

DISSERTATION

ENRICHMENT AS A CONSERVATION TOOL TO ENHANCE BEHAVIOR,  
MORPHOLOGY, GENE EXPRESSION, AND SURVIVAL IN ARKANSAS DARTERS

Submitted by

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## ABSTRACT

### ENRICHMENT AS A CONSERVATION TOOL TO ENHANCE BEHAVIOR, MORPHOLOGY, GENE EXPRESSION, AND SURVIVAL IN ARKANSAS DARTERS

Conservation practitioners often rely on captive breeding programs to supplement wild populations at risk of extinction. While population augmentation has been successful for some taxa, the use of hatchery fish to supplement wild populations can be severely impacted by predation. Elevated predation on hatchery fish may arise because hatchery environments often differ starkly from wild environments, constraining the ability of hatchery fish to phenotypically match the environments in which they are targeted for release. Phenotypic mismatch caused by differences between hatchery and wild environments can limit efforts to conserve fish species at risk of extinction when hatchery-reared fish are used to augment wild populations. Phenotypes adapted to or induced by hatchery environments are thought to be maladapted for life in the wild. Thus, enriching the hatchery environment (abiotically and biotically) to make it more similar to the wild may induce phenotypes, including behavior, morphology, and gene expression profiles, that are better suited to the environments fish will experience after release.

Chapter One explores how hatchery-reared fish respond to novel predators and whether those responses can be enhanced to improve survival. Identifying the presence of innate predator recognition and the capacity for learning to recognize predators can inform conservation management practices. We assessed antipredator behavior (time spent moving and distance from a predator) and the efficacy of predator training for three populations of a species of conservation concern, the Arkansas darter (*Etheostoma cragini*), which is vulnerable to predation by esocid

predators like the introduced northern pike (*Esox lucius*). Arkansas darters demonstrated an innate ability to recognize and respond to a novel esocid predator. Their behavior also changed in response to predator cues (training), though the direction of response to cues was opposite our prediction. Populations differed in their response to the predator treatment, highlighting the potential value of managing populations separately. Our results suggest that antipredator behavior is innate and that exposure to predator cues does affect behavior. This study demonstrates the importance of evaluating enrichment practices and incorporating behavioral observations into conservation programs to guide population-specific management decisions.

In Chapter Two, we used a factorial approach to assess whether abiotic enrichment and biotic enrichment (predator recognition training) increase survival of Arkansas darters during encounters with a novel predator. We also assessed the effects of abiotic enrichment on the expression of behavioral and morphological phenotypes across three populations. Morphology and behavior differed among populations and between abiotic treatments, and populations responded differently to the abiotic treatments. Furthermore, we found that in combination with predator training, abiotic enrichment increased the probability of surviving a first encounter with a predator. We therefore recommend conservation practitioners incorporate abiotic enrichment and predator recognition training in the hatchery, as any increase in survival is expected to benefit efforts to conserve this species.

In Chapter Three, we took a molecular approach (TagSeq) to elucidate how abiotic enrichment and biotic enrichment impacts the whole-brain gene expression of Arkansas darters, comparing the effects in two hatchery populations to a wild reference population. Although, we found no effect of biotic enrichment on gene expression, we did find that abiotic enrichment has the potential to reduce phenotypic mismatch between hatchery and wild fish, indicating that

enrichment may aid current conservation efforts. Overall, these studies suggest a potential role for enrichment in the conservation of imperiled fish, and they highlight the value of a phenotypic approach to managing populations.

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## DEDICATION

*For Noah Hallberg and the punks, up the punx, never give in!*

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# 1. ASSESSING ANTIPREDATOR BEHAVIOR AND THE POTENTIAL TO ENHANCE IT IN A SPECIES OF CONSERVATION CONCERN

## Summary

Animal behavior has the potential to guide the management of populations at risk of extirpation. This includes insights into how animals respond to novel predators and whether those responses can be enhanced to improve survival. Training hatchery-reared fish to recognize predators has been proposed as a way to reduce post-release mortalities in the wild. Identifying the presence of innate predator recognition and the capacity for learning to recognize predators in specific species or populations can inform conservation management practices. Here, antipredator behavior (time spent moving and distance from a predator) and the efficacy of predator training was assessed for three populations of a species of conservation concern, the Arkansas darter (*Etheostoma cragini*), which is vulnerable to predation by esocid predators like the introduced northern pike (*Esox lucius*). Arkansas darters demonstrated some innate ability to recognize and respond to a novel esocid predator and also responded to predator cues (training), though the direction of response to cues was opposite our prediction. Populations differed in their response to the predator treatment, highlighting the value of managing populations separately. While the fitness consequences of exposure to predator cues remain to be tested in Arkansas darters, our results suggest that antipredator behavior is innate and that exposure to predator cues does affect behavior, though whether or how those behavioral changes affect survival is unclear. This study demonstrates the importance of testing enrichment practices and incorporating behavioral observations into conservation programs to guide population-specific management decisions.

## **Introduction**

The study of animal behavior can be an effective tool that conservation practitioners use to successfully manage species of conservation concern (Blumstein and Fernández-Juricic 2010). For example, the study of antipredator behavior can elucidate how animals respond to human-caused changes in the predator community, including the loss and introduction of predators, and whether these changes may pose problems to at-risk populations (Carthey and Blumstein 2018). Further, behavioral studies can aid in the development of tools to enhance the antipredator behavior of animals in conservation breeding programs in order to increase their survival after release (McLean 1996).

Behavioral tools are being explored across multiple taxa to reduce post-release predation of animals reared in captivity (Griffin et al. 2000). For example, Shier and Owings (2007) found that training black-tailed prairie dogs to recognize novel predators prior to release decreased their susceptibility to predation. Similar findings have been documented in other taxa including birds (e.g., Cortez et al. 2015; Guadoso et al. 2011; White Jr. et al. 2005), reptiles (e.g., Burunat-Pérez et al. 2018), amphibians (e.g., Teixeira and Young 2014), and fishes (e.g., D'Anna et al. 2012; Mirza and Chivers 2000; Vilhunen 2006). However, the potential advantages of predator training vary by species and perhaps even population (e.g., Belgrad and Griffen 2016; Blumstein et al. 2019; Greggor et al. 2019; Jolly et al. 2018; Muralidhar et al. 2019; Smith and Blumstein 2012; Tetzlaff et al. 2019; Toscano 2017).

Conservation breeding programs rearing fish for population augmentation may benefit from assessing and enhancing behavioral responses to predators. In many cases, hatchery-reared fish demonstrate high rates of mortality immediately following release (Fraser 2008; Olla et al.

1998), which is thought to be caused in part by high predation rates and a lack of experience interacting with predators (Jackson and Brown 2011; Olla et al. 1998). Because hatchery-reared fish often lack experience with predators, both over their lifetime and across generations, they demonstrate reduced predator recognition, reduced antipredator behavior, and reduced survival compared to their wild counterparts (Crane et al. 2015; Fraser 2008; Jackson and Brown 2011). Experience with predators or predator cues in captivity may improve the development and expression of antipredator behavior (Brown and Laland 2001). Furthermore, introduced and invasive novel predators can be particularly devastating to native populations, as the effect of predation can be very strong due to a lack of co-evolutionary history between predator and prey (Belgrad and Griffen 2016; Sih et al. 2010; Smith and Blumstein 2012).

Predator training has been suggested as a way to increase antipredator behavior and decrease post-release mortality of fish (Brown and Laland 2001; Crane et al. 2015; Jackson and Brown 2011). One method to train fish to recognize and respond to novel predators is to associate predators with conspecific alarm cue, a chemical released from the skin during predation (Brown and Laland 2001; Smith 1979). Fish can learn to recognize a novel predator as a threat through associative learning when alarm cues are detected in combination with an identifying cue of a novel predator, such as with the predator's odor (kairomone) and/or a visual representation of the predator (Brown and Laland 2001; Griffin et al. 2000). This approach could be initiated in conservation hatcheries, potentially increasing survival of trained fish upon release. However, it is necessary to identify the presence of innate predator recognition and the capacity for learning to recognize novel predators (e.g., Kopack et al. 2015, 2016).

The Arkansas darter (*Etheostoma cragini*) is a species of conservation concern in Colorado, USA that inhabits the Arkansas River drainage. It is currently threatened by loss of

habitat and predation from introduced species, including northern pike (*Esox lucius*), a generalist apex predator that has been shown to prey on Arkansas darters and directly reduce darter distribution and abundance in Colorado (Fitzpatrick et al. 2014; Groce et al. 2012; Labbe and Fausch 2000). Captive breeding of Arkansas darters for supplementation into wild populations has been initiated by the state of Colorado (Groce et al. 2012), but its efficacy is still unclear. Fitzpatrick et al. (2014) found that hatchery-reared Arkansas darters contributed very little genetically to wild populations where they had been released. Arkansas darters reproduce in the hatchery, suggesting reproductive barriers are an unlikely cause for their lack of genetic contribution. Instead, it is suspected that hatchery-reared darters cannot reach reproductive age in the wild due to high mortalities from predation (Olla et al. 1998), prompting the state of Colorado to consider the use of predator training as a way to decrease the number of darters lost to predation after release. Little is known about the antipredator behavior of Arkansas darters. To our knowledge, no studies have investigated the response of Arkansas darters to cues of predation or the potential to enhance them in hatchery-reared individuals intended for release.

The goal of this study was to determine if hatchery-reared Arkansas darters have an innate capability to detect and behaviorally respond to the threat of predation from a novel esocid predator (tiger muskellunge [northern pike *E. lucius* X muskellunge *E. masquinongy*]; hereafter “tiger muskie”), and if predator training could be used to enhance the darters’ recognition of, and antipredator responses to, the novel predator. Objectives of the study included determining: i) if Arkansas darters are capable of responding to a non-native predator by reducing movement and maintaining their distance from the threat, ii) if exposing darters to chemical and visual predator cues prior to an encounter with a tiger muskie increases antipredator responses, and iii) if responses to the tiger muskie or predator recognition training differ among three darter

populations that are known to exist with and without esocid predators. Because Arkansas darters have not coevolved with tiger muskie, we predicted that darters would not have an innate predator response to them. We predicted that exposure to alarm cues coupled with tiger muskie kairomones and a visual model would increase antipredator behavior through associative learning. Finally, because populations are known to differ genetically and in the predator community they experience, we expected to find differences among populations in their response to predators as well as perhaps their response to predator training.

## **Methods**

### *Animal Husbandry*

We conducted this experiment using three populations of Arkansas darters: 1) Big Sandy Creek (coordinates 717413, 4269881), 2) Black Squirrel Creek (524117, 4317294), and 3) Horse Creek (613691, 4260607) (hereafter populations 1, 2, and 3). These three populations serve as source populations for conservation hatchery propagation and stocking in Colorado, and we suspected that behavioral responses could differ by population due to genetic differences (Fitzpatrick et al. 2014) and the differences in native and introduced predators known to exist in these populations. Specifically, population 1 experiences a broader community of predators, including an introduced esocid, compared to populations 2 and 3 (Table 1.1).

Both darters and predators for this experiment were sourced from hatcheries in Colorado. One hundred young-of-year Arkansas darters were obtained from each population (ranging from 37 to 58 mm total length with no size differences among populations) from the Colorado Parks and Wildlife (CPW) John W. Mumma Native Aquatic Species Restoration Facility (Alamosa, CO). The genetic integrity of these populations is maintained in the hatchery through careful

breeding practices, including frequent supplementation using wild individuals, meaning hatchery-reared Arkansas darters are only a few generations removed from wild individuals (Fitzpatrick et al. 2014). Seventeen young-of-year tiger muskie (measuring 233 to 295 mm total length) were obtained from the CPW Wray Fish Hatchery (Wray, CO). Tiger muskie were selected as predators because they were readily available, free of disease, easy to maintain, and are a hybrid cross of northern pike and muskellunge.

Arkansas darters and tiger muskie were transported to the CPW Salmonid Disease and Sport Fish Research lab (Fort Collins, CO) where experiments took place. Each species was held for four weeks prior to the start of the experiment in 76-L flow-through tanks (31.75 x 61 x 42 cm) where darters were separated by population and predators were divided into groups of five to six individuals per tank. The water was supplied from a 16-foot well, filtered through a coke ring headbox for degassing and aeration, then held in a head tank before being gravity fed to the tanks at a flow of 7.5 L per minute. Water temperature was maintained at  $13.5 \pm 2^{\circ}\text{C}$  for the duration of the experiment. The lab was illuminated by 32 W fluorescent lights (General Electric Electorlux) with a light cycle of 14:10 hours light:dark, and all experiments and observations took place when lights were on. Darters were fed thawed bloodworms (San Francisco Bay Brand Inc., Newark, CA), while tiger muskie were fed trout feed (Rangen Inc. <sup>TM</sup>, Buhl, ID), ad libitum, once daily. One week prior to conducting any behavioral observations, darters were moved to individual 10-L flow-through tanks (23 x 31 x 19 cm, water depth 17.75 cm) for the remainder of the experiment and continued to be fed thawed bloodworms ad libitum, once daily.

#### *Chemical Cue Extraction*

As part of the predator training treatment, we exposed darters to conspecific alarm cues and kairomones from tiger muskie. Conspecific alarm cues were collected from the epidermal tissue of 51 recently decapitated Arkansas darters (17 from each population, mixed together). Immediately following decapitation, fish were scored 50 times on either side in a crosshatch pattern using a razor blade. Bodies were then rinsed with 10 mL of distilled water filtered through cheesecloth, and the water with the cues was immediately stored in a freezer at -20°C until the day of its use (adapted from Nordell 1998 and following Kopack et al. 2015). Predator kairomones were acquired by collecting water from a closed circulatory 19-L tank that housed two tiger muskie predators for 48 hours. During this time, the predators were fed six darters (two from each population), so the water likely contained conspecific alarm cues in addition to the predator kairomones. The collected water was stored at -20°C until the day it was used (following Kopack et al. 2015).

### *Experimental Design*

This experiment had three parts: 1) we observed darter behavior in the absence of a predator (hereafter “predator-absent” observation) to establish a pre-training baseline, 2) we randomly assigned darters to a treatment (either predator training or a control) and treated them, and 3) we then observed darter behavior in the presence of a predator (hereafter “predator-present” observation; Figure 1). Each individual darter participated in all three parts. In the predator-present observation, the behavior of the control darters represents innate antipredator behavior while the behavior of the darters from the treatment group reflects the added effect of the training.

Both the predator-absent and the predator-present observations were conducted in the same 76-L tank (31.75 x 61 x 42 cm, water depth 30.5 cm), and tanks were emptied and sterilized with iodophor between each trial. The tank was fitted with a permanent, semi-permeable transparent plastic divider in the center to separate darters from the tiger muskie, which allowed water and thus odor to move between the two sides, as well as a removable opaque divider so that visual access to the tiger muskie could be controlled. On the bottom of the tank, on the side occupied by the darter, there was a 30 cm by 30 cm grid (consisting of 36 5-cm<sup>2</sup> blocks), which was used to reference the distance between the darter and the divider (Figure 1). A camera (GoPro Hero 4 Session # CHDHS-101) was mounted above each tank to record behavior, and a blind was placed in front of each tank to hide researcher movements during the experiment.

Predator-absent and predator-present observations of darter behavior were conducted sequentially and identically except that a live predator was present in only the predator-present observation. Bloodworms were withheld from darters for 24-hours prior to the start of the observations. At the beginning of each pre-training, predator-absent observation, nothing (i.e., no tiger muskie predator) was placed on one side of the permanent transparent divider. With the opaque divider in place, we introduced a darter to the opposite side of the tank and allowed 30 minutes for acclimation. After 30 minutes, the opaque divider was removed allowing darters to view the opposite side of the tank through the transparent divider. The video camera then recorded behavior for 20 minutes. During the observations, we introduced food, five thawed bloodworms placed along the darter side of the transparent divider using a 1-ml pipet, in the middle of the 20-minute experiment (after 10 minutes). With no threat of predation on the other

side of the divider, we expected darters to move about the tank normally and approach the divider in an attempt to feed following the introduction of food.

