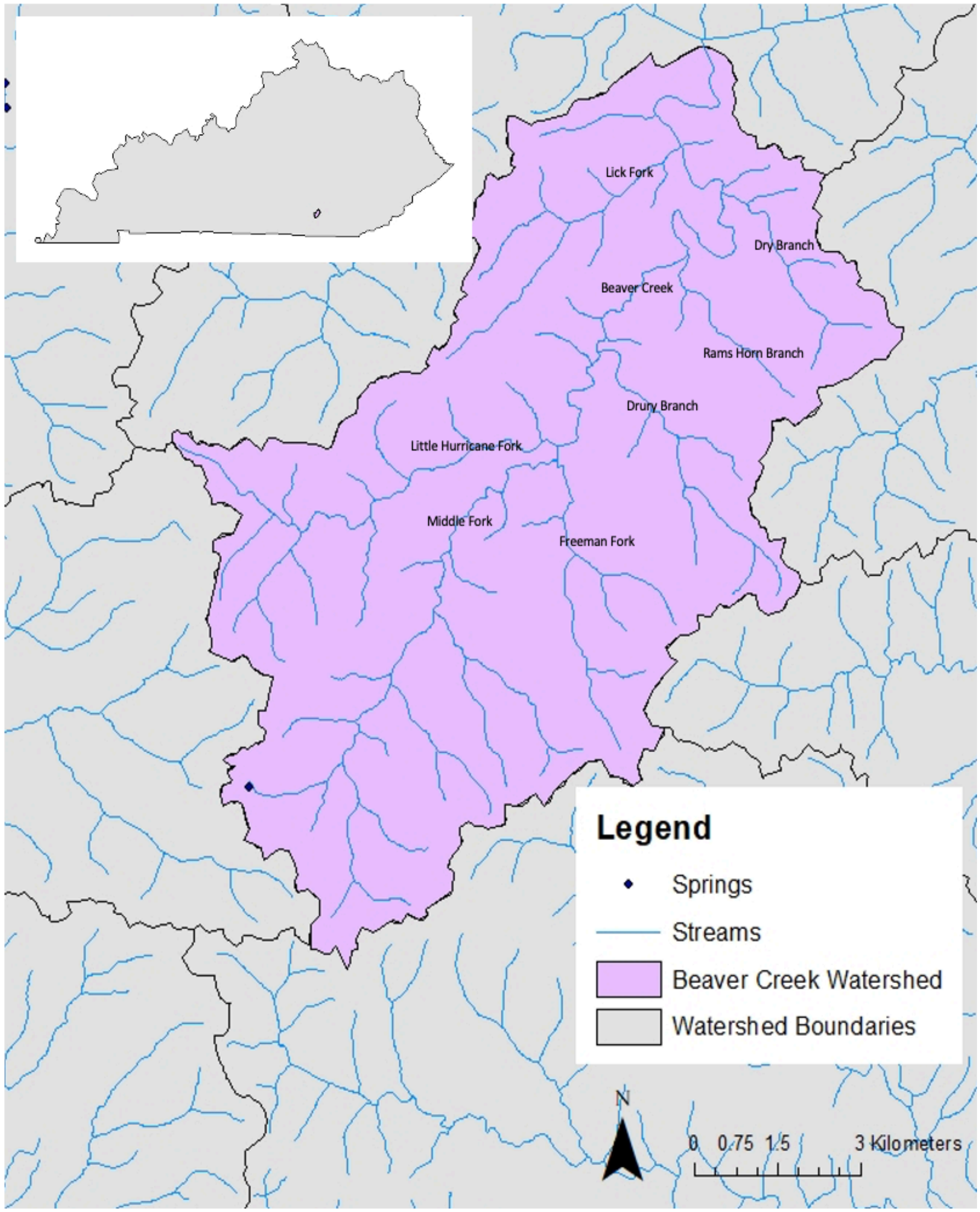

Figure 2. Map of Beaver Creek watershed in McCreary County, Kentucky, showing spring locations and watershed boundaries.

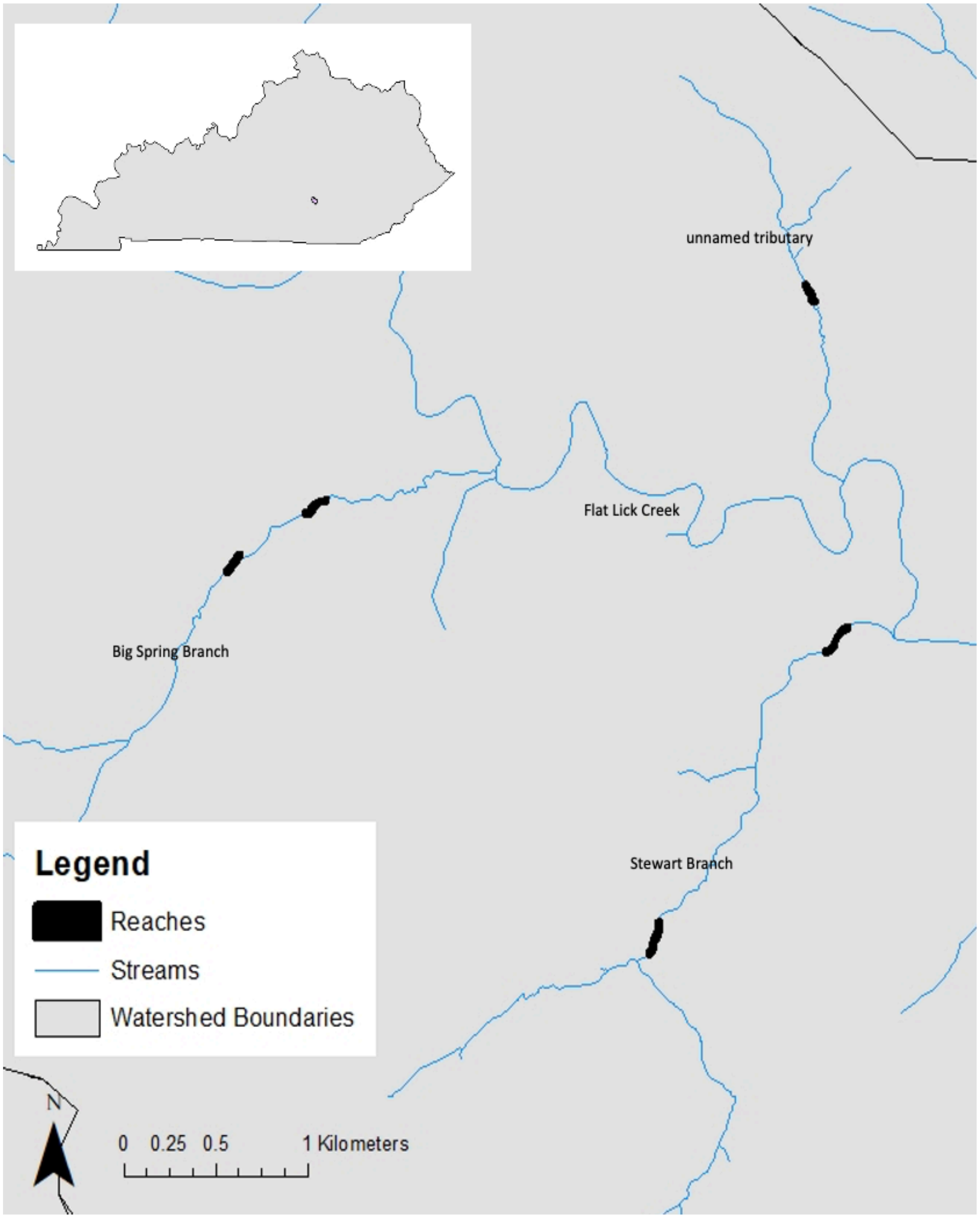

Figure 3. Map of Flat Lick Creek watershed in Pulaski County, Kentucky, showing study reach locations.