After all observations were complete, a trained observer that was not aware of the treatments scored each video to extract two dependent variables: 1) time spent moving and 2) distance from the divider. For movement, the observer recorded the amount of time a darter spent moving (seconds) during the first 10 minutes of the 20-minute observation period (before food introduction) and during the last 10 minutes of the 20-minute observation period (after food introduction). For distance, we used the grid on the bottom of the tank to measure distance from the divider before and after food was added. As a measure of distance before the introduction of food, we measured distance at 0 min and 10 min and averaged the values, and similarly measured distance at 11 min and 20 min and averaged the values following the introduction of food.

Upon completing the initial pre-training behavioral observations in the absence of a tiger muskie, darters were placed back into their individual 10-L tanks for treatment. One week later, half of the individuals from each of the three populations were assigned to a Water Control or Predator Cues treatment. The Water Control treatment received 10 mL of distilled water while the Predator Cues treatment received 5 mL of alarm cue mixed with 5 mL of predator kairomone (previously thawed at room temperature) as well as exposure to a visual predator model. Blinds were placed over all individual tanks to prevent researcher movements from influencing the treatment, and darters were allowed 30 minutes to acclimate following the placement of the blind before initiating the treatment. To add the liquid to each individual tank, air tubing was taped to the front right corner of each tank, extended outward past the viewing blind where the researcher was positioned, and attached to a 10-mL syringe loaded with water or cue. We turned off the

water in the flow-through system to prevent dilution of the cues during the treatment period, and the treatment lasted five minutes. For the visual predator model used in the Predator Cues treatment, a 102 mm plastic lure that resembled a tiger muskie (model # SGK022, Savage Gear USA ®, Ontario, CA) was attached to a wand with fishing line. A researcher standing behind the blind used the wand to introduce the model into the individual tank when the liquid was added (water or cue). After five minutes, the model was removed and the water was turned back on to flush the tank.

Twenty-four hours after darters were treated, they participated in the predator-present observation. We used the same methods described above to record darter behavior, but with one of 17 randomly chosen tiger muskie predators present on the other side of the transparent divider. If darters perceived the predator as a threat, we expected darters to reduce movement and maintain or increase their distance from the divider, rather than approaching the divider in an attempt to feed.

### *Statistical Analysis*

To evaluate if Arkansas darters respond to tiger muskie predators and to assess whether predator training enhanced those responses, darter behavior was measured before (predator-absent observation) and after (predator-present observation) predator training as well as before and after the introduction of food using two metrics: (1) time spent moving and (2) distance from the divider. These behaviors were used as measures of antipredator response, as we expected darters to spend less time moving and to maintain a greater distance from the divider in the presence of a tiger muskie predator if they exhibit normal antipredator behavior typically seen in prey-fish (Crane et al. 2015; Januchowski-Hartley et al. 2011). Time spent moving had a skewed

distribution and unequal variance around the mean (Levene's test of equal variance,  $F_{23,334} = 4.40$ ,  $P < 0.01$ ; "leveneTest" in R, using the car package, Fox and Weisberg 2019) and was therefore standardized using a natural log transformation prior to analysis. During post-training predator-present observations, some tiger muskie breached the transparent divider, consuming darters when they did. A total of 14 darters were consumed during predator-present observations. These trials were excluded from the analyses, resulting in fewer predator-present observations than predator-absent observations (predator-absent:  $N = 120$ ; predator-present:  $N = 106$ ).

We compared antipredator behavior between treatments (Water Control and Predator Cues), among populations (1, 2, 3), before and after food introduction, and in the absence or presence of a tiger muskie in a mixed model. Because each individual was tested with a predator absent, then with a predator present, and before and after the introduction of food, we used a repeated measures analysis of variance (RM ANOVA) for each behavior: movement and distance (lme4 package, Bates et al. 2015; lmerTest package, Kuznetsova et al. 2017). Presence of a tiger muskie (present, absent), treatment (Water Control, Predator Cues), the introduction of food (before, after), and population (1, 2, 3) were treated as fixed effects, while individual ID was treated as a random effect for both analyses (movement and distance). All two-way, tertiary, and quaternary interactions between the fixed effects of behavior were also investigated. We retained and report significant and nonsignificant effects from the full model since each of these fixed effects and interactions were part of our experimental design, we had a priori predictions for all factors in our model, and others have argued against removing nonsignificant effects from models as it can bias the results (e.g., Forstmeier and Schielzeth 2011; Wittingham et al. 2006). Values were reported from the Type III sum of squares. If fixed effects were found to be significant in a model ( $P \leq 0.05$ ), the least squares means method (lsmeans package, Lenth 2016)

was used to determine differences in behavior between treatments, among populations, before and after food introduction, and in the absence and presence of a tiger muskie. All statistical analyses were performed using R statistical software (R Core Team 2017).

## Results

The amount of time darters spent moving in seconds(s) varied in response to the presence of a predator, the introduction of food, and their treatment (exposure to water control or to predator cues). First, darters spent less time moving when there was a tiger muskie predator present compared to when the predator was absent ( $F_{1,258} = 61.84, P < 0.001$ ; Figure 1.2a). This was true for both treatment groups, but treatment also had an effect; on average the group exposed to a water control moved less (mean  $\pm$  SE =  $49.89 \pm 6.14$  s) than the treatment group exposed to predator cues ( $56.51 \pm 6.11$  s;  $F_{1,89} = 4.26, P = 0.04$ ; Figure 1.2a). There was no interaction between the treatment and the presence of a predator ( $F_{1,271} = 1.89, P = 0.17$ ). The introduction of food caused a decrease in movement in the absence of a predator ( $t_{246} = 6.12, P < 0.001$ ) but did not affect the already low levels of movement when a predator was present ( $t_{251} = 0.86, P = 0.39$ ; interaction term  $F_{1,249} = 12.97, P = 0.0004$ ; Figure 1.2b). When a predator was absent, 8.5% of all darters attempted to feed, while 2.1% fed when a predator was present. The three populations also responded differently to the treatment (interaction  $F_{2,88} = 3.48, P = 0.04$ ; Figure 2c), and the main effect of population was marginally significant ( $F_{2,88} = 3.46, P = 0.06$ ). When a predator was present, individuals from population 3 that experienced the Predator Cues treatment spent more time moving than those from the Water Control treatment ( $t_{88} = -2.95, P = 0.004$ ), whereas there were no significant difference in time spent moving between treatments for populations 1 and 2 (population 1:  $t_{90} = -1.36, P = 0.18$ ; population 2:  $t_{86} = 0.78, P = 0.44$ ; Figure

1.2c). Within the Water Control treatment, population 3 spent significantly less time moving in the presence of a tiger muskie than darters from populations 1 ( $t_{89} = 2.38, P = 0.05$ ) and 2 ( $t_{86} = 3.15, P = 0.006$ ). There were no significant differences in time spent moving among populations in the Predator Cues treatment ( $-0.59 \leq t \leq 1.42, P \geq 0.05$ ), as fish from all three populations converged on an intermediate amount of movement when a tiger muskie was present (Figure 1.2c).

Darters changed their distance from the divider in response to predator presence and treatment (Figure 1.3), but population ( $F_{2,89} = 0.13, P = 0.88$ ) and food introduction ( $F_{1,248} = 0.05, P = 0.83$ ) had no effect. When there was a tiger muskie predator present, darters increased their distance from the divider ( $F_{1,269} = 27.90, P < 0.001$ ; Figure 3). Darters in the Predator Cues treatment decreased their distance to the divider compared to darters in the Water Control treatment ( $F_{1,89} = 4.53, P = 0.04$ ; Figure 1.3). There was no interaction between these two main effects ( $F_{1,269} = 0.77, P = 0.38$ ).

## **Discussion**

Our results suggest that Arkansas darters may possess some innate ability to detect, recognize, and respond to the threat of predation from a novel esocid predator. Opposite our prediction, we observed antipredator behavior in the presence of a predator in untrained, control fish. Regardless of the treatment they received, Arkansas darters strongly decreased their time spent moving and increased their distance from the divider when a tiger muskie was present, and both responses are in the direction indicative of antipredator behavior. This suggests an innate ability to recognize and respond to a novel esocid predator, despite never coevolving together (Ferarri et al. 2008; Sih et al. 2010). Innate responses may not be uncommon; Kopack et al.

(2015) found that hatchery-reared rainbow trout (*Oncorhynchus mykiss*) exhibit innate antipredator responses to predator cues. This innate response may reflect the ability of Arkansas darters to generalize predatory threats across suites of species (Ferrari et al. 2008) and may stem from the high predation pressure they experience given their small size at maturity (Jørgensen and Fiksen 2010). Generalizing predatory threats may offer Arkansas darters a way to minimize costs of maintaining species-specific responses given the uncertainty of which predators will be encountered throughout their lives (Ferrari et al. 2008). Future experiments should further explore whether Arkansas darters generalize predatory threats, or if other predatory species elicit greater responses, by observing antipredator behavior in response to training with different native and non-native predators.

Arkansas darters altered their behavior in response to predator training, but it was not in the direction predicted. In the presence of a predator, darters treated with predator cues spent more time moving compared to control fish. A decrease in movement, not an increase, is thought to increase the probability of surviving initial encounters with predators (in rainbow darters *Etheostoma caeruleum*, Crane et al. 2015); however, whether a reduction of movement in the presence of an esocid predator is adaptive in Arkansas darters is unknown. Future studies should directly measure survival, especially since exposure to predator cues does not always translate to increased survival after release (e.g., Kopack et al. 2016) and because the factors correlated with the success of reintroduction programs are diverse (e.g., water quality, prey availability, duration of stocking event; Cochran-Biederman et al. 2015). Further, the magnitude of the treatment effect was small: treatment fish moved for 6.6 seconds more than control fish. It is unclear whether these differences would translate to differences in survival upon initial encounters with a novel predator.

We also found an effect of the predator training on distance from the divider; fish exposed to the predator cues were 28.1 mm closer to the tiger muskie than fish treated with a water control. This effect is small, and we are cautious in our interpretation of this result. There was a slight difference in the predator-absent responses between treatment groups (Predator Cue or Water Control) before we assigned and administered these treatments, potentially due to sampling error and/or variability in individual responses measured during that observation period. This could have affected differences between treatments in our distance measurements when the predator was present after treatment occurred. Additionally, our measure of distance from the divider was an average of only four time points rather than continuous measurements across the entire trial. Despite these limitations, the patterns we detected for distance from the divider mirror our results for movement, providing support for the general result that predator presence and training affected darter behavior.

A similar study in rainbow darters found effects of predator cues on behavior for wild but not hatchery populations (Crane et al. 2015). It is possible that wild Arkansas darters would also be more responsive to a predator cue treatment than the hatchery-reared fish used in our study, and this should be explored in future work, as relaxed and/or reversed selection in captivity could impact the response to predator cues (Brown and Laland 2001; Crane et al. 2015; Fraser 2008). This information could inform the decisions of aquatic resource managers. For instance, if wild Arkansas darters are more responsive than hatchery populations, then continuous and opportunistic supplementation of hatchery populations and their broodstock using wild darters would be necessary to maintain appropriate antipredator behavior in hatchery-reared fish over time.

The three populations differed in their response to the treatment for movement. Exposure to predator cues affected movement for population 3, but did not have a significant effect on the other two populations, highlighting the importance of considering discrete populations independently rather than the species as a single homogenous unit. The cause of this difference among populations is unclear. This pattern does not match population differences in exposure to esocid predators since only population 1 coexists with northern pike. While we know that northern pike do forage on and directly affect abundance and distribution of Arkansas darters (Labbe and Fausch 2000), there are certainly other abiotic and biotic sources of mortality (e.g., pools freezing, Labbe and Fausch 2000) that could affect population-specific treatment efficacy and post-stocking survival among these populations.

To test the tradeoff between safety and foraging, we presented darters with food during observations with and without a predator present. If darters perceived the predator as a threat, it should have affected their foraging behavior. However, very few animals fed during our experiment (8.5% when a predator was absent and 2.1% fed when a predator was present) limiting our ability to make inference from this study. We did find that in the absence of a predator, there was a reduction in movement after food was added, closer to the low levels of movement observed in the presence of a predator. This could indicate that the addition of food had a startling effect that induced antipredator behavior or it could reflect a reduction in exploratory behavior over time in the tank. Unfortunately, we cannot tease these two alternatives apart or draw conclusions about potential tradeoffs between foraging and antipredator behavior from our study, but future work should incorporate foraging/antipredator tradeoffs to more closely mimic the decisions facing wild individuals.

Our study demonstrates how behavioral observations can be applied in conservation programs to guide the decisions of managers. Our experimental design allowed us to mimic what Arkansas darters experience during initial encounters with non-native esocid predators after release and gain insight into how they respond. Arkansas darters may have an innate potential to respond to novel esocid predators, which managers could leverage, but ideally these would be compared to the antipredator responses of wild darters in future studies. Predator recognition training has been suggested as a way to increase post-release survival of captive-reared, predator-naïve species of conservation concern (Brown and Laland 2001; Crane et al. 2015). To our knowledge, our study is the first to investigate the potential to enhance antipredator behavior in Arkansas darters. While we did find effects of predator training, the behavioral responses were not in the direction predicted and varied across populations; further it is unclear whether or how these responses might affect survival and fitness. This highlights the importance of testing enrichment strategies before implementing them widely in hatcheries. There are also several other methods that could potentially increase antipredator behavior and survival in this species that have yet to be explored, such as multiple training events, training in groups rather than individually (which would be more logistically feasible in a hatchery), or different forms of predator training (e.g., multiple predator species, exposure to live predators, etc.). Further, because hatcheries rarely mimic natural environments, abiotic enrichment is a promising future research avenue (Lamothe and Drake 2019). Fish must not only recognize predators but also be able to escape them, and both abiotic (e.g., refugia, water flow, temperature) and biotic (e.g., competition for resources, predatory threats) environmental factors can shape behavioral, morphological, and physiological traits associated with surviving predation (Conover and Baumann 2009; D'Anna et al. 2012; Sgró and Hoffmann 2004). The use of multiple populations

in this experiment allowed us to detect population-level variation in behavior, validating the importance of continuing to manage Arkansas daters at the population level. This study lays important groundwork for assessing the efficacy of predator training that future research can build upon to inform the management of behavior in hatcheries.

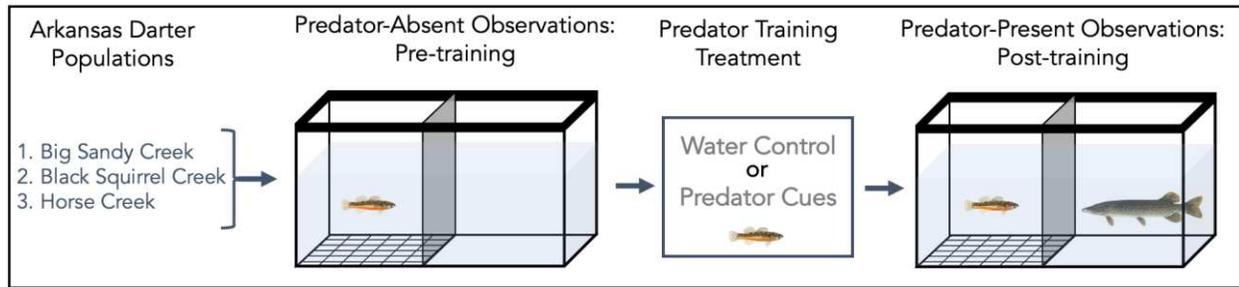
### **Contributors**

Coauthors on the publication produced from this chapter include E. Dale Broder, Eric R. Fetherman, Ryan M. Fitzpatrick, and Lisa M. Angeloni. The authors would like to thank D. Winkelman, T. Smith and P. Foutz for the use of facilities, logistical support and expertise; R. Osmundson, T. Hubbard, B. Avila, K. Dick, T. Klee, and F. Horne for assistance with setup and data collection; and the staff at the CPW John W. Mumma Native Aquatic Species Restoration Facility for fish, equipment and supplies. The authors have no conflict of interest to declare. Animals were handled and cared for following the requirements and guidelines of Colorado State University's Institutional Animal Care and Use Committee (protocol no. 16-6518A). Funding for this project was provided by Colorado Parks and Wildlife Species Conservation Trust Fund SCTF911C.

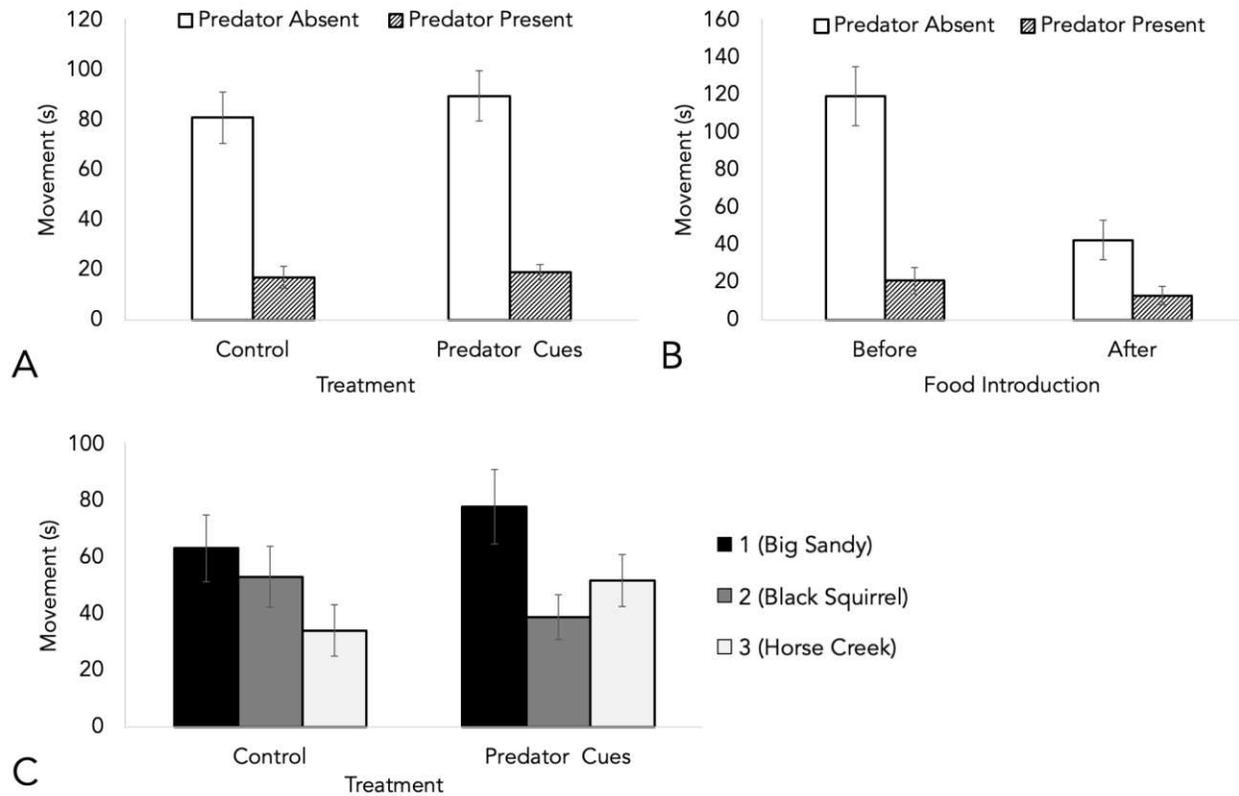
**Table 1.1:** Predator species known to exist within each population of Arkansas darters. In each column (darter populations 1, 2, and 3), an asterisk (\*) indicates that the predator species has been detected in that population of darters. Unpublished data provided by Colorado Parks and Wildlife.

Predator Species	1 (Big Sandy Creek)	2 (Black Squirrel)	3(Horse Creek)
Yellow Perch ( <i>Perca flavescens</i> )†	*		
Northern Pike ( <i>Esox lucius</i> )†	*		
Black Bullhead ( <i>Ameiurus melas</i> )	*	*	*
Green Sunfish ( <i>Lepomis cyanellus</i> )	*	*	*
Largemouth Bass ( <i>Micropterus salmoides</i> )†	*		
Channel Catfish ( <i>Ictalurus punctatus</i> )	*		*
Orangespotted Sunfish ( <i>Lepomis humilis</i> )	*		

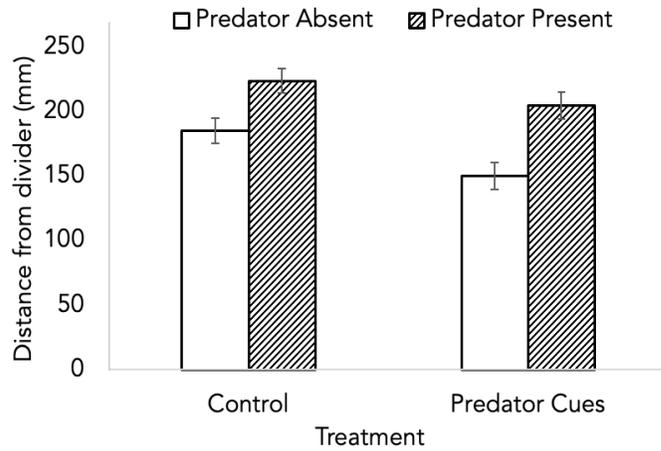
Obelus (†) indicates predator is not native.



**Figure 1.1:** Flow diagram of experimental procedures. We evaluated the antipredator behavior of three populations of Arkansas darter ( $n = 180$ ). First, we measured behavior without a predator (predator-absent; 20 minutes). Next, darters were randomly assigned to either a predator recognition training (predator cues) or a control (water control) treatment. Finally, we measured the same behaviors in response to a live tiger muskie predator (predator-present; 20 minutes). Food was introduced after 10 minutes during both the predator-absent and predator-present observations. Pre- and post-training observations were conducted in a 76-L tank and training was conducted in individual 10-L tanks.



**Figure 1.2:** (A) Time darters spent moving in seconds (s; mean  $\pm$  SE bars) during predator-absent observations (empty bars) and predator-present observations (hashed bars), before (left) and after (right) darters received the Water Control (Control) or Predator Cues treatment. Both main effects were significant but not their interaction. (B) Time darters spent moving in seconds (mean  $\pm$  SE bars) during predator-absent observations (empty bars) and predator-present observations (hashed bars), before (left) and after (right) the introduction of food halfway through the observation. There was a significant interaction between treatment and food introduction. In panels A and B all populations are pooled. (C) When predators were present, the time darters spent moving in seconds (mean  $\pm$  SE bars) between Water Control and Predator Cues treatments differed among populations (see Tables S1.3 and S1.4). In A, B, and C error bars show standard error (n=180).



**Figure 1.3:** Distance from the divider (mm; mean  $\pm$  SE bars) when the predator was absent (empty bars) and present (hashed bars) between the Water Control or the Predator Cue treatments. All populations are pooled. Both main effects were significant though the interaction was not.

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## 2. THE EFFECTS OF ENVIRONMENTAL ENRICHMENT ON THE BEHAVIOR, MORPHOLOGY, AND SURVIVAL OF A SPECIES OF CONSERVATION CONCERN

### **Summary**

In response to the global biodiversity crisis, conservation practitioners rely on captive breeding programs to supplement wild populations at risk of extinction. While population augmentation has been successful for some taxa, predation often limits the ability of hatchery fish to survive and contribute genetically to wild populations where released. Hatchery environments often differ starkly from wild environments, constraining the ability of hatchery fish to phenotypically match the environments in which they are targeted for release. Here, we took a factorial approach to assess whether abiotic enrichment and predator recognition training increase survival of a species of conservation concern, the Arkansas darter (*Etheostoma cragini*) during encounters with a novel predator. We also assessed the effects of abiotic enrichment on the expression of behavioral and morphological phenotypes across three populations. We found that morphology and behavior differed across populations, between abiotic treatments, as well as how populations responded to abiotic treatments. Furthermore, we found that in combination with predator training, abiotic enrichment increased the probability of surviving first encounters with a predator. We therefore recommend conservation practitioners incorporate abiotic enrichment and predator recognition training in the hatchery, as any increase in survival is expected to benefit efforts to conserve this species.

### **Introduction**

As biodiversity declines globally, conservation programs that supplement wild populations offer one possible solution for maintaining or increasing the abundance of threatened and endangered species (Mallinson 1995). For such conservation efforts to be successful, captive breeding programs must produce animals capable of surviving and reproducing in the wild. While there are several examples of successful captive breeding and reintroduction efforts among terrestrial animals (e.g., black-footed ferret: Biggins et al. 1999; California condor: Utt et al. 2010; Golden lion tamarins: Stonski et al. 2003; Caribbean rock iguanas: Alberts 2007), aquatic conservation efforts struggle to produce self-sustaining, wild populations of fish (Fraser 2008; Griffin et al. 2000; Shumway 1999). Though conservation hatcheries produce adequate quantities of fish for supplementation in their native ranges, survival after release is often very low (Brown and Laland 2001; Crane et al. 2015; Fitzpatrick et al. 2014; Fraser 2008; Griffin et al. 2000; Hawkins et al. 2008; Jackson and Brown 2011).

One reason why hatchery-raised fish do poorly after release is that the hatchery environment favors traits better suited for life in captivity than in the wild. For example, fish reared in captivity often demonstrate riskier behaviors like increased time spent exploring and foraging when a predator is present (Crane et al. 2015). Additionally, hatchery fish tend to exhibit greater morphological abnormalities (Belk 2008; Hutchison et al. 2012; Saraiva and Pompeu 2016) and physiological limitations that further constrain the probability of surviving after release (Chittenden et al. 2010; Fuss and Byrne 2002; Hutchison et al. 2012). As such, the genetic contribution of hatchery-reared fish to wild populations can be lacking (Fitzpatrick et al. 2014), and predation (often from introduced species) is suspected to be the leading cause of mortality immediately following release (Hutchison et al. 2012; Mesquita and Young 2007; Olla et al. 1998).

Predator recognition training and abiotic enrichment have been suggested as ways to promote antipredator behavior and increase post-release survival by incorporating more natural conditions into the hatchery rearing environment that closely mimic those experienced following release (Crane et al. 2015; Hawkins et al. 2008; Saraiva and Pompeu 2016; Vilhunen 2006). Behavioral, morphological, and physiological traits associated with detecting, recognizing, avoiding, and escaping predators can be highly correlated and often work in tandem to increase the breadth of an animal's response to the threat of predation, as well as its probability of surviving encounters with predators (Chittenden et al. 2010). For example, training fish to recognize predators by exposing them to predator cues can increase antipredator behavior (Becker and Gabor 2012; Crane et al. 2015; but see Kopack et al. Chapter 1), and fish can learn to associate a visual cue, like a model of a predator, with danger when paired with conspecific alarm cue and/or an identifying predator odor (Becker and Gabor 2012; Brown and Laland 2001; Olla et al. 1998; Vilhunen 2006). Similarly, abiotic enrichment to make the captive environment more natural shows promise for increasing expression of more wildtype phenotypes associated with surviving predator encounters after release (Belk 2008; Chittenden et al. 2010; Fuss and Byrne, 2002; Hutchison et al. 2012; Saraiva and Pompeu 2016; Ullah et al. 2017). Subjecting hatchery-reared fish to variable temperatures, variable flow, and/or structure (e.g., artificial plants and refugia) can shift traits like thermal tolerance (Blair and Glover 2019), metabolic rate (Cook et al. 2018), muscle development (Evans et al. 2015), swimming performance (Chittenden et al. 2010; Higham et al. 2015; Bergendahl et al. 2017), neural plasticity (Salvanes et al. 2013), and stress levels (Zhang et al. 2020) to more closely match those observed in wild fish. Shifts in one or more of these traits may improve the ability of fish to survive initial predator encounters (D'Anna et al. 2012; Higham et al. 2015).

A more complete understanding of the effects of predator recognition training and abiotic enrichment on behavior and survival is required before recommending the adoption of these tools by conservation practitioners. While studies have traditionally focused on the effects of either abiotic enrichment (e.g., Belk 2008; Chittenden et al. 2010; Fuss and Byrne 2002; Hutchison et al. 2012; Saraiva and Pompeu 2016; Ullah et al. 2017) or predator recognition training (e.g., Brown 2003; Kopack et al. 2015, 2016) individually, few have evaluated both simultaneously to determine their relative contributions and potential interactions in shaping antipredator behavior and survival (but see D'Anna et al. 2012; Tave et al. 2019). Furthermore, populations within a species may differ in their responses to training due to underlying genetic variation and/or differences in predation pressure experienced among them (Mery and Burns 2010). As such, conservation efforts may benefit from considering population-level effects of training during initial assessments of its efficacy.

Here, we assessed the effect of abiotic enrichment on morphology and behavior, as well as the relative contributions of abiotic enrichment and predator recognition training, on the survival of Arkansas darters (*Etheostoma cragini*) when encountering a predator for the first time. The Arkansas darter is a species of conservation concern in the state of Colorado due to population declines observed since the 1980s (Fitzpatrick et al. 2014; Groce et al. 2012; Labbe and Fausch 2000). In an effort to conserve the species, Colorado Parks and Wildlife (CPW) began an artificial propagation program for the Arkansas darter in 1999 (Groce et al. 2012). Despite intense propagation and supplementation efforts, hatchery-reared Arkansas darters have contributed little genetic material to wild populations where released, suggesting they experience high mortality following release (Fitzpatrick et al. 2014), perhaps facilitated by a lack of

experience with predators and/or an inability to survive in wild stream environments due to being reared in the hatchery environment.

Hatchery-reared Arkansas darters inhabit an environment that differs drastically from the wild, which may affect their behavioral and morphological development and contribute to their low post-release survival. For example, wild environments offer refugia, high water flow, and temperatures that shift daily, while hatchery environments typically lack structural complexity, provide minimal water flow, and maintain constant water temperatures over time (Piper et al. 1982). Hatcheries also lack predators, which can inhibit the proper development and expression of antipredator behaviors and predator recognition in prey (Crane et al. 2015). As such, hatchery-reared Arkansas darters may benefit from both abiotic enrichment and predator recognition training prior to release if such strategies increase their chances of surviving predation. The objectives of this study were to determine: (1) if abiotic enrichment altered feeding behavior and/or morphology of hatchery-reared darters; (2) if populations differed in their responses to abiotic enrichment; and (3) the relative contributions, if any, abiotic enrichment and predator recognition training had on surviving an initial encounter with a novel predator.

## **Methods**

### *Animal Husbandry and Abiotic Enrichment*

We obtained 300 fully grown and developmentally mature young-of-year Arkansas darters (ranging from 37 to 58 mm in total length) from three source populations (100 darters from each) from the CPW John W. Mumma Native Aquatic Species Restoration Facility (Alamosa, CO). The three populations (Big Sandy Creek, Black Squirrel Creek, and Horse Creek) are maintained as separate brood stocks in the hatchery breeding program. Genetic

differences among these populations are high due to a lack of gene flow in the wild. To maintain genetic integrity, hatchery brood stocks are often supplemented using wild individuals. As a result, hatchery-reared darters are likely only a few generations removed from wild populations (Fitzpatrick et al. 2014).