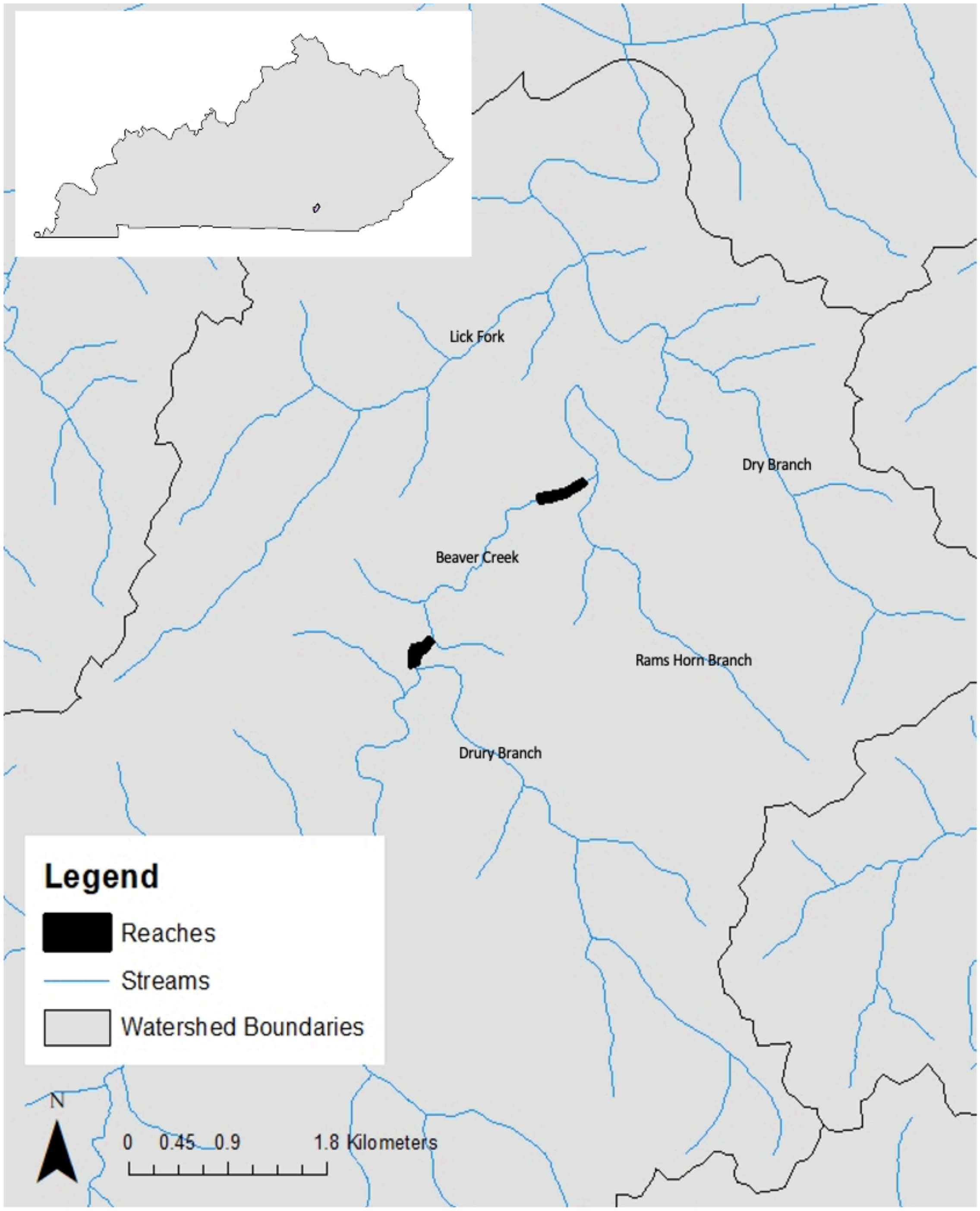

Figure 4. Map of Beaver Creek watershed in McCreary County, Kentucky, showing study reach locations.

Nest Habitat Measurements

Beginning the first week of April 2021, each reach was checked weekly for darter nests. Reaches were walked, starting at the downstream end, and potential nest rocks were identified. When a slab rock was observed, the rock was gently turned over and searched for darter eggs (Kornman 1980). The rock was then lowered back down to the bottom of the stream, being careful to place it back in its original position. If a nest was found it was flagged using flagging tape tied to the bank directly in line with the nest. At Beaver Creek, if a nest was found, it was observed after replacement for a Striped Darter to ensure it was a Striped Darter nest and not a different species of darter. Once the entire reach was checked and every potential nest rock was searched, the reach was walked again, and habitat variables were measured at the darter nests.

At each nest site, eight habitat variables were measured, including nest rock size (cm^2), water depth (cm), distance to nearest streambank (m), wetted-channel width (m), flow velocity (cm/s), water temperature (°C), conductivity (µS), pH, dissolved oxygen (mg/L), canopy cover (Mattingly and Black 2013), and substrate type. Nest rock size was measured with a meter stick, measuring the longest diameter (cm) and the shortest diameter (cm) through the center of the rock, taking care to not disturb the nest underneath. These measurements were then multiplied to get the area of the nest rock (cm^2). Water temperature (°C), conductivity (µS), pH and dissolved oxygen (mg/l) were measured with a YSI Pro Plus Multiparameter instrument (Yellow Springs Inc, OH) at each nest location directly above the nest rock. Next, looking at the area surrounding the nest, we visually observed the substrate type, using a modified Wentworth scale: fines/sediment <0.06 mm; sand 0.06–2mm; gravel 2–15 mm; pebble 16–63 mm; cobble 64–256 mm; boulder >256 mm; and bedrock. Using a meter tape, distance to the nearest streambank (nearest 0.1 m) from the center of the nest was measured perpendicular to the flow of the stream. Wetted channel width was also measured with the meter tape (nearest 0.1 m), measuring a transect perpendicular to the stream flow and running directly over the center of the nest rock. Canopy cover was visually measured using a transect running the width of the stream that crossed directly over the nest. Four equally spaced points were chosen along the transect to measure canopy cover. Each nest was given a canopy cover rank, 0-4, based on how many points along the transect gave a positive cover rating from a visual observation (0 = no canopy cover, 1 = 25% cover, 2 = 50% cover, 3 = 75% cover, and 4 = 100% cover) (Mattingly & Black 2013).

Water depth was measured using a meter stick (cm) at four locations directly surrounding the nest (upstream, downstream, to the right, and to the left) (Mattingly & Black 2013). Flow velocity was measured by placing a meter stick on the surface of the water, parallel with stream flow, and counting how many seconds it took the surface water to travel the length of the meter stick. All nest habitat variables were measured weekly for each nest except nest rock size, which was measured once when the nest was first observed.

Once a nest was located and its habitat variables were measured, the flagging tape was left to ensure it could be found during subsequent weeks. Previously located nests had their habitat variables measured weekly for four weeks, along with any new nests found, and were not checked again until the fourth week. This allowed for the nests to not be disturbed during the eggs' incubation period (Lake 1936). On the fourth week, the nests were carefully checked for eggs. If the nest was still active, the rock was gently placed back down, and its habitat variables were measured. Nests that were still active after four weeks were checked each week until no eggs were found. Once a nest was deemed inactive, the flagging tape was removed, and habitat measurements were no longer recorded at that location.

Habitat Availability Measurements

Available nest habitat was compared between reaches. Twelve one-meter-wide transects, perpendicular to stream flow, were randomly placed throughout the reach. At each transect, we measured the wetted channel width of the stream (m) and recorded the number of available nest rocks. Available nest rocks from each transect were then added together and divided by the total area of the transects to get available rocks/m^2. Next, the average length of all the transects was determined and multiplied by 120m to get the area of the reach. This number was then multiplied by the available rocks/m^2 to get the total number of available rocks in that reach. This calculation was done for each reach to determine how many nestable rocks were available in each reach.