We held darters in 76-L flow-through tanks in the CPW Salmonid Disease and Sport Fish Research Lab (Fort Collins, CO), maintained water temperatures of  $13.5 \pm 2^\circ\text{C}$ , and fed them thawed bloodworms (San Francisco Bay Brand Inc., Newark, CA) ad libitum, once daily. Populations were kept separate, with two tanks for each population (40 darters in each tank). Within each population, one tank was not enriched (“abiotic control”), while the other was enriched by incorporating structural cover, increased flow, and diurnal temperature cycles (“abiotic enrichment”). For structural cover, we added three, three-inch long,  $\frac{3}{4}$ -inch diameter PVC tubes (as cave structures) and nine artificial plants (multi-pack B1; Marineland®, Blacksburg, VA) dispersed throughout the tanks. Plants were secured to the bottom of tanks using suction cups attached to plant bases via zip ties. Enriched tanks also included two powerheads, each producing constant flows of 155 L per minute (Hydor USA Inc., Sacramento, CA) mounted side by side at one end of the tank, as well as two in-tank 100 W water heaters (Eheim™, Buffalo, NY) mounted in the middle of the long side of the tanks and connected to timers. We set the heater timers to turn on after sunrise (8 am) following the natural light cycle and shut off ten hours after turning on. On average, tank temperatures fluctuated by  $3 \pm 0.5^\circ\text{C}$  daily. Fish were exposed to these conditions for 90 days before commencement of morphological evaluations and predator training.

Seventeen tiger muskellunge (*Esox masquinongy* x *E. Lucius*; hereafter “tiger muskie”) predators were obtained from the CPW Wray Fish Hatchery (Wray, CO) for use in survival

trials. We selected tiger muskie because they were readily available, free of disease, easy to maintain, and are the hybrid cross of muskellunge (*E. masquinongy*) and northern pike (*E. lucius*), and are an invasive predator in some Arkansas darter habitats. Predators ranged from 233 to 295 mm total length. We housed tiger muskies in five 76-L flow-through tanks maintained at  $13.5 \pm 2^\circ\text{C}$  and fed them trout feed (Rangen Inc. <sup>TM</sup>, Buhl, ID) ad libitum, once daily.

### *Behavior*

Feeding trials were conducted seven weeks into the abiotic enrichment experiment to determine if enrichment influenced feeding behavior of Arkansas darters (i.e., feeding and/or moving), as increased temperatures and flows may increase activity (Maynard et al. 2004; Stoner 2004), metabolism (Stoner 2004; Cook et al. 2018), and calorie intake needs (Jørgensen and Jobling 1993; Stoner 2004), while the presence of structure might provide a greater sense of safety by allowing for greater concealment (Moberg et al. 2011; D'Anna et al. 2012; Ullah et al. 2017). We withheld food from darters in both the abiotic enrichment and control tanks for 24 hours prior to the start of the feeding trials to ensure darters were not satiated and to encourage them to feed during the experiment. On the day of a feeding trial, we introduced one thawed bloodworm cube to each tank. An observer recorded the number of fish feeding and/or moving during instantaneous scans conducted every two minutes over a 30-minute observation period (15 scans in each tank). We repeated this process two more times every other week, for a total of three feeding trials per tank. We expected darters in abiotic enrichment tanks to move and feed more than darters in abiotic control tanks because enrichment typically increases movement and feeding due to the high energetic demand of the enriched environment (Braithwaite and Salvanes 2005; Higham et al. 2015).

## *Morphology*

Body shape in fish has been shown to influence swimming behavior and escape performance (Saraiva and Pompeu 2016). Thus, to test if the abiotic treatment altered body shape, we photographed fish at the end of the 90-day treatment period with a high-definition camera (Coolpix AW100, Nikon Inc., Melville, NY) mounted to a tripod and facing down on a white board illuminated by two 30-cm, 415-lumen LED light bars mounted on either side. For each photograph, a researcher (CJK) netted each fish out of its abiotic treatment tank, positioned it horizontally on the board above a 16-cm ruler for scale, and fanned the caudal and first-dorsal fins with a wet paintbrush. We photographed 20 fish from each population (10 from each abiotic treatment), but all photographs of the Big Sandy Creek population were lost, as well as two individuals from the abiotic treatment group in the Black Squirrel Creek population, due to an error in camera operation. From each remaining photograph, 13 linear morphological measurements were recorded (Table 2.1) using Image J software (Abramoff et al. 2004), from which nine ecomorphological attributes were calculated and assessed (following Saraiva and Pompeu 2016; Pessanah et al. 2015; Table 2.2). We used proportional ratios of the morphological measurements (Table 2.1) to generate nine ecomorphological attributes that control for differences in body size and reflect an individual's ability to perform under various ecological contexts, including predator-prey interactions (Saraiva and Pompeu 2016; Table 2.2). We chose attributes considered ecologically relevant in high flow environments and potentially important for escaping predators after release (Table 2.2). If these traits exhibit plasticity in response to the abiotic environment, we expected darters exposed to abiotic enrichment to decrease the Ventral Flattening Index (VFI), Relative Height of the Body (RH), and Relative

Height of the Head (RHH), and increase all other attributes, based on patterns associated with high flow environments and/or swimming ability (Pessanha et al. 2015; Saraiva and Pompeu 2016; Table 2.2).

### *Predator Training*

At the end of the 90-day abiotic treatment period, darters were moved to 10-L flow-through group-tanks for the remainder of the experiment. For each population, 16 group-tanks housed five darters each, eight tanks for darters from the abiotic enrichment treatment and eight for darters from the abiotic control treatment, that continued to be fed thawed bloodworms *ad libitum*, once daily. Next, for each population we assigned four of its eight abiotic treatment tanks (and all individuals contained therein) to a predator control treatment (exposed to distilled water only), and we assigned the other four tanks to a predator training treatment (exposed to conspecific alarm cue, predator kairomone and a predator model) following Kopack et al. (Chapter 1), so that groups of individuals in each abiotic and predator treatment were maintained separately, without mixing. We tagged darters with a color-coded mark on either side of their dorsal fins using Visible Implant Elastomer (VIE; Northwest Marine Technology Inc., Shaw Island, WA) to identify each individual's treatment combination ("abiotic control" vs. "abiotic enrichment") and predator treatment ("predator control" vs. "predator training").

Conspecific alarm cue was collected by scoring the epidermis of 51 recently decapitated Arkansas darters (17 from each population, mixed together) with a razor blade. Bodies were then rinsed with 10 mL of distilled water, which was filtered through cheesecloth and immediately stored in a freezer at -20 °C until the day of its use (following Kopack et al. 2015 and adapted from Nordell 1998). We obtained predator kairomone by collecting water from a 19-L non-

circulating tank that housed two tiger muskie predators for 48 hours. During this time, the predators were fed six darters (two from each population), so the water likely contained conspecific alarm cues in addition to the predator kairomone. The collected water was stored at 20°C until the day it was used (following Kopack et al. 2015).

After experimental darters were moved to 10-L group tanks, they were allowed to acclimate for one week before starting predator treatments. Twenty-four hours prior to the start of survival trials, we treated darters with a five-minute exposure to predator cues or control conditions in their group tanks following Kopack et al. (Chapter 1). To administer the treatments, 10 mL syringes, loaded with either distilled water (“predator control”) or 5 mL of alarm cue mixed with 5 mL of predator kairomone thawed at room temperature (“predator training”), were connected to air tubing taped to the front right corner of each tank. We placed a blind over all tanks to prevent darters from seeing the researcher and turned off the water flow to prevent dilution of cues during the treatment period. Following placement of the blind, we allowed darters 30 minutes to acclimate before adding the 10 mL of liquid through the air tubing. For fish in the predator training group, we additionally exposed each tank to a predator model, a 102 mm plastic lure that resembled a tiger muskie predator (Savage Gear USA®, Ontario, CA), for five minutes immediately following the introduction of chemical cues. The model was attached to a wand with fishing line so that it could be introduced and removed from the tank by a researcher standing behind the blind. After the five-minute exposure period, we removed the model and turned on the system’s water flow to flush out any remaining chemical cues.

### *Survival*

Prior to the start of survival trials, five tiger muskie predators were assigned to one of five 151-L predator encounter tanks, where they were housed for the remainder of the experiment. Each encounter tank included 12 artificial plants and three PVC caves for structure, two powerheads for generating flow, and two in-tank heaters for fluctuating temperatures, replicating conditions in the abiotic enrichment tanks and simulating conditions darters are expected to experience during release. Survival trials were conducted for each population separately, one population at a time, using a 2x2 factorial design consisting of 20 individuals per abiotic and predator treatment combination (“control” = abiotic control + predator control; “enrichment” = abiotic enrichment + predator control; “training” = abiotic control + predator training; and “enrichment + training” = abiotic enrichment + predator training). Twenty-four hours after receiving predator treatments, darters from a given population were assigned to one of the five encounter tanks, so that each encounter tank included four darters, one from each treatment combination. On the day of survival trials, darters were collected into a small caddy and released into their respective predator encounter tanks on the side opposite the predator, marking the start of a trial. A trial ended once the tiger muskie consumed a darter, at which point the observer removed the tiger muskie predator from the tank to prevent further consumption, retrieved the three surviving darters, and then returned the tiger muskie to the tank. Next, using the VIE tags to identify the three remaining survivors, we identified the treatment combination of the consumed individual. We then placed each of the three survivors into one of four 10-L holding tanks, separated by treatment combination. We repeated this process daily until every darter had been through one encounter trial with a predator, conducting one trial per predator, per day, to prevent predator satiation.

Once every surviving fish within a given population had completed an initial survival trial, one fish from each of the four holding tanks (representing each treatment combination) was randomly selected to participate in a second predator encounter trial. This process was repeated for each population until one of the four treatment combinations went extinct. Up to three rounds of trials were conducted within a population before this occurred, allowing us to record the first treatment combination to go extinct for each population.

### *Statistical Analyses*

All statistical analyses were performed using R statistical software version 4.0.2 (R Core Team 2020), except cumulative survival, which was assessed using Program MARK (White and Burnham 1999). To compare Arkansas darter feeding behavior between abiotic treatments (tanks with or without enrichment) and among populations, we conducted a linear mixed model (lme4 package: Bates et al. 2014; lmerTest package: Kuznetsova et al. 2017) for each of the two behaviors recorded: (1) the number of individuals feeding in any given scan and (2) the number of individuals moving in any given scan. Because these were count data, we treated them as Poisson distributions. We included abiotic treatment, population, and their interaction as fixed effects and feeding trial as a random effect in both analyses. We reported Wald Chi-Squared values, identified significant fixed effects, and used the estimated marginal means method (emmeans package: Lenth 2020) to determine significant differences for interactions between treatments and populations.

To assess morphological differences across abiotic treatments and populations, we performed a Redundancy Analysis (RDA; adapted from Borcard et al. 2011; following Marques et al. 2019) using the vegan package (Oksanen et al. 2020), followed by a non-parametric,

permutational multivariate analysis of variance (PERMANOVA; Legendre and Legendre 2012), as it is particularly robust when sample sizes are limiting. Briefly, this approach allowed us to reduce complex multivariate variation into a few axes, analyze these axes of variation for differences among treatments and populations, and then reveal direction and magnitude of any changes to individual ecomorphological attributes by using scores obtained from the RDA in MANOVAs (adapted from Borcard et al. 2011; following Marques et al. 2019). Before conducting the analysis, we partitioned the data into two distinct data sets, one representing individual response variables (ecomorphological attributes; Table 2.2) and the other, a data frame of their corresponding predictor variables (abiotic treatment and population). Next, the data were submitted to the RDA, from which permutation tests (999 permutations) were conducted to determine the relative contribution of each main effect and their interactions to the variance partitioning of each constrained canonical axis (McArdle and Anderson 2001; Legendre et al. 2010). While all nine unconstrained canonical axes were retained from the redundancy analysis (Table 2.4), of the three constrained canonical axes produced, the first axis (RDA1) explained 51% of the variation observed and the second axis (RDA2) explained 34%, cumulatively representing 85% of the total variation observed. Permutation tests performed on the eigenvalues of the constrained canonical axes found that a significant proportion of observed variation across all attributes was explained by RDA1 (variance = 0.96,  $F_{3,34} = 4.60$ ,  $P = 0.004$ ) and RDA2 (variance = 0.65,  $F_{3,34} = 3.12$ ,  $P = 0.008$ ) but not RDA3 (variance = 0.29,  $F_{3,34} = 1.39$ ,  $P = 0.20$ ). To determine the effects of treatment, population, and their interaction on the variance partitioning for each constrained canonical axis found to be significant in the RDA, we performed an ANOVA using the ecomorphological attribute scores obtained from the analysis. Last, to assess changes to individual attributes in response to abiotic treatment, we conducted a

non-parametric, permutational MANOVA (999 permutations; “adonis2” in R, Oksanen et al. 2020) using a Euclidean distance matrix of the ecomorphological attributes (Legendre and Anderson 1999; McArdle and Anderson 2001). We included treatment, population, and their interaction as fixed effects in all models, reporting statistics from the type III sum of squares, followed by pairwise comparisons of means for interactions found to be statistically significant (McArdle and Anderson 2001; RVAideMemoire package, Hervé 2021).

Lastly, to determine survival differences among abiotic and predator treatments, survival rates were calculated and compared across their combinations, both within and among trials. Using a binomial distribution, with one indicating survival and zero indicating mortality, we calculated instantaneous survival rates for each trial using a generalized linear model and compared survival differences among the trials using an RM ANOVA, with trial, treatment, and population included as fixed effects and predator identification as a random effect. However, because no differences were detected among populations ( $\chi^2 = 2.25$ ,  $P = 0.33$ ), instantaneous survival rates were pooled for comparison. Additionally, because the model’s random effect variance was estimated to be at or near zero, predator identification was dropped as a random effect to prevent overfitting the model. Because we had a 100% recapture probability and we could identify the treatment combination of the consumed individual immediately after a trial was completed, cumulative survival rates were estimated across all trials using a known fate capture-recapture estimator in Program MARK (White and Burnham 1999). Cumulative survival rates were estimated for the four treatment combinations only, with data from all three populations combined into the same analysis, as there were not enough encounters overall to parse out population effects. The model set included an intercept model, models with treatment and trial effects, and models with the additive and interactive combinations of those effects.

Cumulative survival ( $\pm$  unconditional standard error) is reported as a model-averaged derived parameter from the known fate model set.

## Results

### *Feeding Behavior*

In our assessment of feeding behavior, abiotic enrichment had no effect on the number of darters feeding overall ( $X^2 = 1.38$ ,  $P = 0.24$ ), but the number of individuals feeding differed by population ( $X^2 = 13.03$ ,  $P = 0.002$ ), as the Big Sandy Creek ( $z = 3.89$ ,  $P < 0.001$ ) and Horse Creek ( $z = -2.80$ ,  $P < 0.05$ ) populations fed more than the Black Squirrel Creek population but did not differ from each other ( $z = 1.01$ ,  $P > 0.1$ ). There was a significant interaction between abiotic treatment and population ( $X^2 = 7.49$ ,  $P = 0.02$ ), as abiotic enrichment caused an increase in feeding for the Horse Creek population ( $z = 4.44$ ,  $P < 0.001$ ) but not the Big Sandy Creek ( $z = 1.18$ ,  $P > 0.1$ ) or Black Squirrel Creek populations ( $z = 2.04$ ,  $P > 0.1$ ; Figure 2.1a; Table 2.5).

Fewer individuals were observed moving in abiotic enrichment tanks than the control tanks ( $X^2 = 174$ ,  $P < 0.001$ ). Movement also differed by population ( $X^2 = 61.60$ ,  $P < 0.001$ ), as the Black Squirrel Creek ( $z = -5.32$ ,  $P < 0.001$ ) and Horse Creek ( $z = -4.81$ ,  $P < 0.001$ ) populations moved more than the Big Sandy Creek population but did not differ from one another ( $z = 0.32$ ,  $P > 0.1$ ). There was a significant treatment-population interaction ( $X^2 = 77.45$ ,  $P < 0.001$ ); abiotic enrichment caused a decrease in movement in the Big Sandy Creek ( $z = -13.19$ ,  $P < 0.001$ ) and Black Squirrel Creek ( $z = -12.95$ ,  $P < 0.001$ ) populations but not the Horse Creek population ( $z = -2.10$ ,  $P > 0.1$ ; Figure 2.1b; Table 2.6).

## *Morphology*

In our assessment of morphology, we found treatment ( $F_{3,34} = 2.62$ ,  $P = 0.01$ ), population ( $F_{3,34} = 3.43$ ,  $P = 0.001$ ), and their interaction ( $F_{3,34} = 3.06$ ,  $P = 0.01$ ) were significant predictors, explaining 55%, 72%, and 64% of the variation observed across all ecomorphological attributes, respectively. Our Redundancy Analysis produced three constrained canonical axes, two of which (RDA1 and RDA2) explained a significant proportion of variation across ecomorphological attributes. The first constrained canonical axis (RDA1) was attributed to differences in Aspect Ratio of the Caudal Fin (ARCF), Relative Length of the Head (RLH), Relative Height of the Head (RHH), Relative Area of the Pectoral Fin (RAPTF), Relative Length of the Caudal Peduncle (RLCP), and Aspect Ratio of the Pectoral Fin (ARPTF), while the second axis (RDA2) was attributed to differences in ARPTF, and Aspect Ratio of the Caudal Fin (ARCF). An ANOVA attributed variation in RDA1 to the main effects of abiotic treatment ( $F_{3,34} = 3.98$ ,  $P = 0.05$ ) and population ( $F_{3,34} = 6.52$ ,  $P = 0.02$ ), while the variation in RDA2 was associated with the effects of abiotic treatment ( $F_{3,34} = 40.02$ ,  $P < 0.001$ ), population ( $F_{3,34} = 25.14$ ,  $P < 0.001$ ), and their interaction ( $F_{3,34} = 52.60$ ,  $P < 0.001$ ; Figure 2.4). An analysis of the significant interaction for RDA2 revealed that populations responded differently to abiotic treatment, with Black Squirrel Creek increasing ( $t = -6.33$ ,  $P < 0.001$ ) and Horse Creek decreasing ( $t = 3.86$ ,  $P = 0.002$ ) overall expression of attributes when exposed to abiotic enrichment.

To understand the multivariate RDA, we analyzed individual ecomorphological attributes using scores obtained from the RDA in MANOVAs. This revealed an effect of abiotic treatment, population and/or their interaction for several of the attributes examined (Tables 2.2 and 2.3). Counter to our predictions, abiotic enrichment caused a decrease in ARPTF for all darters (Table 2.3), though the decrease was greater in the Black Squirrel Creek population ( $F_{3,34} = 10.71$ ,  $P <$

0.05; Figure 2.2). Also counter to predictions, there was a non-significant trend where RAPTF and ARCF decreased when exposed to abiotic enrichment (Table 2.2; Figure 2.2). The two populations were found to differ morphologically from one another regardless of the abiotic treatment they received, with Black Squirrel Creek having a lower RLCP ( $F_{3,34} = 12.45$ ,  $P = 0.001$ ), but higher RLH ( $F_{3,34} = 8.09$ ,  $P = 0.008$ ) and RHH ( $F_{3,34} = 4.29$ ,  $P = 0.04$ ) compared to Horse Creek (Table 2.3). Populations also responded differently to abiotic treatment with respect to Relative Area of the Caudal Fin (RACF), as abiotic enrichment caused an increase in RACF for Horse Creek ( $F_{3,34} = 51.04$ ,  $P = 0.006$ ), which matched expectations, but a trend for a reduced RACF for Black Squirrel Creek ( $F_{3,34} = 6.76$ ,  $P < 0.1$ ; Table 2.3).

### *Survival*

The combination of abiotic enrichment and exposure to predator cues significantly increased survival of darters in the presence of a predator immediately after training ( $z = 8.27$ ,  $P = 0.04$ ; Figure 2.3a). During the first survival trial (trial 1), those that received abiotic enrichment and predator training treatments (“enrichment + training”) had significantly higher instantaneous survival than the other three treatment combinations ( $z = 2.04$ ,  $P = 0.04$ ; Figure 2.3a), which did not differ from each other ( $0.00 \leq z \leq 1.67$ ,  $P > 0.05$ ). Overall, cumulative survival, calculated across trials, was also highest in fish receiving both abiotic enrichment and predator training, but it did not differ among treatment combinations (Figure 2.3b). Darters that received only abiotic enrichment (“enrichment”) were the first treatment to go extinct during survival trials for the Big Sandy Creek and Black Squirrel Creek populations, whereas those that received only predator training (“training”) were the first to go extinct in the Horse Creek population.

## Discussion

Preparing captive-reared animals for the environments they will encounter after release has been suggested to increase survival and improve the success of conservation breeding programs (D'Anna et al. 2012, Tave et al. 2019). The results of this study suggest that abiotic enrichment, including physical structure, increased water flow, and varied temperatures, can alter the behavior and morphology of Arkansas darters, and the combination of abiotic enrichment and predator training can increase the probability of surviving initial encounters with novel predators. Additionally, as predicted, populations differed in their behavioral and morphological responses to abiotic enrichment; however, populations did not differ in survival.

### *Feeding Behavior*

We assessed the effect of abiotic enrichment on rates of feeding and movement to determine whether changes in temperature, flow, and structure might alter Arkansas darter behavior within hatcheries. We expected abiotic enrichment to increase both movement and feeding if darters increased their caloric intake in response to the high energetic demands associated with greater physical activity in the enriched environment (Braithwaite and Salvanes 2005; Brown et al. 2003). However, Arkansas darters exposed to abiotic enrichment moved less and, in some populations, fed more compared to those in abiotic control tanks. The decrease in movement, as well as the slight increase in feeding in abiotic enrichment tanks may have been the result of food dispersion caused by increased flow produced by powerheads (Gu et al. 2018). Flows were observed to increase dispersion of bloodworms (food) throughout the enriched tanks, allowing darters more opportunities to encounter prey and feed without needing to move. Similarly, Gu et al. (2018) found benthic Chinese sturgeon (*Acipenser sinensis*) experienced

greater foraging efficiency on drifting prey in a lotic system with high flows, because of an increase in prey encounter rates and a decrease in prey escape ability.

In contrast, the lack of flow in abiotic control tanks caused food to remain concentrated in the location it was introduced, requiring darters to move in order to feed. This may also have caused individuals to cluster around the food, increasing competition and decreasing foraging efficiency, while potentially increasing their susceptibility to predation (Lima and Dill 1990; Tyler 1993). Increased intraspecific competition caused by higher rearing densities, as well as relaxed and reversed selection in hatcheries, is thought to contribute to the bolder and riskier behaviors hatchery-reared fish often express compared to their wild counterparts, potentially leading to their high predation rates and low survival immediately following their release, especially if they fail to discriminate novel predatory threats (Braithwaite and Salvanes 2005; D'Anna et al. 2012; Moberg et al. 2011; Roberts et al. 2011). Thus, abiotic enrichment may offer conservation practitioners a way to reduce the maladaptive effects of traditional hatchery environments and impart a survival advantage if the behaviors observed here persist after release into the wild (Braithwaite and Salvanes 2005; D'Anna et al. 2012; Moberg et al. 2011; Roberts et al. 2011).

Populations differed in their response to the abiotic treatment. For the number of fish feeding, only Horse Creek was significantly impacted by the abiotic treatment with more fish feeding in enriched tanks compared to the control. However, this increase (approximately a difference of one fish) was so slight, it may not be biologically meaningful. For movement, the Horse Creek population did not differ between treatments while the other two populations moved much less in the abiotic treatment compared to the control (approximately a difference of 10 individuals). The greater movement associated with abiotic enrichment for Horse Creek

individuals could increase conspicuousness and translate to higher susceptibility to predation after release (Lima and Dill 1990) if behavioral differences persist in the wild. As such, we suggest conservation managers continue to explore the effects of enrichment on behavior of Arkansas darters at the population level, while continuing efforts to prevent esocid predators from establishing themselves where darters are particularly vulnerable to predation.

### *Morphology*

In our assessment of Arkansas darter morphology, we found that abiotic enrichment affected four of the nine ecomorphological attributes, indicating some degree of morphological plasticity, particularly for traits associated with the caudal and pectoral fins. Darters exposed to abiotic enrichment had a lower ARPTF (Aspect Ratio of the Pectoral Fin), lower ARCF (Aspect Ratio of the Caudal Fin), and lower RAPTF (Relative Area of the Pectoral Fin) compared to those in abiotic control tanks. However, these differences contradicted our adaptive plasticity prediction; in other words, they were not in the direction thought to increase swimming performance (Saraiva and Pompeu 2016). It is important to note that the original study that examined the ecological relevance of these attributes (i.e., whether they were adaptive and increased swim performance) did so in 33 species, of which only four were darters (Gatz Jr. 1979). The difference between our predictions and our findings may reflect the benthic lifestyle of darters and their lack of a swim bladder. Movement is more energetically costly for darters, which do not actively swim as much as other species and/or those with swim bladders (Gatz Jr. 1979). Because darters rely on their pectoral and caudal fins to maintain their position in space (Carlson and Lauder 2010), the reductions to ARCF, RAPTF, and ARPTF we observed in abiotic treatment tanks may allow darters to combat the effects of increased drag resulting from

having larger fins in higher flows, thereby reducing the amount of energy and activity required to maintain their position in space (Carlson and Lauder 2010; Franssen et al. 2013b). Indeed, the fact that darters in the abiotic enrichment with predator exposure treatment had the highest survival suggests that the morphological differences we observed may be adaptive. Furthermore, the lack of change observed in other attributes may be partially due to the inclusion of structure in abiotic treatment tanks, as darters could have used these structures as refuge to shelter themselves from the physical stressors that accompany high flows, providing potential opportunities to overcome the high energetic cost of shifting morphological traits in response to the environment (Liao 2007; Carlson and Lauder 2010).

Some ecomorphological attributes differed between populations regardless of which abiotic treatment they received and are likely the result of the genetic differences that exist between them. Because various biotic and abiotic factors contribute to selection pressures that shape morphology, differences between the environments of the source populations (e.g., differences in available prey or flow regimes), may have led to morphological divergence and the significant effect of population we detected in this study (Franssen et al. 2013a, 2013b; Pessanha et al. 2015). Furthermore, we anticipated the possibility that populations would morphologically differ from one another due to the high genetic variation and lack of gene flow known to exist between them (Fitzpatrick et al. 2014). As with the feeding experiment, we also detected differences in how the populations responded to the abiotic treatment (gene-by-environment interaction). Specifically, for RACF (Relative Area of the Caudal Fin) Horse Creek differed in the expected direction as it was larger in the enrichment treatment, while the response was in the opposite direction in the Black Squirrel Creek population. Differences in plasticity among populations should be explored in future work by investigating differences in gene

expression among populations in response to various treatments like abiotic enrichment and predator exposure. Because phenotypic and genetic differences exist, conservation efforts would benefit from knowing the degree to which the abiotic and biotic environments of the source populations work independently and/or in tandem to shape the morphological differences among them, as understanding the environmental forces that shape these differences may allow practitioners to design captive environments that promote the morphological variation observed in the wild.

### *Survival*

In our assessments of survival, we found that the abiotic and biotic enrichment interacted synergistically to increase the probability of surviving first encounters with an esocid predator. Additionally, although not statistically significant, the higher cumulative survival observed for darters exposed to the combination treatment suggests abiotic enrichment in combination with predator recognition training may provide lasting effects that span beyond first encounters with a novel predator. Our results suggest that prey must be able to both recognize (enhanced via predator training) and escape (enhanced via abiotic enrichment) a predator in order survive, and this aligns with other work. The post-release survival of hatchery-reared white seabream (*Diplodus sargus*) doubled when they were conditioned to recognize predators and reared with refugia (D'Anna et al. 2012), and methods to rear endangered Rio Grande silvery minnows (*Hybognathus amarus*) in semi-natural environments with both abiotic and biotic stressors (including predation) showed promise for increasing survival after release (Tave et al. 2019). To our knowledge, our study is the first to measure survival in Arkansas darters exposed to abiotic and biotic enrichment, and we found that both types of enrichment together increased survival

despite using a single predator training event and making minimal shifts to the abiotic environment (e.g., 3°C daily shift in temperature) compared to the wild (e.g., 10°C daily shifts). Considering that thousands of hatchery-reared Arkansas darters are released into the wild annually, even a small survival difference could benefit efforts to conserve this species.

We were not able to detect statistical differences among populations in survival, likely due to small sample sizes. Because Arkansas darters are a threatened species, it is difficult to acquire animals for research purposes, which was a challenge for this research. Among populations, the darters that experienced only abiotic enrichment went extinct first for the Big Sandy Creek and Black Squirrel Creek populations, while darters that experienced only predator training were the first to go extinct for Horse Creek, suggesting that there may be some differences in survival among populations and treatments. We suggest conservation managers, therefore, continue to explore population differences to determine if population-specific management is necessary for the persistence of darters in the wild. We tested survival for each population independently; future research could test all populations simultaneously during survival trials to explore whether particular Arkansas darter populations are more likely to survive predation. Knowing if certain populations are better at surviving predation could help managers determine which populations are the best sources for translocations and population augmentation.

## **Conclusions**

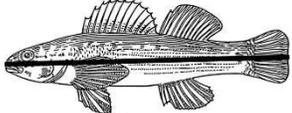
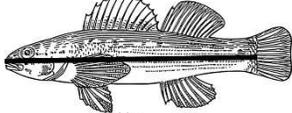
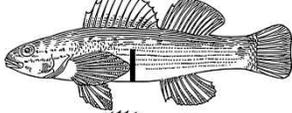
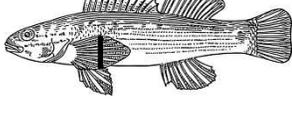
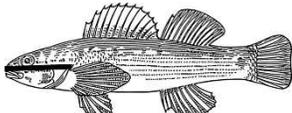
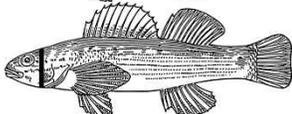
This study can inform conservation practices. By mimicking a predatory encounter that darters might experience after release in our survival experiment, we gained valuable insight into how the biotic and abiotic environments interact to influence survival. We found that abiotic

enrichment altered both behavior and morphology of Arkansas darters, and that in combination with a predator treatment, abiotic enrichment increased survival during an initial encounter with a novel predator. Thus, these types of hatchery enrichment practices show promise for increasing Arkansas darter survival after release. We recommend that conservation practitioners employ predator training and abiotic enrichment at the hatchery level by incorporating powerheads, in-tank heaters, artificial structure, and other forms of enrichment. Because we detected differences among populations, we suggest that practitioners continue to manage Arkansas darters at the population level. While it may seem challenging to implement abiotic enrichment and predator training at the hatchery level, simple methods like those we tested here have the potential to make a big impact, and any increase in survival will bolster efforts to conserve this species.