Seasonal Water Temperature Measurements

In early April 2021, temperature and conductivity data loggers (Onset HOBO U24 Data loggers, Cape Cod, MA) were placed at two locations along three of the four study tributaries (Big Spring Branch, Stewart Branch, and the unnamed tributary), one directly upstream of each study reach. In Beaver Creek, one logger was placed directly upstream of the upstream reach. These loggers recorded water temperature (°C) and conductivity (µS) at fifteen-minute intervals throughout the entire spawning season. Data were downloaded from each logger every other month and a weekly average was calculated for each. These data were used to determine changes in temperature and conductivity throughout the season and temperature's effect on timing of spawning events between the darter populations.

Data Analysis: Nest Habitat Variables

For each quantitative nest habitat variable (nest rock size (cm^2), water depth (cm), distance to nearest bank (m), wetted-channel width (m), flow (cm/s), temperature (°C), conductivity (µS), pH, and dissolved oxygen (mg/L)), measurements were taken at each nest for the duration of the nest being active. Measurements from the first active week only for each nest were combined from each reach into populations to avoid pseudoreplication from multiple measurements at each nesting site. A Shapiro-Wilk test was then run on these data to test for normality. Data were found to be non-normal and showed unequal variances, and thus, each variable was compared between the introduced and established Buck Darter populations and between the established Buck Darter and Striped Darter populations using a non-parametric Mann Whitney U test. Box plots were also created to show differences in mean, median, and range values, showing the upper and lower quartile along with the highest and lowest non-outlier values, between the populations for all measurements taken throughout the spawning season. Canopy cover and substrate type were taken for each population and frequencies of value or type were compared between populations.

Data Analysis: Nest Density Measurements

The average width of each reach was calculated using the wetted-channel width measured at each transect and was multiplied by the 120-m length of the reach to determine the area (m^2) of each reach. The weekly number of active nests in each reach was then divided by the area of that reach to determine the weekly nest density for each reach (active nests/m^2). Weekly nest densities were then plotted for comparison between each stream to show trends in density and spawning season length.

Data Analysis: Physiochemical Variables

Mean weekly values of water temperature and conductivity were calculated from the data downloaded from the HOBO dataloggers. These averages were then plotted for each reach to show changes in temperature and conductivity throughout the spawning season for each population. A quadratic regression was then run for each reach to determine the effect of temperature on nest density. Quadratic regression was chosen because, if correlated, nest density should increase, reach its maximum, and then decrease as temperature continues to increase throughout the spawning season. Average weekly temperature was then plotted with nest densities to show trends between the two datasets within each reach. Statistical significance was evaluated at $\alpha = 0.05$ for all analyses. All Analyses were completed in the R environment (R CoreTeam 2020).

Chapter III: Results

Habitat Variables

When available nest rocks were counted using transects, the established Buck Darter reaches averaged 670 ± 291 available rocks/reach (1.71 ± 0.67 available rocks/m^2) and the introduced Buck Darter population only had 30 available rocks/reach (0.11 available rocks/m^2). The Striped Darter reaches had a much higher availability of nest rocks with an average of 1,975 ± 276 available rocks/reach (2.10 ± 0.42 available rocks/m^2).

Habitat variable means for the first week of spawning at each nest location showed differences in distance to the nearest streambank, wetted channel width, and flow velocity between the established and introduced Buck Darter populations (Table 1). The introduced population nested closer to the streambank, in a smaller wetted-channel width and in a slower flow velocity than the established population (Figure 5). Both populations showed similarities in average nest depth and nest rock size. The introduced population's nests were found in a higher mean conductivity and lower mean dissolved oxygen; however, ranges for both populations were highly overlapping. Mean values for nest depth, pH, and nest rock size were similar; however, the introduced population's nesting range for average depth was much smaller than that of the established population. Canopy cover and substrate type also showed differences between the two populations. Almost half (47%) of the established population's nests had 100% canopy cover, and 41% of nests had 0% canopy cover, with an overall average of 53 ± 48% canopy cover across all nests. The introduced population had 100% of its nests with 100% canopy cover. Substrate type for the established Buck Darter population's nests were a mix of 38% bedrock, 32% fines, and 28% pebble. The introduced population's nests had 78% bedrock and 22% fines as their most frequent substrate types throughout the season. Nesting water temperature for the established population was an average of 18.3 ± 3.0°C and slightly lower than the temperature of the introduced population's nests (19.4 ± 2.6°C).

Table 1. Summary of Mann Whitney U test results comparing nest habitat variables between the established Buck Darter population in Big Spring Branch and Stewart Branch and the introduced Buck Darter population in the unnamed tributary, Buck Creek system, Kentucky.

Habitat Variable	Established Buck Darter Population Mean (Standard Deviation)	Introduced Buck Darter Population Mean (Standard Deviation)	W	p
Average Depth (cm)	23.46 (8.30)	24.72 (4.69)	352	0.386
Distance to Nearest Streambank (m)	1.24 (0.68)	0.81 (0.34)	625.5	0.022
Channel Width (m)	3.77 (1.36)	2.74 (0.52)	665	0.006
Velocity (cm/s)	9.12 (7.45)	2.31 (3.52)	665	0.005
Conductivity (µS)	298.81 (37.30)	332.69 (106.99)	325.5	0.241
pH	8.32 (0.32)	8.34 (0.16)	367	0.488
Dissolved Oxygen (mg/L)	7.81 (2.55)	7.60 (2.74)	444.5	0.849
Rock Size Area (cm^2)	393.86 (305.72)	380.89 (199.55)	393	0.694

Based on a Mann Whitney U test comparing established (n = 95) and introduced (n = 9) Buck Darter populations.