### **Contributors**

Coauthors on the publication produced from this chapter include Eric R. Fetherman, E. Dale Broder, Ryan M. Fitzpatrick, and Lisa M. Angeloni. The authors would like to thank H. Crockett, P. Foutz, G. Schisler, A. Treble, T. Smith, and the staff of the Colorado Parks and Wildlife John W. Mumma Native Aquatic Species Restoration Facility and Wray Fish Hatchery for providing fish, equipment and supplies, logistic support, and expertise. The authors would also like to thank B. Avila, E. Vigil, R. Osmundson, T. Hubbard, K. Dick, F. Horne, and T. Klee for help with setup, animal husbandry, and data collection. Fish were cared for under the guidelines and requirements of the Colorado State University Animal Care and Use Committee (Protocol no. 16-6518A). Funding for this work was provided by Colorado Parks and Wildlife's Species Conservation Trust Fund (Grant no. SCTF911C) and a Graduate Research Fellowship awarded to C. Kopack by the National Science Foundation.

**Table 2.1:** Morphological measurements, their description, and illustration (following Saraiva and Pompeu 2016).

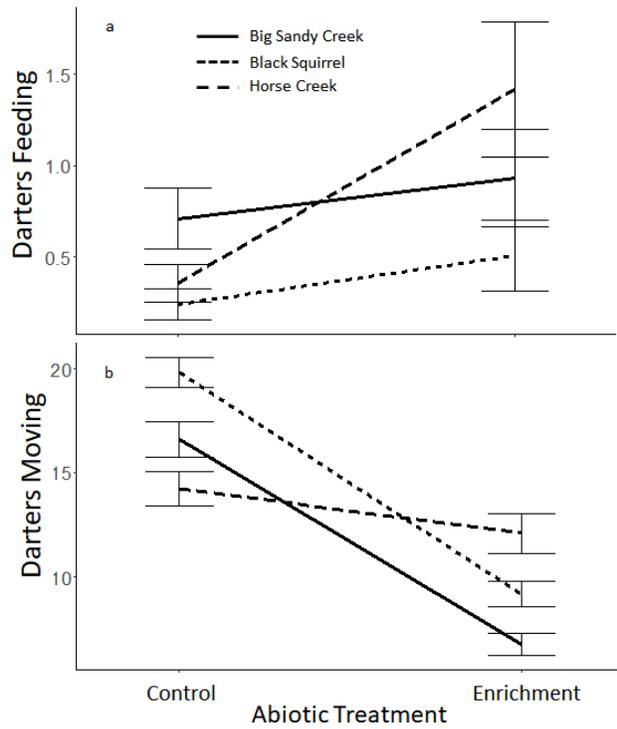
<u>Morphological Measurements</u>		
Measurement	Description	Illustration
Total Length (TL)	The distance from the tip of the snout to the tip of the caudal fin.	
Standard Length (SL)	The distance from the tip of the snout to the tip of the caudal peduncle.	
Body Height (BH)	The greatest distance between the ventral and dorsal sides of the body, perpendicular to its length.	
Average Body Height (ABH)	The greatest vertical distance from the abdomen to the lateral line.	
Body Area (BA)	The area of the body plus the area of the caudal fin.	
Caudal Peduncle Length (CPL)	The distance from the posterior end of the anal fin to the tip of the caudal peduncle.	
Caudal Fin Width (CFW)	The greatest distance between the anterior and posterior ends of the fully extended caudal fin.	
Caudal Fin Area (CFA)	The area of the fully extended caudal fin.	
Pectoral Fin Area (PFA)	The area of the fully extended pectoral fin.	
Pectoral Fin Length (PFL)	The greatest distance between the base and tip of the pectoral fin.	
Pectoral Fin Width (PFW)	The greatest distance from the dorsal end to the ventral end of the fully extended pectoral fin, perpendicular to its length.	
Head Length (HL)	The distance from the tip of the snout to the posterior end of the operculum.	
Head Height (HH)	The distance from the ventral side of the mandible to the dorsal side of the cranium.	

**Table 2.2:** Ecomorphological attributes, their calculation, ecological significance, and the direction of change expected and observed for each population (Black Squirrel Creek: “BSQ” and Horse Creek: “HRS”) in response to abiotic enrichment. Direction of change is indicated by an increase (“+”) or decrease (“-”), with “X” representing no change in attribute expression, in response to the abiotic enrichment treatment (obtained from Pessanha et al. 2015; Saraiva and Pompeu 2016).

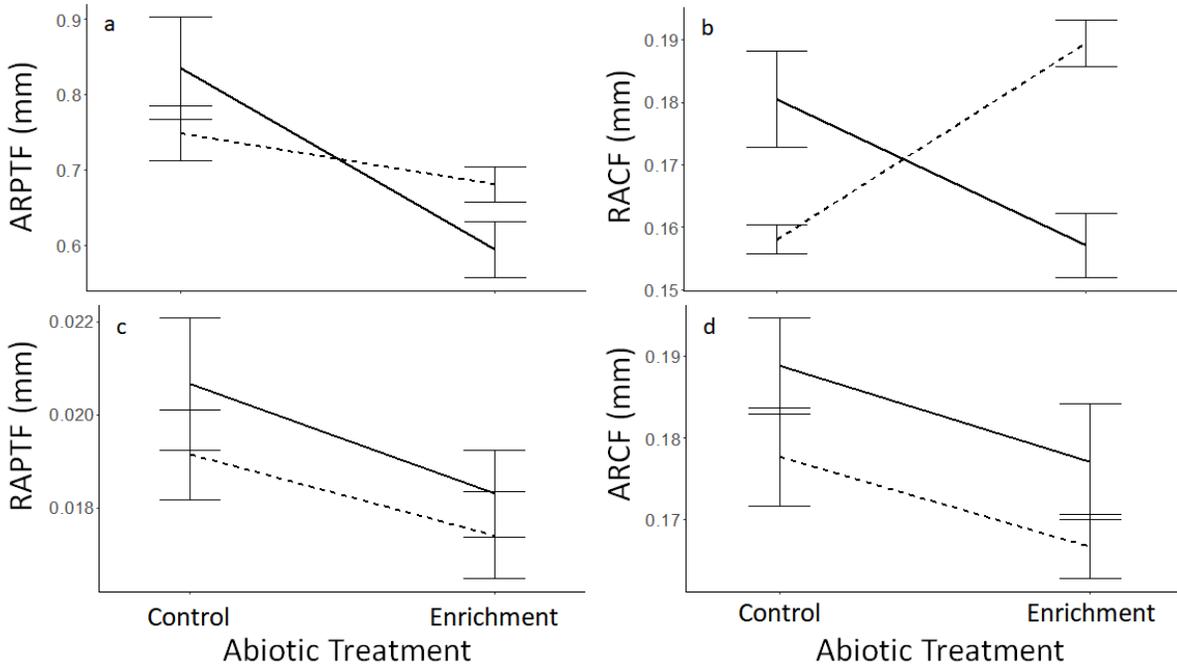
Attribute	Ecomorphological Attributes		Direction of Change		
	Calculation	Ecological Significance	Expected	BSQ	HRS
Ventral Flattening Index (VFI)	$VFI = ABH/BH$	Lower values reflect fish living in high flows with a greater ability to maintain position without swimming.	-	X	X
Relative Height of the Body (RH)	$RH = BH/SL$	Lower values reflect fish living in high flows.	-	X	X
Relative Length of the Caudal Peduncle (RLCP)	$RLCP = CPL/SL$	Higher values reflect fish living in high flows with better swimming ability.	+	X	X
Relative Area of the Caudal Fin (RACF)	$RACF = CFA/BA$	Higher values reflect fish with a benthic lifestyle and greater burst swimming capabilities.	+	-	+
Aspect Ratio of the Caudal Fin (ARCF)	$ARCF = CFW/CFA$	Higher values reflect fish that swim more.	+	-	-
Relative Area of the Pectoral Fin (RAPTF)	$RAPTF = PFA/BA$	Higher values reflect fish living in high flows with greater maneuverability.	+	-	-
Aspect Ratio of the Pectoral Fin (ARPTF)	$ARPTF = PFL/PFW$	Higher values reflect fish living in high flows with longer fins and greater swimming ability.	+	-	-
Relative Length of the Head (RLH)	$RLH = HL/SL$	Higher values reflect fish living in high flows.	+	X	X
Relative Height of the Head (RHH)	$RHH = HH/SL$	Lower values reflect fish living in high flows.	-	X	X

**Table 2.3:** PERMANOVA results showing the effect of abiotic treatment, population, and their interaction for each ecomorphological attribute (adapted from Saraiva and Pompeu 2016). P-values in bold represent those that are significant at the level of  $\alpha = 0.05$ . Asterisks (\*) represent a non-significant trend (p-value < 0.1).

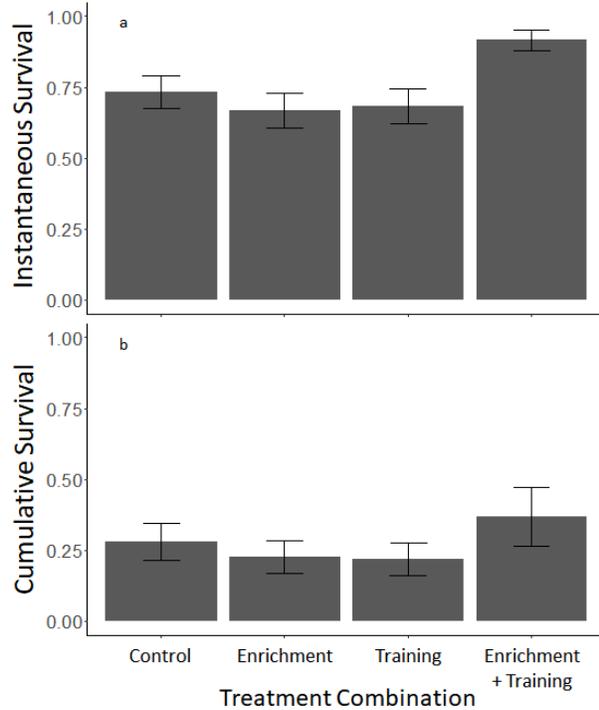
Attribute	Treatment			Effect			Interaction		
	R <sup>2</sup>	F <sub>(1,34)</sub>	p-value	R <sup>2</sup>	F <sub>(1,34)</sub>	p-value	R <sup>2</sup>	F <sub>(1,34)</sub>	p-value
VFI	0.012	0.428	0.541	<0.001	0.019	0.911	0.006	0.222	0.660
RH	0.016	0.611	0.422	0.006	0.205	0.665	0.066	2.461	0.115
RLCP	0.012	0.586	0.461	0.265	12.511	<b>0.003</b>	0.004	0.196	0.650
RACF	0.017	1.207	0.266	0.027	1.862	0.169	0.464	32.130	<b>0.001</b>
ARCF	0.082	3.319	0.062*	0.082	3.324	0.067*	<0.001	0.005	0.950
RAPTF	0.090	3.485	0.070*	0.033	1.287	0.273	<0.001	0.084	0.796
ARPTF	0.252	12.875	<b>0.002</b>	<0.001	0.016	0.919	0.084	4.304	<b>0.038</b>
RLH	0.065	2.976	0.104	0.196	9.060	<b>0.005</b>	0.003	0.119	0.744
RHH	0.001	0.029	0.887	0.108	4.155	<b>0.049</b>	0.009	0.341	0.552



**Figure 2.1:** The mean ( $\pm$  SE bars) number of darters feeding (panel a) and moving (panel b) during instantaneous scans made every two minutes during a 30-minute observation period for fish treated with (“Enrichment”) and without (“Control”) abiotic enrichment in each population (Big Sandy Creek, Black Squirrel Creek, and Horse Creek). There was a significant interaction between treatment and population in both a and b.



**Figure 2.2:** The mean ( $\pm$  SE bars) Aspect Ratio of the Pectoral Fin (ARPTF, panel a), Relative Area of the Caudal Fin (RACF, panel b), Relative Area of the Pectoral Fin (RAPTF, panel c), and Aspect Ratio of the Caudal Fin (ARCF, panel d) for Arkansas darters from two populations (Horse Creek: dotted line; Black Squirrel Creek: solid line) reared with ("Enrichment") and without ("Control") abiotic enrichment. There was a significant interaction between treatment and population for a and b and trends for treatment effects for c and d (Table 2.6).



**Figure 2.3:** The mean ( $\pm$  SE bars) instantaneous (panel a) and cumulative ( $\pm$  unconditional SE bars; panel b) survival of Arkansas darters that received no abiotic enrichment and no predator training (“Control”), only abiotic enrichment (“Enrichment”), only predator training (“Training”), or received both abiotic enrichment and predator training (“Enrichment + Training”). Panel a shows instantaneous survival for only the first encounter trial with a tiger muskie predator. In a, the Enrichment + Training treatment was significantly different from all other treatments, and there were no differences in b.

**Table 2.4:** Redundancy analysis (RDA) eigenvalues, proportion of variation explained, and cumulative proportion of variation explained by each unconstrained canonical axis of the RDA model.

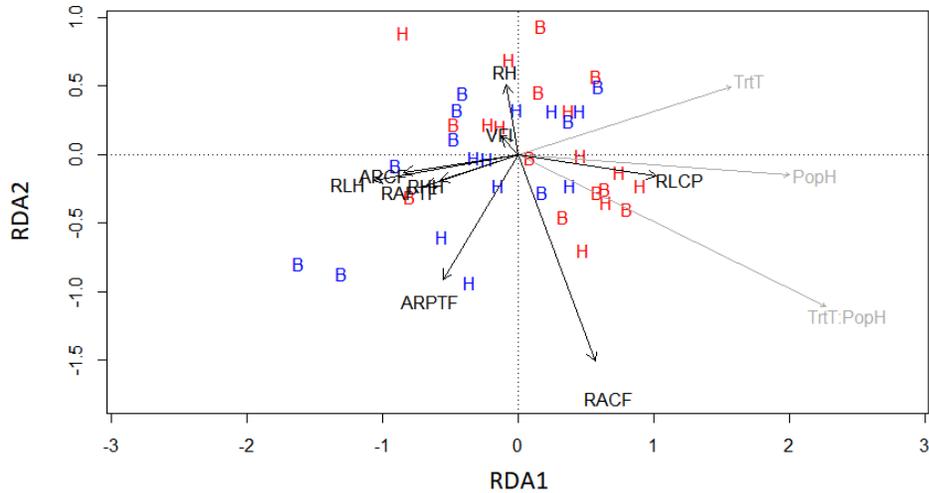
	<u>Unconstrained Axes</u>								
	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
Eigenvalue	2.0517	1.2522	1.0040	0.9190	0.5389	0.4549	0.4085	0.3285	0.1402
Proportion Explained	0.2891	0.1764	0.1414	0.1295	0.0759	0.0641	0.0576	0.0463	0.0197
Cumulative Proportion	0.2891	0.4655	0.6069	0.7364	0.8123	0.8764	0.9340	0.9803	1.0000

**Table 2.5:** Pairwise comparisons of estimated marginal means for the interaction between the number of darters feeding in each population (Big Sandy Creek = “BGS”, Black Squirrel Creek = “BSQ”, Horse Creek = “HRS”) and abiotic treatment (Abiotic Control = “C”; Abiotic Enrichment = “E”), presented on the log scale. Tukey adjusted p-values in bold represent those that are significant at the level of  $\alpha = 0.05$ .