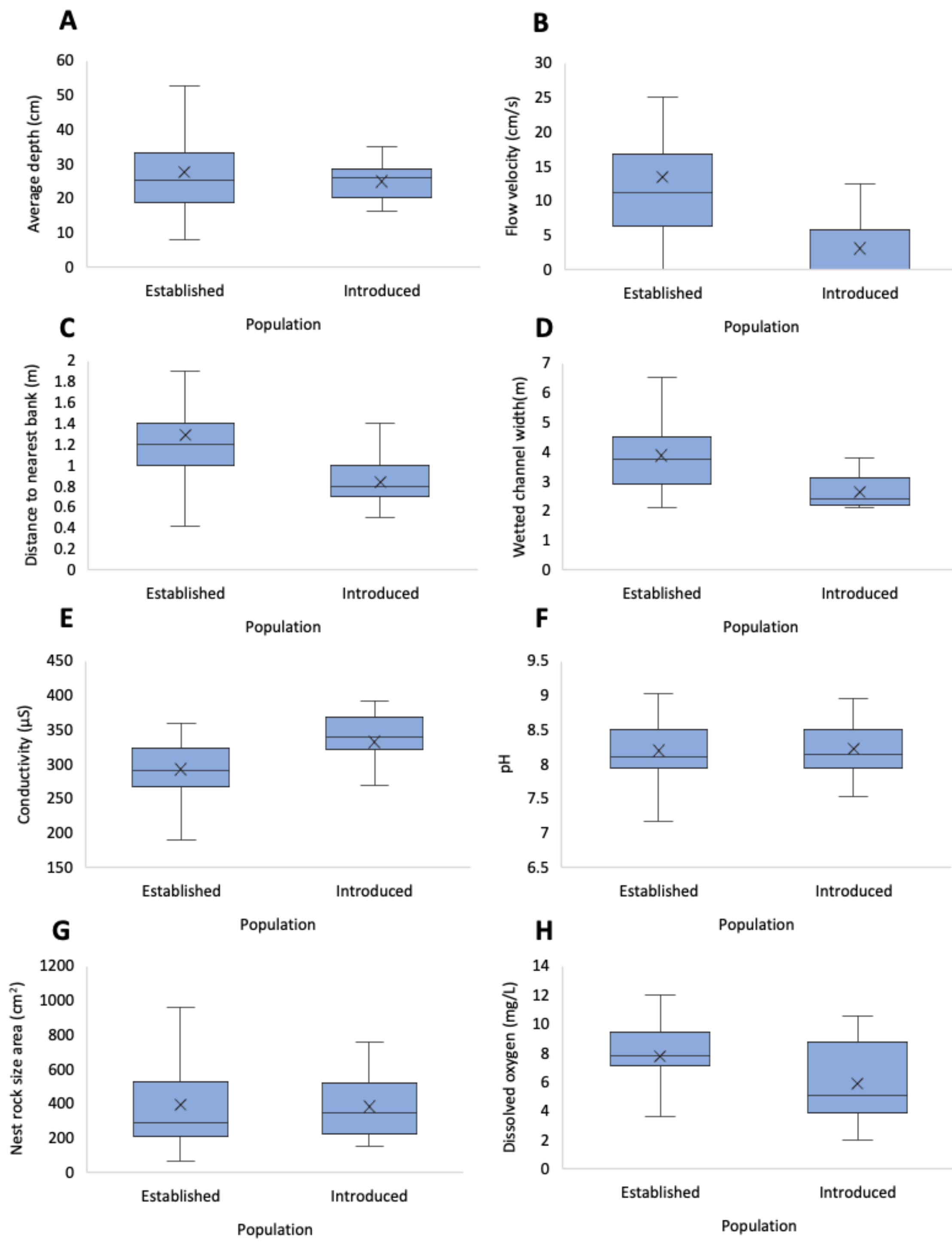

Figure 5. Comparison of nest habitat variables (A: average depth; B: flow velocity; C: distance to the nearest bank; D: wetted-channel width; E: conductivity; F: pH; G: nest rock size area; H: dissolved oxygen), showing mean (represented with an X), median, upper and lower quartile, and maximum and minimum non-outlier values between the established Buck Darter population in Big Spring Branch and Stewart Branch and the introduced Buck Darter population in the unnamed tributary, Buck Creek system, Kentucky.

The established Buck Darter and Striped Darter populations had more differences in nest habitat variables than the established and introduced Buck Darter populations. Comparison of these two populations showed differences in distance to the nearest streambank, wetted-channel width, conductivity, and dissolved oxygen (Table 2). The Striped Darter population chose nests that were farther from the streambank with a larger wetted-channel width, lower conductivity, and lower dissolved oxygen than the established Buck Darter population, although the Striped Darter nests' maximum range for dissolved oxygen reached close to that of the established population's nests. (Figure 6). Both groups did show similarities in average nest depth, flow velocity, pH, and rock size. Striped Darter nests did have a slightly lower mean flow velocity; however, the flow velocity ranges for the Buck and Striped Darter populations were very similar. The Striped Darter population also had 81% of all nests with 100% canopy cover and only 14% with 25% canopy cover. Overall, the Striped Darter nests had an average of 88 ± 26% canopy cover, higher than the established Buck Darter nests' average of 53 ± 48% canopy cover. The dominant substrate type for the Striped Darter nests were fines, found at 56% of nests, followed closely by pebble, which was found at 44% of nesting sites. The Striped Darter population showed no bedrock nesting sites compared to the established Buck Darter population which had bedrock as its most frequent substrate type. Water temperature for the Striped Darter nests was an average of 20.6 ± 1.3°C for the entire spawning season, which was slightly warmer than the 18.3 ± 3.0°C average for the established Buck darter nests.

Table 2. Summary of Mann Whitney U test results comparing nest habitat variables between the established Buck Darter population in Big Spring Branch and Stewart Branch, Buck Creek System, and the Striped Darter population in Beaver Creek, Cumberland River, Kentucky.

Habitat Variable	Established population mean	Striped Darter population mean	W	p
Average Depth (cm)	23.46 (8.30)	26.52 (7.00)	480	0.095
Distance to Nearest Streambank (m)	1.24 (0.68)	2.58 (0.99)	131	<0.001
Channel Width (m)	3.77 (1.36)	8.44 (2.82)	61	<0.001
Flow Velocity (cm/s)	9.12 (7.45)	7.78 (10.14)	787.5	0.261
Conductivity (µS)	298.81 (37.30)	51.39 (3.18(	1330	<0.001
pH	8.32 (0.32)	8.13 (0.35)	876	0.057
Dissolved Oxygen (mg/L)	7.81 (2.55)	5.93 (2.41)	965.5	0.007
Rock Size Area (cm^2)	393.86 (305.72)	336.93 (197.38)	724	0.596

Based on a Mann Whitney U test comparing established Buck Darter (n = 95) and Striped Darter populations (n = 14).

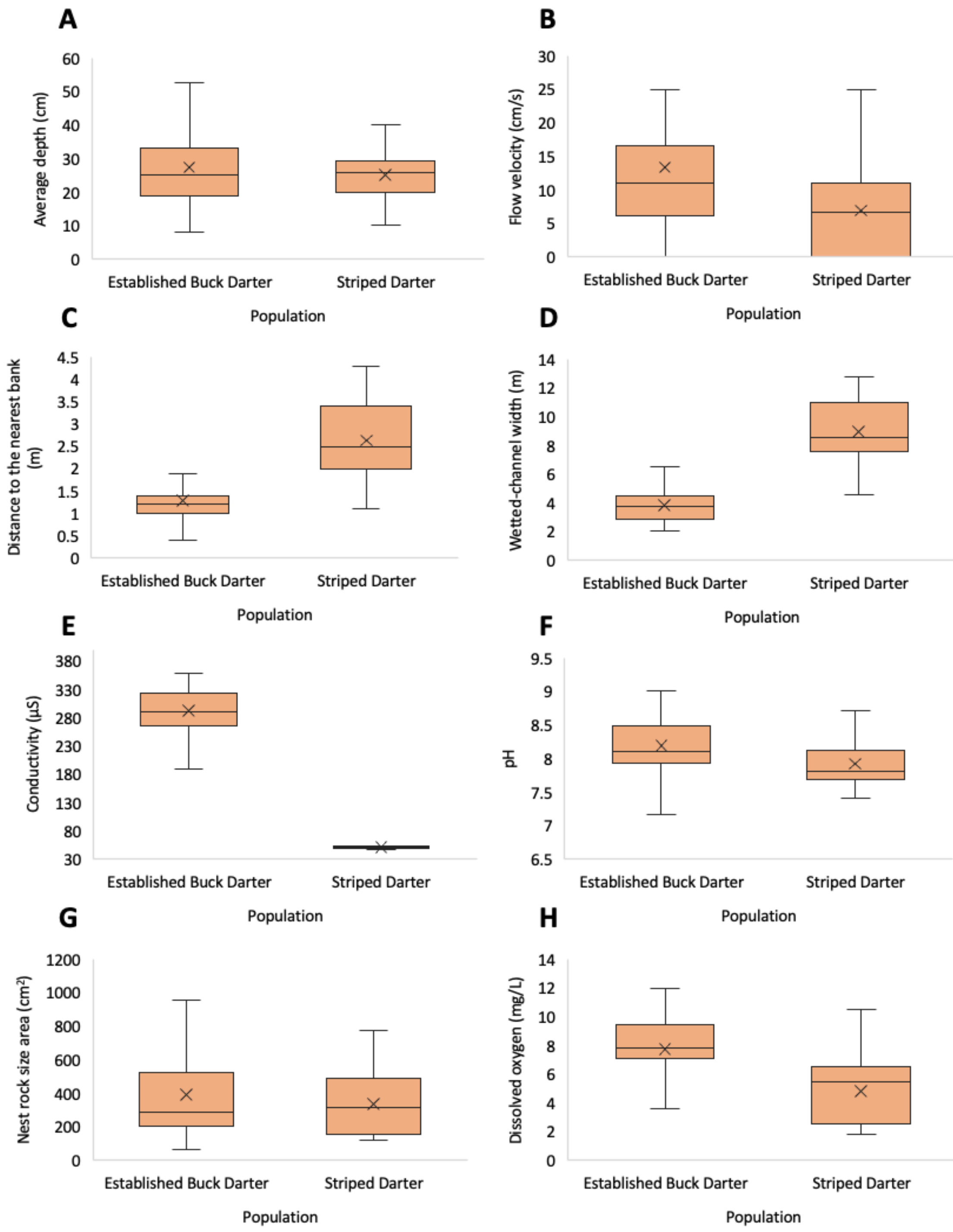

Figure 6. Comparison of nest habitat variables (A: average depth; B: flow velocity; C: distance to the nearest bank; D: wetted-channel width; E: conductivity; F: pH; G: nest rock size area; H: dissolved oxygen), showing mean (represented with an X), median, upper and lower quartile, and maximum and minimum non-outlier values between the established Buck Darter population in Big Spring Branch and Stewart Branch, Buck Creek System, and the Striped Darter population in Beaver Creek, Cumberland River, Kentucky.