Contrast	Estimate	Standard Error	z-ratio	p-value
BGS(E) – BGS(C)	0.272	0.232	1.175	0.8490
BGS(E) – BSQ(E)	0.602	0.256	2.353	0.1730
BGS(E) – BSQ(C)	1.340	0.334	4.010	<b>0.0009</b>
BGS(E) – HRS(E)	-0.318	0.214	-1.484	0.6743
BGS(E) – HRS(C)	0.965	0.290	3.330	<b>0.0112</b>
BGS(C) – BSQ(E)	0.330	0.270	1.225	0.8250
BGS(C) – BSQ(C)	1.068	0.345	3.097	<b>0.0240</b>
BGS(C) – HRS(E)	-0.590	0.230	-2.560	0.1073
BGS(C) – HRS(C)	0.693	0.302	2.295	0.1960
BSQ(E) – BSQ(C)	0.738	0.362	2.040	0.3197
BSQ(E) – HRS(E)	-0.920	0.255	-3.609	<b>0.0042</b>
BSQ(E) – HRS(C)	0.363	0.321	1.130	0.8690
BSQ(C) – HRS(E)	-1.658	0.333	-4.973	<b>&lt;1.00e-04</b>
BSQ(C) – HRS(C)	-0.375	0.386	-0.970	0.9275
HRS(E) – HRS(C)	1.283	0.289	4.440	<b>0.0001</b>

**Table 2.6:** Pairwise comparisons of estimated marginal means for the interaction between the number of darters moving in each population (Big Sandy Creek = “BGS”, Black Squirrel Creek = “BSQ”, Horse Creek = “HRS”) and abiotic treatment (Abiotic Control = “C”; Abiotic Enrichment = “E”), presented on the log scale. Tukey adjusted p-values in bold represent those that are significant at the level of  $\alpha = 0.05$ .

Contrast	Estimate	Standard Error	z-ratio	p-value
BGS(E) – BGS(C)	-0.896	0.0679	-13.191	<b>&lt; 1.00e-04</b>
BGS(E) – BSQ(E)	-0.303	0.0755	-4.018	<b>0.0008</b>
BGS(E) – HRS(E)	-0.606	0.0773	-7.835	<b>&lt;1.00e-04</b>
BGS(E) – HRS(C)	-0.743	0.0695	-10.684	<b>&lt;1.00e-04</b>
BGS(C) – BSQ(E)	0.593	0.0613	9.671	<b>&lt;1.00e-04</b>
BGS(C) – BSQ(C)	-0.177	0.0496	-3.579	<b>0.0046</b>
BGS(C) – HRS(E)	0.290	0.0635	4.569	<b>0.0001</b>
BGS(C) – HRS(C)	0.153	0.0538	2.844	<b>0.0508</b>
BSQ(E) – BSQ(C)	-0.770	0.0595	-12.946	<b>&lt; 1.00e-04</b>
BSQ(E) – HRS(E)	-0.302	0.0715	-4.228	<b>0.0003</b>
BSQ(E) – HRS(C)	-0.440	0.0631	-6.971	<b>&lt;1.00e-04</b>
BSQ(C) – HRS(E)	0.468	0.0618	7.568	<b>&lt;1.00e-04</b>
BSQ(C) – HRS(C)	0.330	0.0517	6.386	<b>&lt;1.00e-04</b>
HRS(E) – HRS(C)	-0.137	0.0652	-2.103	0.2857



**Figure 2.4:** Dispersion plot of the Redundancy Analysis (RDA) results. Each letter represents a data point from one of two populations (Black Squirrel Creek = “B”, Horse Creek = “H”) in each abiotic treatment (red = abiotic enrichment, blue = abiotic control). Black arrows represent the direction (sign of the correlation) and magnitude (arrow length) of ecomorphological attribute change, relative to the effects of abiotic treatment (“TrtT”), population (“PopH”), and their interaction (“TrtT:PopH”; grey arrows), with significant correlation to the first two constrained canonical axes (RDA1 and RDA2).

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### 3. THE EFFECT OF ENVIRONMENTAL ENRICHMENT ON WHOLE-BRAIN GENE EXPRESSION IN AN IMPERILED FISH

#### **Summary**

Phenotypic mismatch caused by differences between hatchery and wild environments can limit efforts to conserve fish species at risk of extinction when hatchery-reared fish are used to augment wild populations. Phenotypes adapted to or induced by hatchery environments are thought to be maladapted for life in the wild. Thus, enriching the hatchery environment (abiotically and biotically) to make it more similar to the wild may induce phenotypes, including gene expression profiles, that are better suited to the environments fish will experience after release. Here, we took a molecular approach (TagSeq) to elucidate how abiotic and biotic (predator training) enrichment impacts the whole-brain gene expression of a species of conservation concern, the Arkansas darter (*Etheostoma cragini*), comparing the effects in two hatchery populations to a wild reference population. Although we found no effect of biotic enrichment on gene expression, we found that abiotic enrichment has the potential to reduce phenotypic mismatch between hatchery and wild fish, indicating that enrichment may aid current conservation efforts.

#### **Introduction**

As biodiversity declines, conservation practitioners must creatively use new and existing tools to conserve populations and assess the efficacy of their efforts. One frequently used tool is population augmentation, which involves supplementing populations with individuals from another location or from captivity (Mallinson 1995). However, programs that use captive animals

to supplement wild populations often struggle to produce individuals capable of surviving and reproducing after release (Brown and Laland 2001; Brown and Day 2002; Fitzpatrick et al. 2014; Fraser 2008; Griffin et al. 2000; Hawkins et al. 2008; Hutchison et al. 2012; Jackson and Brown 2011; Mallinson 1995; Mesquita and Young 2007; Olla et al. 1998). Stark differences between captive and wild environments may contribute to the poor performance of captive individuals after release into the wild, prompting a critical examination of the effects of the captive environment on phenotypes (Brown and Day 2002; Crane et al. 2015; Evans et al. 2015; Jackson and Brown 2011; Lavoie et al. 2018; Stringwell et al. 2014). This is particularly important for aquatic hatcheries that produce vast numbers of fish of conservation concern for supplementation (Brown and Laland 2001; Brown and Day 2002; Jackson and Brown 2011; Lavoie et al. 2018; Stringwell et al. 2014). The hatchery environment can cause captive fish to differ phenotypically from wild populations, through both evolutionary change (Brown and Laland 2001; Brown and Day 2002; Bull et al. 2022; Christie et al. 2012; Christie et al. 2016; Lavoie et al. 2018; Stringwell et al. 2014) and phenotypic plasticity (Brown and Laland 2001; Brown and Day 2002; Crane et al. 2015; Lavoie et al. 2018; Stringwell et al. 2014). The phenotypic mismatch between hatchery and wild fish can be vast (Brown and Laland 2001; Lavoie et al., 2018; Stringwell et al. 2014), encompassing morphology (e.g., Belk 2008; Hutchison et al. 2012; Kihlslinger et al. 2006; Saraiva and Pompeu 2016), physiology (e.g., Barcellos et al. 2018; Chittenden et al. 2010; Fuss and Byrne 2002; Hutchison et al. 2012; Salvanes et al. 2013), behavior (e.g., Barcellos et al. 2018; Brown and Day 2002; Crane et al. 2015; D'Anna et al. 2012; Salvanes et al. 2013), life history (e.g., McDermid et al. 2007), and even individual personality (e.g., Johnsson et al. 2014; Snell-Rood 2013).

One potential tool to address this phenotypic mismatch is to enrich the hatchery environment to better match the wild environments that individuals will experience after release (Brown and Laland 2001; Brown and Day 2002; D'Anna et al. 2012; Kopack et al. Chapters 1 and 2; Stringwell et al. 2014; Salvanes et al. 2013; Tave et al. 2019; Ullah et al. 2017; Vilhunen 2006). This may involve enhancing the hatchery environment with abiotic enrichment, including an increase in environmental complexity (D'Anna et al. 2012; Johnsson et al. 2014; Kopack et al. Chapter 2; Salvanes et al. 2013; Tave et al. 2019; Ullah et al. 2017), and/or biotic enrichment, including experience with foraging and predator avoidance (Brown and Laland 2001; Brown and Day 2002; D'Anna et al. 2012; Kopack et al. Chapters 1 and 2; Sanogo et al. 2011). Enrichment interventions can cause plastic effects across phenotypes (e.g., morphology, physiology, behavior, etc.), making it challenging to holistically quantify their impacts. Measuring changes in gene expression is one way to reveal plasticity across a broad array of traits in a single assay while also elucidating the mechanisms underlying phenotypic change. For example, Salvanes et al. (2013) found that abiotic enrichment in the hatchery increased expression of certain genes in the forebrain related to cognitive performance in Atlantic salmon (*Salmo salar*). Here, we use gene expression to assess the impacts of abiotic and biotic enrichment for a species of conservation concern, the Arkansas darter (*Etheostoma cragini*). While previous studies have used gene expression to identify differences in phenotypes among populations/individuals, ours is the first, to our knowledge, that uses gene expression to better understand how we might reduce phenotypic differences between captive animals of conservation concern and their wild counterparts.

The Arkansas darter is a small freshwater fish endemic to North America that is threatened at the state level in Colorado. Current efforts to conserve this species include using

hatchery populations to supplement wild populations from which they were originally sourced. However, the genes from stocked fish have not persisted in wild populations, suggesting that supplementation efforts are not effective (Fitzpatrick et al. 2014). We have explored both abiotic and biotic enrichment as possible solutions (Kopack et al. Chapters 1 and 2), with some encouraging results. When combined, the two forms of enrichment caused an increase in survival upon a first encounter with a live predator (Kopack et al. Chapter 2). However, nothing is known about how abiotic or biotic enrichment affects gene expression in Arkansas darters.

We used Tag-based sequencing (TagSeq; Lohman et al. 2016; Matz 2018) to assess plasticity of gene expression in response to abiotic and biotic enrichment in hatchery-reared darter populations. We also assessed gene expression of a wild population as a comparison to determine whether enrichment shifts phenotypes to be more similar to those of wild fish. In this chapter we address three main questions: (1) Does abiotic enrichment affect gene expression of hatchery-reared darters?; (2) Does biotic enrichment (predator training) affect gene expression of hatchery-reared darters?; and (3) If so, do changes in gene expression of hatchery-reared darters make them more similar to the gene expression of wild darters? We expected both types of enrichment to cause changes in gene expression profiles, making them more similar to the profiles of wild fish.

## **Methods**

### *Experimental design*

The design of our experiment allowed us to determine whether enrichment affected gene expression of neural tissue in both hatchery-reared and wild Arkansas darters. This design was used in a second parallel experiment that examined the effects of abiotic and biotic enrichment

on survival in these same darters (Kopack et al. Chapter 2). Briefly, we reared two populations of captive, hatchery darters in two abiotic treatments (with and without enrichment) to evaluate whether abiotic enrichment affected gene expression. Second, we exposed a subset of these fish to two biotic treatments (with and without predator training) to examine if biotic experience affects gene expression (Figure 3.1). A wild population not previously exposed to the abiotic treatments was added to the biotic enrichment portion of the experiment to determine if fish in the treatments that mimic the wild environment (abiotic enrichment and predator training) would have gene expression profiles that were more similar to the profiles of wild fish. Following these treatments, we sacrificed darters, collected their neural tissue, and used a Tag-based sequencing approach to identify genes that were differentially expressed among populations and treatments.

### *Experimental animals*

We used three populations of Arkansas darters for this experiment: two hatchery-reared populations, Horse Creek and Big Sandy Creek, and a wild population collected from the May Valley Ditch (hereafter ‘Wild’). We obtained 100 young-of-year fish per population that were approximately 10 months old and developmentally mature from the John W. Mumma Native Aquatic Species Restoration Facility (Alamosa, CO) in March 2018 and 20 fish from the May Valley Ditch (Lamar, CO) in June 2018. Hatchery-reared and wild fish were transported to the Colorado Parks and Wildlife (CPW) Salmonid Disease and Sport Fish Research Laboratory (Fort Collins, CO) for experimentation.

### *Abiotic and biotic enrichment*

The Horse Creek and Big Sandy Creek fish were randomly divided into two abiotic treatments (with and without enrichment) and reared for 90 days (see Kopack et al. Chapter 2 for details). For the control treatment, each population was housed in a separate 76-L flow-through tank supplied with filtered well water at a flow rate of 7.5 L per minute and an average ( $\pm$  SD) temperature of  $13.5 \pm 2^\circ\text{C}$ . Abiotic enrichment was added to two additional 76-L tanks, one per population, which included structure (caves made from PVC pipe and artificial plants; multi-pack B1; Marineland®, Blacksburg, VA), temperature variation (daily fluctuations of  $3 \pm 0.5^\circ\text{C}$  created by two 100 W in-tank heaters; Eheim™, Buffalo, NY), and flow (two Hydor powerheads 155 L per minute; Hydor USA Inc., Sacramento, CA). The lab was illuminated by 32 W fluorescent lights (General Electric Electrolux) with a light cycle of 14:10 hours light:dark.

Darters were then exposed to biotic enrichment treatments that consisted of either a predator training treatment or a control. Three populations, the two populations of hatchery darters that had been given abiotic enrichment or not, and the Wild population, were exposed to biotic enrichment. We randomly selected darters from the Wild population and from both abiotic treatment groups of the two hatchery populations and assigned them to one of two predator treatments such that there were five darters per population and treatment combination (Table 3.1). Twenty-four hours before administering the predator treatment, we moved darters to individual 10-L flow-through tanks receiving the same filtered well water (see Kopack et al. Chapter 2 for details). Then the control treatment received a water control while the predator treatment received three stimuli simultaneously: 5 mL of conspecific alarm cue, 5 mL of predator kairomone (tiger muskie [*Esox masquinongy* x *E. lucius*]; used as a surrogate for northern pike [*E. lucius*]), and a 102 mm 3D tiger muskie model (Savage Gear USA, Ontario, CA; following Kopack et al. Chapter 1). Alarm cue was extracted from decapitated darters by

scoring the skin with a razor blade and rinsing with distilled water (following Kopack et al. Chapter 1, adapted from Nordell 1998), and predator kairomone was collected from 19-L non-circulating tanks that housed two adult Tiger Muskie that were fed darters and held for 48 hours (following Kopack et al. Chapter 1; see Kopack et al. Chapter 2 for details). The 3D model was attached to a wooden dowel via fishing line so that the researcher could place it in the individual darter tank from behind a blind. A researcher administered the predator treatments (water control and predator cues with predator model) from behind a blind and waited 5 minutes before removing the model and turning on the flow-through system to flush any residual cues from the tanks.

#### *Neural tissue collection (whole brain samples)*

We collected whole brains from five darters per population and treatment combination (Table 3.1). We focus on gene expression in brain tissue because this is the organ that is directly involved with changes in behavioral phenotypes (Salvanes et al. 2013). Exactly one hour after biotic enrichment, each individual fish was netted out of its tank, placed on a sterilized (90% ethanol) surface, decapitated, followed by pithing of the brain. Next, we cut the head along its bilateral axis and used sterile forceps (90% ethanol) to collect tissue from the cranium, which we immediately transferred into RNAlater (Ambion; Lohman et al. 2016) and stored at -20°C until RNA extraction (55 to 62 days later). All tissues were collected within 60 seconds of netting fish out of their treatment tank.

#### *TagSeq sample preparation and cDNA library synthesis*

We used an RNeasy ® Lipid Tissue Mini Kit (cat. No. 74804; Qiagen) to extract RNA from whole brains, following the standard protocols provided with the kit. Quality of RNA isolated was quantified using a Qubit 2.0 fluorometer (Invitrogen; Life technologies). However, we failed to recover RNA from individuals within the abiotic enrichment treatment of the Big Sandy Creek population (many of the samples had quantities of RNA that were below a threshold to analyze); thus, abiotic treatment inferences could only be made for the Horse Creek population (see Table 3.1). Isolated RNA samples with concentrations of 10 µl or more were then sent to the University of Texas Austin Genome Sequencing and Analysis Facility (GSAF) where they were sequenced on a single lane of an Illumina HiSeq 2500, 2x100 (following Lohan et al. 2016 and Aglyamova et al. 2019).

#### *Gene expression data preparation and analysis*

We assessed sequencing quality using FastQC v. 0.11.9 and multiQC v. 1.12 (Andrews 2010; Ewels et al. 2016). Following quality control, we aligned reads to the published *E. cragini* genome (CSU\_Ecrag\_1.0; GCF\_013103735.1; Reid et al. 2013) using STAR v. 2.7.10a. with default parameters and in quantification mode (Dobin et al. 2013). Gene-level counts generated using quantification mode were then compiled and used for downstream analyses.