Nest Density Measurements

The average nest densities across the entire spawning season for the established Buck Darter and the introduced Buck Darter populations were similar, with the established population having a slightly lower average, 0.015 ± 0.008 nests/m^2, than the introduced population, 0.02 nests/m^2. The Striped Darter population had a much lower season average with 0.004 ± 0.002 nests/m^2. Of all the reaches, Big Spring Branch had the highest nest density during one week (0.037 nests/m^2) and the unnamed tributary had the second highest (0.03 nests/m^2) (Figure 7). Beaver Creek had the lowest nest densities, having its peak week in mid-June and producing 0.007 nests/m^2. Beaver Creek also showed the shortest nesting period out of all the streams, only having active Striped Darter nests for 6 weeks out of the season, from June 3rd to July 6th. Big Spring Branch, Stewart Branch, and the unnamed tributary had longer nesting periods, producing nests for 17, 16, and 14 weeks, respectively. Buck Darters began nesting in all three streams during the week of April 15th, with the unnamed tributary ending July 13th, Stewart Branch ending July 29th, and Big Spring Branch ending August 4th.

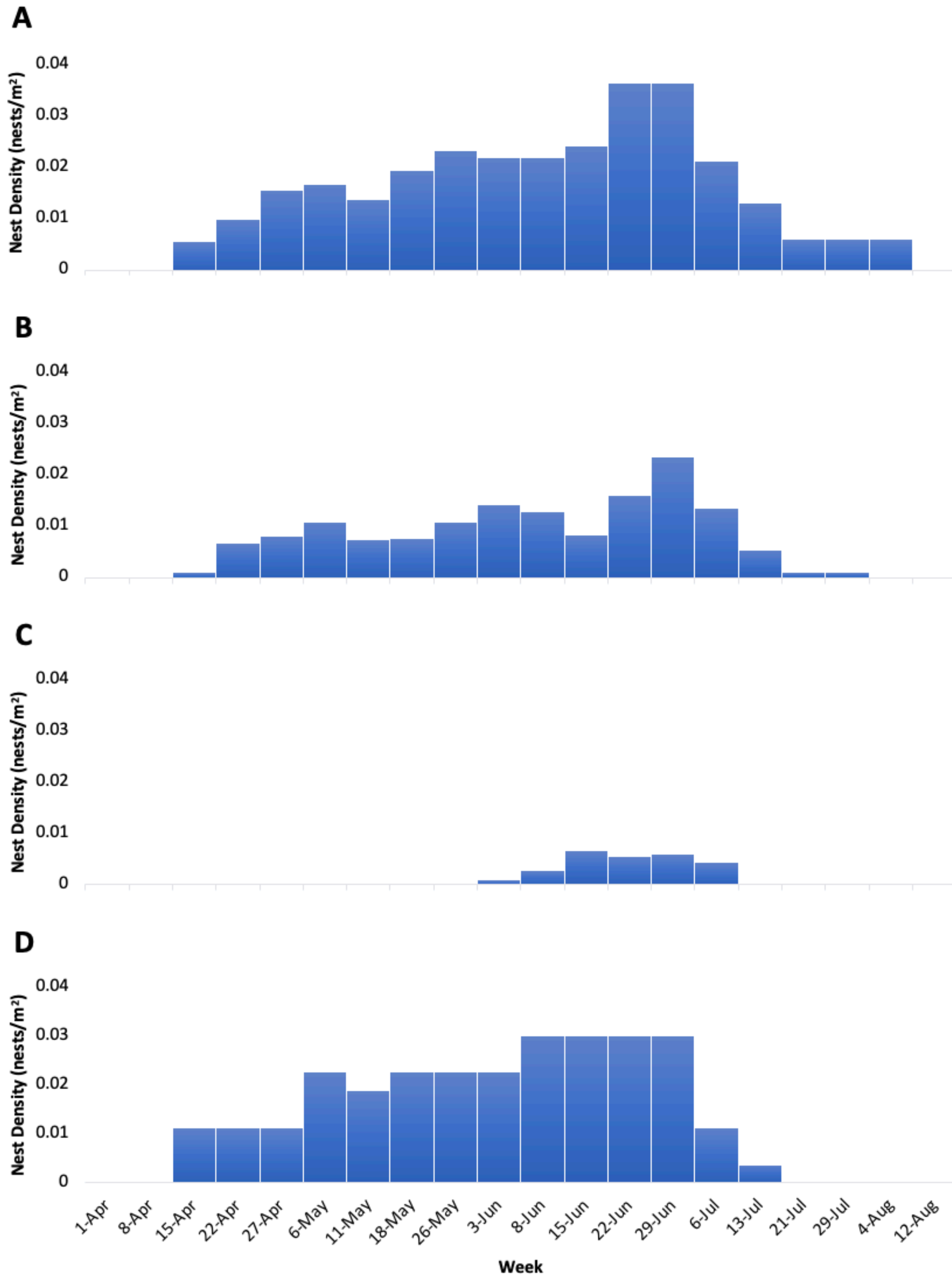

Figure 7. Comparison of weekly nest densities for each study stream (A: Big Spring Branch; B: Stewart Branch; C: Beaver Creek; D: unnamed tributary) throughout the spawning season of 2021 in the Buck Creek system and Beaver Creek, Cumberland River, Kentucky.

Physicochemical Variables

The established and introduced Buck Darter reaches showed a similar trend in temperature throughout the season starting at approximately 13°C and ending at approximately 19-22°C, with Stewart Branch upstream having the lowest temperature throughout the entire season (Figure 8). Beaver Creek started at the lowest temperature, 9°C, and ended at the highest temperature, 22.5°C, having the steepest increase in temperature throughout the season compared to all the Buck Darter reaches.

At the upstream location of each Buck Darter reach and one location in Beaver Creek, weekly average water conductivity was recorded throughout the entire spawning season (Figure 9). The introduced and established Buck Darter population reaches started the season around the same conductivity, between 210 and 240 µS, with the Stewart Branch reaches increasing the least, reaching approximately 330 µS, and the unnamed tributary and Big Spring Branch increasing the most, reaching between 360 and 420 µS, by the end of the spawning season. The one location logged in Beaver Creek showed a much lower conductivity than any of the Buck Darter locations, starting the season at 16 µS and ending the season at 52 µS.

Water temperature had no statistical relationship with nest density in six of the reaches (Table 3). The only reach where temperature had a statistically significant relationship with nest density was the unnamed tributary ($R^2 = 0.5807$, F-stat. = 6.233, $p = 0.020$). Nesting did show a similar trend in all 7 reaches in relationship to water temperature at the start and end of the nesting season. Nesting started between 13-

14°C in all Buck Darter reaches and in the upstream reach of Beaver Creek (Figure 10). The downstream reach of Beaver Creek showed a higher start temperature of 17.5°C. Nesting ended for all reaches between 20-22°C, except for Stewart Branch Upstream, where nesting ended at 18.6°C.

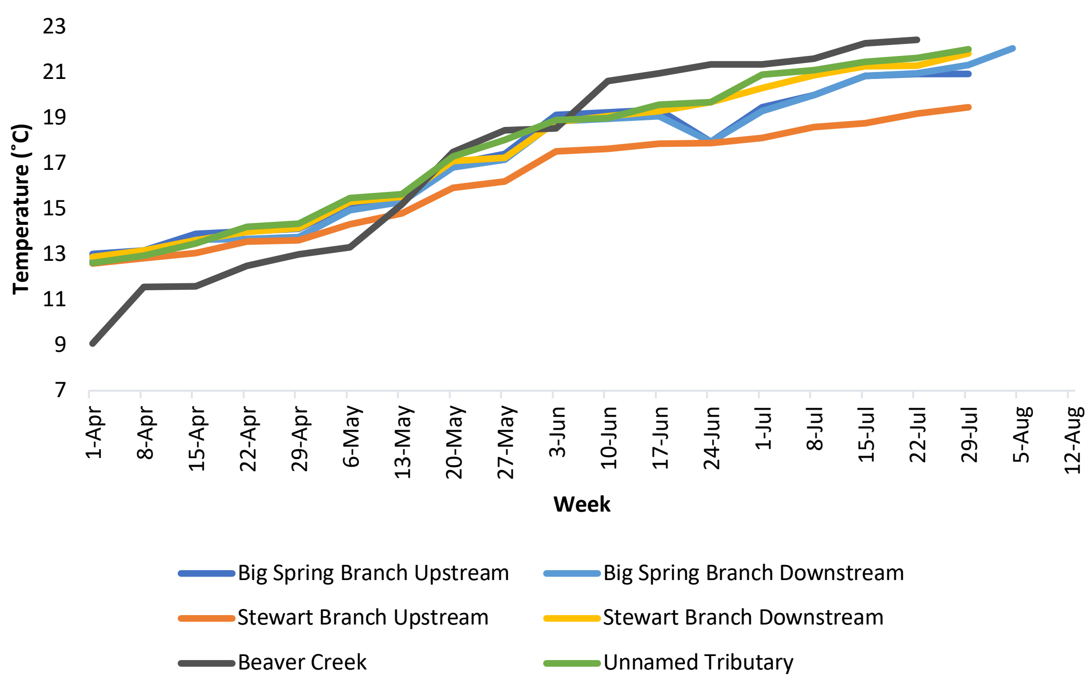

Figure 8. Weekly average water temperature for all study reaches from early April to early August, 2021, in Big Spring Branch, Stewart Branch, and the unnamed tributary, Buck Creek system, and Beaver Creek, Cumberland River, Kentucky.

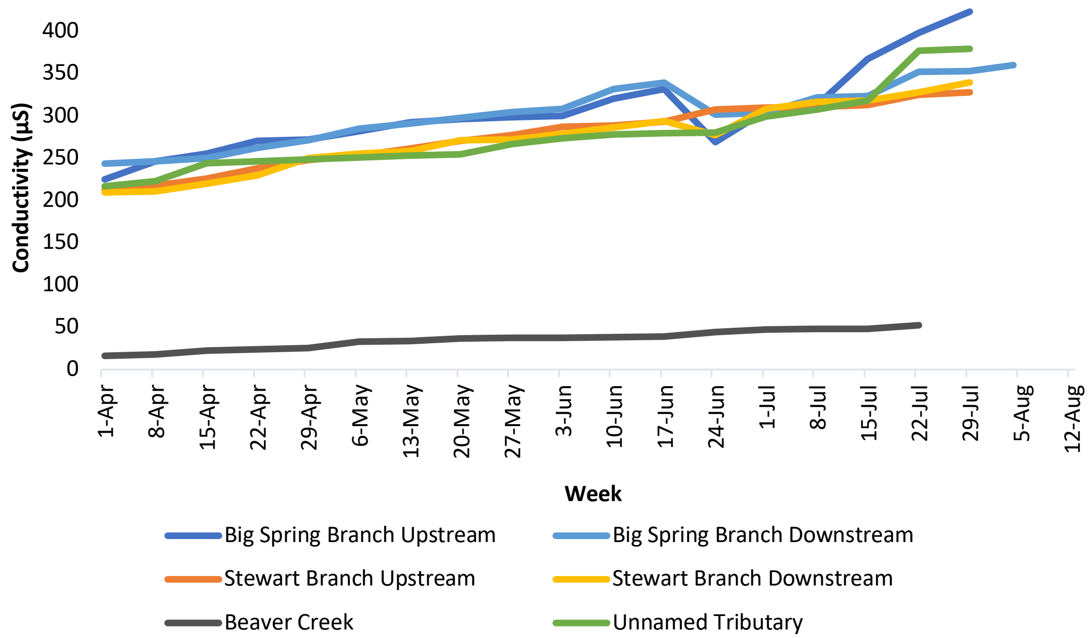

Figure 9. Weekly average water conductivity for all study reaches from early April to early August, 2021, in Big Spring Branch, Stewart Branch, and the unnamed tributary, Buck Creek system, and Beaver Creek, Cumberland River, Kentucky.

Table 3. Summary of quadratic regression results showing the effect of water temperature on nest density for all study reaches of Buck Darter and Striped Darter populations in Big Spring Branch, Stewart Branch, and the unnamed tributary, Buck Creek system, and Beaver Creek, Cumberland River, Kentucky.

Reach	R^2	F	p
Big Spring Branch Upstream	0.219	1.681	0.227
Big Spring Branch Downstream	0.264	1.614	0.252
Stewart Upstream	0.215	1.234	0.336
Stewart Downstream	0.382	3.397	0.071
Beaver Creek Upstream	0.257	1.207	0.354
Beaver Creek Downstream	0.415	1.063	0.448
Unnamed Tributary	0.581	6.233	0.020

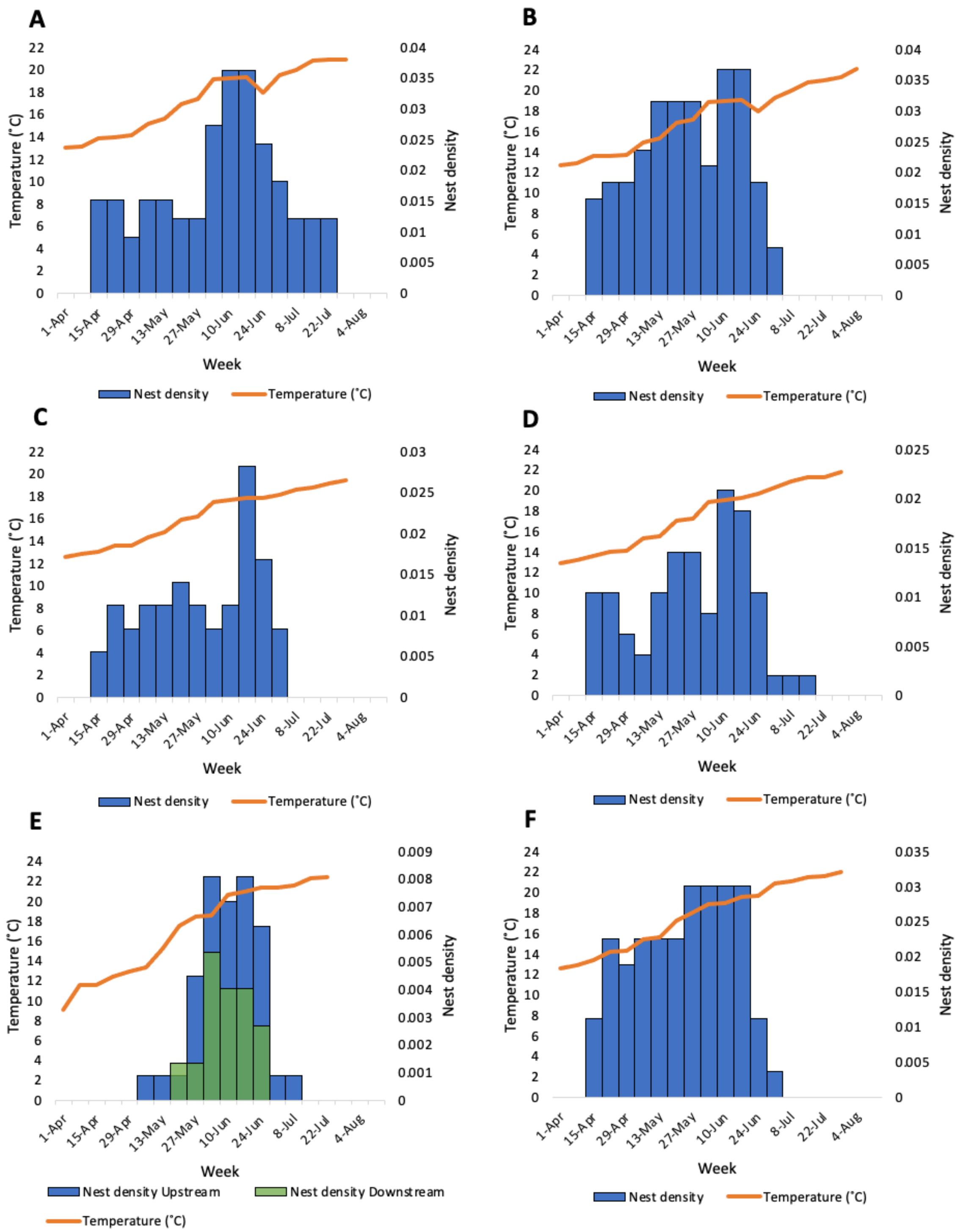

Figure 10. Comparison of weekly nest density to water temperature between each reach (A: Big Spring Branch Upstream, B: Big Spring Branch Downstream, C: Stewart Branch Upstream, D: Stewart Branch Downstream, E: Beaver Creek Upstream and Downstream, F: Unnamed Tributary) throughout the spawning season in the Buck Creek system and Beaver Creek, Cumberland River, Kentucky

Chapter IV: Discussion

Habitat Variables

Overall, the Buck Darter was found to nest in slower-moving, pool areas of the stream, like other Catonotus species (Page and Burr 1976, Kornman 1980). Its nest habitat was similar to the non-nesting habitat variables described for the Buck Darter by Black (2018), with the main difference being a smaller substrate type found in our study. The smaller substrate type for nesting locations could be due to the nest habits of Catonotus males and the need for a substrate type that allows them to dig a nest opening under the slab rock. For the introduced population, we saw similar nesting habitat as the established population, with most of the nests being in slower-moving, pool areas. The introduced population did have nests that were closer to the streambank; however, they also had an average wetted-channel width that was smaller than that of the established population. The average nest's distance to the nearest bank was 33% of the overall wetted-channel width for both populations indicating that both populations nest the same distance from the bank relative to the overall width of the stream at that location. The established and introduced nests also showed similar conductivity and dissolved oxygen; however, the introduced population's means showed a higher amount of dissolved solids and lower available oxygen than the established populations. Fish can survive at dissolved oxygen levels as low as those found in the unnamed tributary; however, darters, along with other benthic fish, avoid low dissolved oxygen levels because of physical limitations and their location away from the oxygenated surface (Doudoroff & Shumway 1970, Killgore &

Hoover 2001). The introduced Buck Darters having to nest in a low, unpreferred dissolved oxygen range could be a factor of low nest availability forcing them to nest in undesirable locations. Lower dissolved oxygen in the unnamed tributary could also be due to the slightly warmed water temperatures and slower moving water (Mackay & Fleming 1969). The major variable differences seen between the established and introduced population were flow velocity and canopy cover. These differences could have come from low nest availability in the unnamed tributary causing the introduced population to nest in unpreferred habitat compared to the established population which had more nest locations to choose from and a higher choice of nests in varying habitat. The differences in canopy cover could be a factor of the location of the reaches within the stream, with the unnamed tributary's reach having 100% canopy cover overall, whereas the established reaches varied between locations.

Nest habitat between the established Buck Darter population and the Striped Darter population showed more variability. We saw lower water quality in Beaver Creek when comparing dissolved oxygen, with the Striped Darter nest locations dipping down into the harmful dissolved oxygen range that we saw for the unnamed tributary. We did however see higher water quality for Beaver Creek with conductivity. This is most likely a factor of the protected forest surrounding Beaver creek leading to little runoff of nutrients into the stream, compared to Big Spring Branch and Stewart branch, which are completely surrounded by agricultural land (Cicerello and Butler 1985, Taboada-Castro et al. 2004, Pramual & Kuvangkadilok 2009). Striped Darter nests were also in sandy substrate, matching the substrate type found by Kornman

(1980) and differing from the bedrock around the established Buck Darter nests. Distance to the nearest streambank and wetted-channel width also were significantly different for the two groups; however, looking at the means of the two variables shows that the average nest's distance to the nearest streambank was 31% of the overall channel width for the Striped Darter. This is lower than the 33% shown for the established Buck Darter populations and indicates that the Striped Darter nests are slightly closer to the bank, relative to the overall channel width. Both groups did show similarities in nest depth, which matched those found by Kornman (1980) for Striped Darters in Clear Creek, and nest rock size; although both species' rock sizes were larger than those found for other Catonotus species (Hansen et al. 2006).

Water temperature at each nest location was not compared using the Mann-Whitney Test due to potential biases from measurements taken at different times of the day. Temperature variability was also seen to directly correlate with shaded and non-shaded areas within the established Buck Darter streams, which has shown to affect water temperatures (Rutherford et al. 1997). The upstream reaches of Big Spring Branch and Stewart Branch had the lowest overall canopy cover and had a nesting water temperature average of 1.7°C warmer than the downstream reaches of the two streams, which had the highest overall canopy cover (Appendix B). The unnamed tributary also showed 100% canopy cover but a higher water temperature, indicating there are other variables impacting the average nesting water temperature more than canopy cover. The two reaches in Beaver Creek had 100% and 50% canopy cover but showed nesting temperatures only 0.5°C apart and higher than those of the established and introduced Buck Darter populations, possibly an outcome of differing amounts of spring water entering the Flat Lick Creek and Beaver Creek systems.

Nest Density Measurements

Average nest densities during the entire spawning season were similar for the established and introduced populations of Buck Darters; however, established populations of Buck Darters showed a higher nest density than Striped Darter populations. Overall, we found the highest nest density of Buck Darters in Big Spring Branch, which corresponds to where the highest population estimate was recorded for the established Buck Darter population by Black (2018). One factor that could have impacted nest density in Beaver Creek is the presence of other *Catonotus* species that would be using the same nest rocks as the Striped Darters compared to the three Flat Lick Creek tributaries where the Buck Darter is the only *Catonotus* species (Near & Thomas 2015). This could decrease the number of available rocks and lead to a decrease in nest density in those areas. Kornman (1980) also found Striped Darter nests in close proximity to one another, within 20cm, and found that higher densities could be related to lower nest rock availability. This would explain why the Buck Darter population, which had lower nest rock availability, showed a higher nest density than the Striped Darter populations with higher nest rock availability. Conversely, the unnamed tributary was affected by low nest rock availability. Because the number of available rocks was so low, most nest rocks were active for much longer than the four-week incubation period. It is unknown if this is because more females kept coming to the same male or if new males would take over the rock as soon as the first male's nest hatched. This shows that a lack of potential nest rocks led to many nest rocks being re-used for longer periods of time. This would lower the overall nest density of the unnamed tributary because the lack of available rocks led to not as many nests being active as there could have been.

Flooding events also had an impact on weekly nest densities within the Buck Darter reaches. Large rainfall led to murky, turbid water that made the bottom of the stream not visible. Because of this, possible nest rocks were not able to be checked during those weeks and new nests could not be found, but former active nests still had their habitat variables measured. This could have led to lower weekly nest densities for Big Spring Branch, where this occurred three weeks out of the season, and the unnamed tributary, where rain and construction led to murky water for four weeks out of the season. Flooding also led to lower nest densities due to emptying a nest before its four-week incubation period was over, from either being washed away or the male abandoning the nest in the flood. Nests were checked every week after flooding to determine nests lost from rain. Three Buck darter nests were empty before the end of incubation during flooding throughout the study with all three being in Big Spring Branch; one of which was a nest found on the underside of a board, with the board unable to be found after the flood event.

Physicochemical Variables

The introduced Buck Darter population was the only group that showed temperature having a significant effect on nest density. The larger streams for the other populations could have added more variables that affected nest density, other than just temperature. Kornman (1980) explained how sunlight amount and intensity, depth, water flow, and turbidity all play a role in when the spawning season starts and ends for the Striped Darter population in Clear Creek. We may have seen a higher impact of temperature on nest density because of the lower water flow, lower depth, and higher canopy cover blocking the sunlight in the unnamed tributary compared to the streams of the other populations. We also found that for all populations, nesting started around 13-14°C and ended around 19-21°C, similar to start and end temperatures found by Kornman (1980). The nesting period for Striped Darters however, started later in the year and ended earlier than the Buck Darter populations, coinciding with the temperature in the Beaver Creek reaches that started the season off lower and had a steeper increase throughout the season, reaching 21°C an average of three weeks before all Buck Darter reaches. The higher start temperature and shallower increase throughout the season for the Buck Darter established and introduced reaches is most likely a factor of the higher number of springs that feed into these streams (Black 2018), which have shown to decrease the overall change in temperature throughout varying seasons (Tague et al. 2007). This may have been why the Buck Darter populations' nesting periods were an average of three weeks longer than those of the Striped Darter population.

Conductivity collected above each reach showed no major difference between the established and introduced Buck Darter populations but did show a major difference between the Buck and Striped Darters. Beaver Creek was found to have less dissolved solids in its water than the Buck Darter streams, which could be seen in the clarity of the water while in the stream. The high measurements of conductivity in the Buck Darter streams could also be playing a major role in the decline of this population since a similar Darter species showed declines in population abundance as conductivity rose above 261 µS (Hitt et al. 2016). The higher amount of dissolved solids in the Buck Darter reaches is most likely a factor of the agricultural land that surrounds the streams (Cicerello & Butler 1985, Taboada-Castro et al. 2004, Pramual & Kuvangkadilok 2009), or from an increase in sediment deposits from the large number of springs and high rainfall events (Toran et al. 2006). Runoff from the agricultural land also had a noticeable impact on the Buck Darter's nesting habits. Starting in mid-June, reaches surrounded by agricultural land and having low canopy cover saw a major increase in aquatic plant growth, including algae growth on many of the suitable nest rocks within the reaches. Nest densities within these reaches started to sharply decline at this point in the season, with the Stewart Branch Upstream reach, the reach that was impacted the most by algae growth, ending its nesting season an average of two weeks before the other established Buck Darter reaches. Within our stream observations we also noted no active nests under rocks whose tops were 100% covered in algae and only one active nest under a rock that was partially covered in algae.

Conclusions

More research between the Striped Darter and Buck Darter and the established and introduced Buck Darter populations would provide us with a greater understanding of how they are similar and how they both survive in very different locations. Continued observations using more samples and replication would allow for a better understanding of how the introduced population is surviving compared to the established population and how the Buck Darter species compares to the closely related Striped Darter. This would give us a greater insight into how the species relate to one another and what makes them unique enough to live apart.

This study provides us with an outline of the nesting habitat for the established Buck Darter population and the newly introduced population. Knowing these habitat variables will help us find potential locations for new populations, like the introduced population in the unnamed tributary, in streams that provide habitat ranges that are similar to those found in our study. Streams within Buck Creak that are known to be historic locations for the Buck Darter can also be observed as potential locations for reintroduction. Habitat at these locations can be compared to our findings and potential habitat restoration can be done to create suitable streams for new populations of Buck darters. It is still unknown why no other darter species have been found in the Buck Darter streams, but the nesting habitat variables found in our study could provide us with the information as to why the Buck Darter survives in this watershed. This could help lead researchers to compare locations inhabited by other darter species to the Buck Darter streams and potentially figure out why the Buck Darter is the only darter that can survive in these locations. Observing differences in nest habitat between the declining Buck Darter population and the persisting Striped Darter population gives insight into which habitat variables could have the greatest impact on the Buck Darter and the best ways to restore this habitat to help the species survive. With this information, we now know the Buck Darter nesting habitat and non-nesting habitat (Black 2018) and can use this to hopefully increase the range and size of the population. Introducing new groups to different streams within the Buck Creek Drainage could provide this species with the resources to grow its population and hopefully alleviate the decline of the Buck Darter population that has occurred over the last 40 years (Cicerello & Butler 1985, Thomas & Brandt 2013, Near & Thomas 2015).

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APPENDICES

Appendix A: Description of survey reaches in Big Spring Branch, Stewart branch, and the unnamed tributary, Buck Creek system, and Beaver Creek, Cumberland River, Kentucky.

Appendix A: Description of survey reaches in Big Spring Branch, Stewart Branch, and the unnamed tributary, Buck Creek system, and Beaver creek, Cumberland River, Kentucky.

Reach	Location Description	Latitude/Longitude
Big Spring Branch Upstream	Billy Vaught Farm	37.1592/-84.5135
Big Spring Branch Downstream	~310m downstream from Billy Vaught Farm	37.1614/-84.5099
Stewart Branch Upstream	Stewart Farm	37.1452/-84.4933
Stewart Branch Downstream	Shopville Community Park	37.15598/-84.4851
Unnamed Tributary	~800m upstream from the Flat Lick Confluence	37.1692/-84.4852
Beaver Creek Upstream	~500m upstream of Rams Horn Branch	36.9090/-84.4338
Beaver creek Downstream	~60m downstream of Drury Branch	36.9185/-84.4235

Appendix B: **Mean habitat variables for each study reach throughout the 2021 spawning season in Big Spring Branch, Stewart Branch, and the unnamed tributary, Buck Creek system, and Beaver Creek, Kentucky.**

Appendix B: Mean habitat variables for each study reach throughout the 2021 spawning season in Big Spring Branch, Stewart Branch, and the unnamed tributary, Buck Creek system, and Beaver Creek, Kentucky.

Population	Reach	Substrate Type	Depth (cm)	Nearest bank (m)	Channel width (m)	Canopy cover	Velocity (cm/s)	Conductivity (µS)	pH	Dissolved oxygen (mg/L)	Rock size area (cm^2)	Temperature (℃)
Established population	Big Spring Branch Upstream	Fines	29.9 (8.9)	1.2 (0.3)	2.9 (0.5)	0.0	14.9 (9.7)	302.9 (35.8)	8.3 (0.4)	8.0 (1.9)	304.7 (137.3)	19.4 (3.3)
Established population	Big Spring Branch Downstream	Bedrock	22.9 (10.1)	1.3 (0.5)	4.2 (0.6)	4.0	14.5 (9.6)	295.8 (28.5)	8.2 (0.4)	8.1 (1.6)	534.8 (426.2)	17.6 (2.5)
Established population	Stewart Branch Upstream	Fines	23.6 (12.3)	0.8 (0.3)	3.1 (0.8)	1.0	13.1 (23.4)	292.5 (36.1)	8.1 (0.7)	7.1 (3.1)	295.4 (249.5)	19.0 (3.2)
Established population	Stewart Branch Downstream	Bedrock	35.0 (12.4)	1.8 (0.9)	5.1 (1.7)	3.0	9.6 (12.3)	271.6 (39.5)	8.2 (0.3)	7.4 (2.7)	387.0 (229.6)	17.4 (2.9)
Introduced population	Unnamed Tributary	Pebble	24.8 (4.7)	0.8 (0.2)	2.6 (0.5)	4.0	3.0 (6.6)	331.7 (54.4)	8.2 (0.3)	5.9 (2.6)	380.9 (199.6)	19.4 (2.6)
Striped Darter population	Beaver Creek Upstream	Fines	27.1 (7.2)	2.8 (0.8)	9.2 (2.1)	4.0	8.6 (5.0)	51.1 (3.6)	7.9 (0.4)	4.7 (2.3)	270.6 (165.3)	20.4 (1.4)
Striped Darter population	Beaver Creek Downstream	Bedrock	19.3 (4.7)	2.2 (1.1)	8.1 (2.7)	2.0	5.9 (9.6)	51.8 (2.0)	8.0 (0.3)	5.0 (2.4)	502.8 (188.5)	20.9 (1.2)

Data are represented as mean (standard deviation).