All analyses were performed using R statistical software (R Core Team 2020). Prior to analysis, we summed counts across columns to account for technical replicates. Next, the count data were prefiltered by removing all genes with a mean count less than ten (following Love et al. 2014). We then conducted a Principal Coordinate Analysis (PCoA) followed by a formal analysis of differentially expressed genes using DESeq2 (Love et al. 2014). For both analyses,

we compared the impact of abiotic and biotic enrichment on gene expression in whole-brain tissues.

When assessing the effects of abiotic enrichment on gene expression, we initially included darters from the biotic enrichment portion of the experiment that had received just the water control treatment to eliminate any effects of biotic enrichment. However, after discovering that biotic enrichment had no effect on gene expression (see below), we performed a post-hoc analysis for the effect of abiotic enrichment that included individuals from both the water control and predator cues treatment groups. These are the results we report below. To assess the effect of abiotic enrichment, we compared the number of differentially expressed genes (DEGs) between the two Horse Creek abiotic treatment groups and the Wild population, with population as a factor nested within abiotic treatment. We considered genes with adjusted (Benjamini-Hochberg) p-values less than 0.05 to be significantly differentially expressed (DEGs).

To assess the effect of biotic enrichment, we compared the number of DEGs between biotic treatment groups (Water Control and Predator Cues) and among populations (Horse Creek, Big Sandy Creek, and Wild), with both included as fixed effects in a mixed model. We report post-hoc pairwise comparisons across treatments and populations.

## **Results**

Abiotic enrichment had a significant impact on gene expression, causing Horse Creek fish to have a gene expression profile that was more similar to the Wild population (Figure 3.2). From the PCoA, the first axis (MDS1) explained approximately 37% of the variation observed and was attributed to differences among groups (Wild, Abiotic Enrichment, Abiotic Control), while the second axis (MDS2) explained only 9% of the variation captured and was attributed to

interindividual variation (Figure 3.2). Pairwise comparisons using DESeq2 revealed more DEGs between the Abiotic Control and Wild fish than between the Abiotic Treatment and Wild fish (Figure 3.3). In total, we found 4,162 DEGs (out of 27,486 expressed genes) between Wild fish and fish from the Abiotic Control treatments, with 1,620 genes that increased in expression and 2,542 that decreased expression for the Abiotic Control treatments (Figure 3.3). Between the Wild fish and Abiotic Enrichment treatments, a total of 398 DEGs were found, with 140 genes increasing in expression and 258 decreasing in expression for the Abiotic Enrichment treatments (Figure 3.3). We also found 1,336 DEGs between Abiotic Control and Abiotic Enrichment treatments, with 405 increasing in expression and 931 decreasing in expression for the Abiotic Enrichment treatment (Figure 3.3).

The biotic enrichment treatment had no effect on DEGs; however, genes were differentially expressed across populations (Figure 3.4). From the PCoA, the first axis (MDS1) captured a significant proportion (38%) of the variation and was attributed to differences among populations (Figure 3.4). The second axis (MDS2) explained approximately 8% of the variation and was attributed to effects of predator training (water control and predator cues; Figure 3.4), though these effects were not significant. Pairwise comparisons using DESeq2 found zero differentially expressed genes between the fish in the Predator Cues and Water Control treatments (Figure 3.4). We found a total of 3,093 genes to be significantly differentially expressed among the three populations (Figure 3.5). Between the Wild and Horse Creek populations, we found 1,182 of those genes increased in expression, while 1,911 decreased in expression for the Horse Creek population (Figure 3.5). Between the Wild and Big Sandy Creek populations, we found 1,246 genes that increased expression and 1,847 genes that decreased expression for the Big Sandy Creek population (Figure 3.5). Lastly, we found 1,926 genes

increased expression, while 1167 genes decreased expression in the Horse Creek hatchery population compared to the Big Sandy Creek hatchery population (Figure 3.5).

## **Discussion**

In this study, we measured differences in whole-brain gene expression between hatchery and wild populations of Arkansas darters and assessed whether abiotic and biotic enrichment could be used as a conservation tool to reduce those differences. We detected population differences in gene expression, with the Horse Creek hatchery population differing most from the Wild population. We also detected effects of abiotic enrichment on gene expression, which induced plastic responses in the Horse Creek population that reflected expression profiles of wild individuals. This suggests that abiotic enrichment may be used to reduce phenotypic mismatch between wild and hatchery fish, potentially priming fish for success in the environment where they will be released. Applying abiotic enrichment at the hatchery level would not be logistically difficult as our efforts involved adding simple structure, flow, and temperature variation with readily available materials. Furthermore, we might have elicited an even greater change in gene expression if we had reared darters with abiotic enrichment for a longer time period than 90 days, as darters may spend 10 to 12 months in the hatchery before being released. Future research should investigate the plastic effects of longer-term or cross generational enrichment (West-Eberhard 1989).

We found no evidence that our biotic enrichment induced phenotypic plasticity, and there were zero differentially expressed genes between predator treatments. This suggests that the form of predator training we used may not be useful in eliciting observable phenotypic change, supporting previous research that predator training did not have a strong effect on antipredator

behavior in Arkansas darters, perhaps because antipredator behavior is innate (Kopack et al. Chapter 1). However, this response could also result from the relatively short time fish were exposed to predator cues (acute vs chronic exposures; Snell-Rood 2013) and further research is needed to assess the effects of other forms of predator training (Rittschof and Hughes 2018). While single, acute-exposure experiments have been shown to elicit behavior (Kopack et al. 2015), Sanogo et al. (2011) induced differences in gene expression by subjecting stickleback fish (*Gasterosteus aculeatus*) to longer exposure times during development. Furthermore, the one-hour duration of time between treatment and tissue collection may not have been long enough to detect changes in expression profiles, and longer durations may be needed to detect meaningful differences. However, combined with abiotic enrichment, exposure to predator cues increased survival in Arkansas darters (Kopack et al. Chapter 2), suggesting there could be an advantage to giving darters experience with predator cues prior to their first encounter with a novel predator. As such, the potential for biotic enrichment to increase survival should continue to be investigated.

We did find differences in gene expression among darter populations, suggesting they differ in regulatory genes with potential impacts on a range of phenotypes, which is also supported by prior research that found genetic and phenotypic differences among populations (Fitzpatrick et al. 2014; Kopack et al. Chapters 1 and 2). However, it is unclear if these population differences reflect adaptive differences stemming from the variability between wild or hatchery environments, are a product of drift in their wild source populations, or resulted from bottleneck or drift in the hatchery setting. Comparisons with the wild populations from which the hatchery populations were sourced could elucidate the origins of these differences. Thus, it may be important to manage darters at the level of the population and maintain genetically separate

lineages within the hatchery, as there could be potential adaptive value among them. If these phenotypes are found to be adaptive, future conservation efforts could use them as migrant populations into those that are targeted for genetic rescue (Funk et al. 2019). Additionally, the variation among populations and individuals that we detected is the additive genetic variation that managers could select upon if a particular profile is found to be adaptive in the wild (Christie et al. 2016; Dingemanse et al. 2009; Funk et al. 2019).

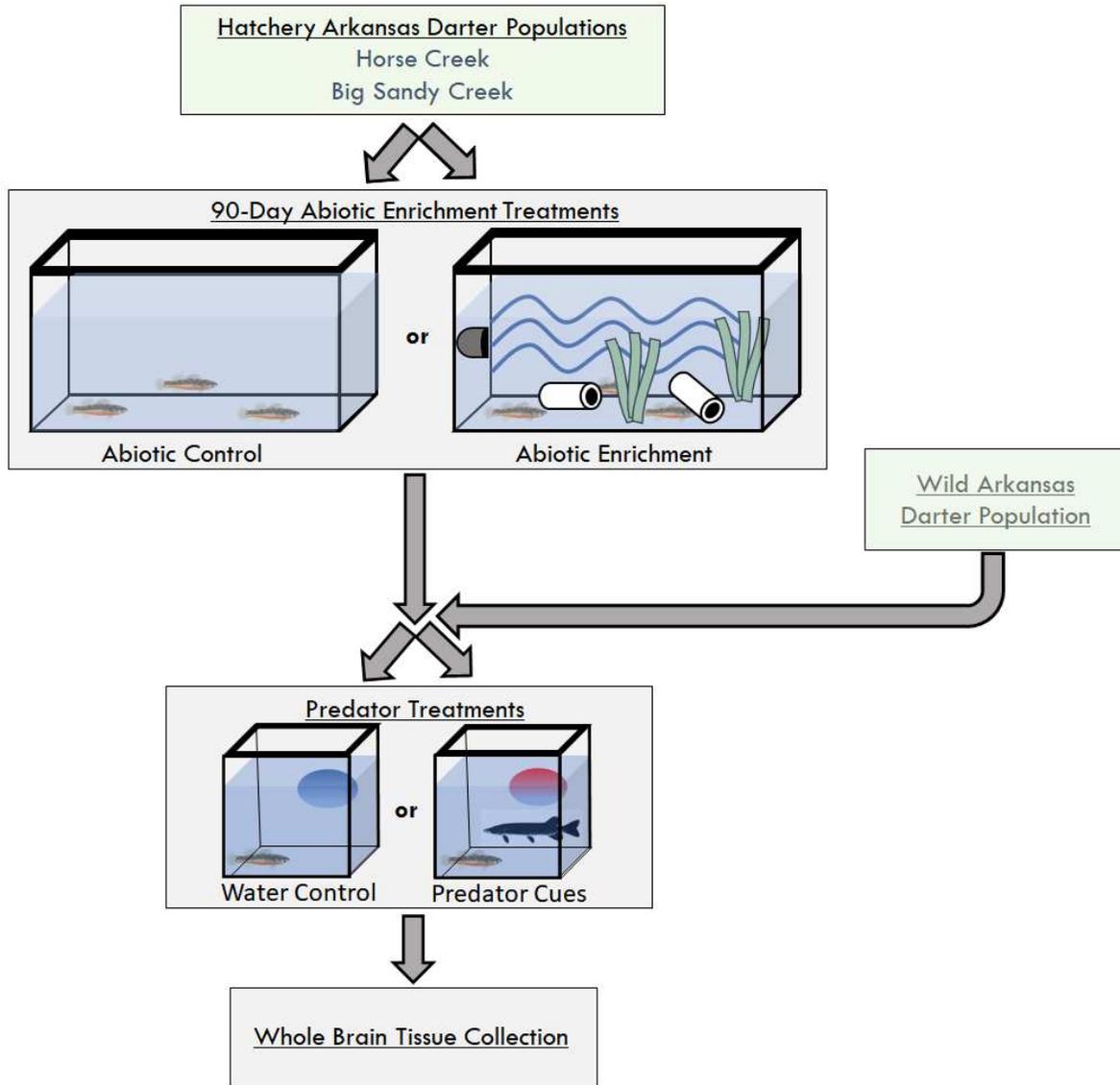
## **Conclusions**

We demonstrated the value of measuring gene expression and using enrichment as possible tools in the conservation of an imperiled fish. In doing so, we were able to determine that: 1) abiotic enrichment has the potential to reduce phenotypic mismatch between hatchery and wild populations; 2) darters show no evidence of plasticity in response to biotic enrichment (predator training); 3) there are strong population differences that should be taken into consideration; and 4) the many candidate genes that potentially underly phenotypic differences among populations provide valuable information that can be leveraged in conservation efforts. We hope this work inspires others to use gene expression as a tool to measure and reduce phenotypic mismatch between captive and wild animals of conservation concern, and we encourage others to consider novel applications of gene expression to solve conservation problems.

## **Contributors**

Coauthors on the publication produced from this chapter include Coby McDonald, E. Dale Broder, Eric R. Fetherman, Ryan M. Fitzpatrick, Kim Hoke, and Lisa M. Angeloni. The

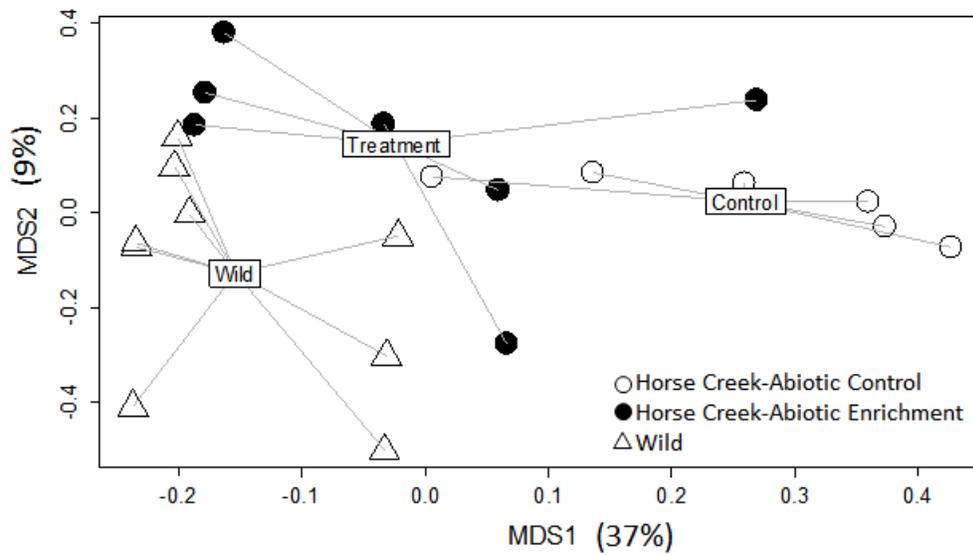
authors thank S. Fitzpatrick, R. Moran, L. Stein, M. Matz, H. Crockett, P. Foutz, G. Schisler, A. Treble, T. Smith, and the staff of the Colorado Parks and Wildlife John W. Mumma Native Aquatic Species Restoration Facility and Wray Fish Hatchery for providing fish, equipment and supplies, logistic support, and expertise. The authors would also like to thank B. Avila, R. Cheek, E. Vigil, T. Swarr, R. Osmundson, T. Hubbard, K. Dick, F. Horne, and M. Whedbee for help with setup, animal husbandry, and data collection.



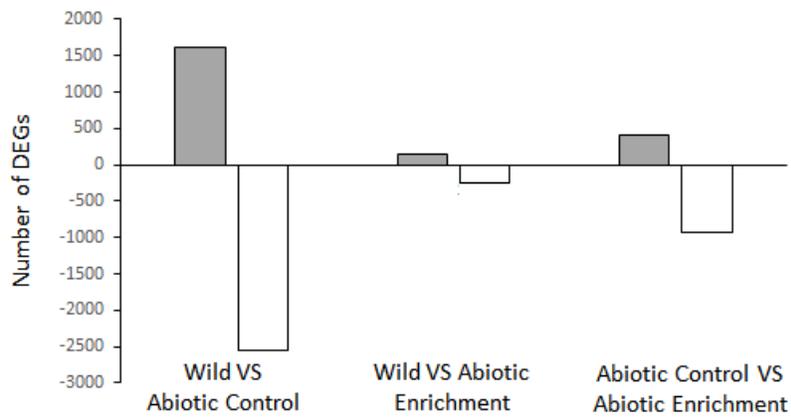
**Figure 3.1:** Schematic of experimental design and procedures. Horse Creek and Big Sandy Creek hatchery populations were exposed to abiotic treatments for 90 days. The Abiotic Enrichment treatment tanks included high flows, changing temperatures, artificial plants, and PVC caves. Following abiotic treatments, both hatchery and wild darters were exposed to predator treatments. The Predator Cues treatment included a combination of conspecific alarm cue, predator kairomone, and a predator model. After treatments, whole brain tissues were collected for gene expression analysis.

**Table 3.1:** Number of individual Arkansas darters (N=50 attempted, 5 per cell) from which we recovered RNA by population and enrichment treatments. Note that many of the samples had quantities of RNA that were below a threshold to analyze.

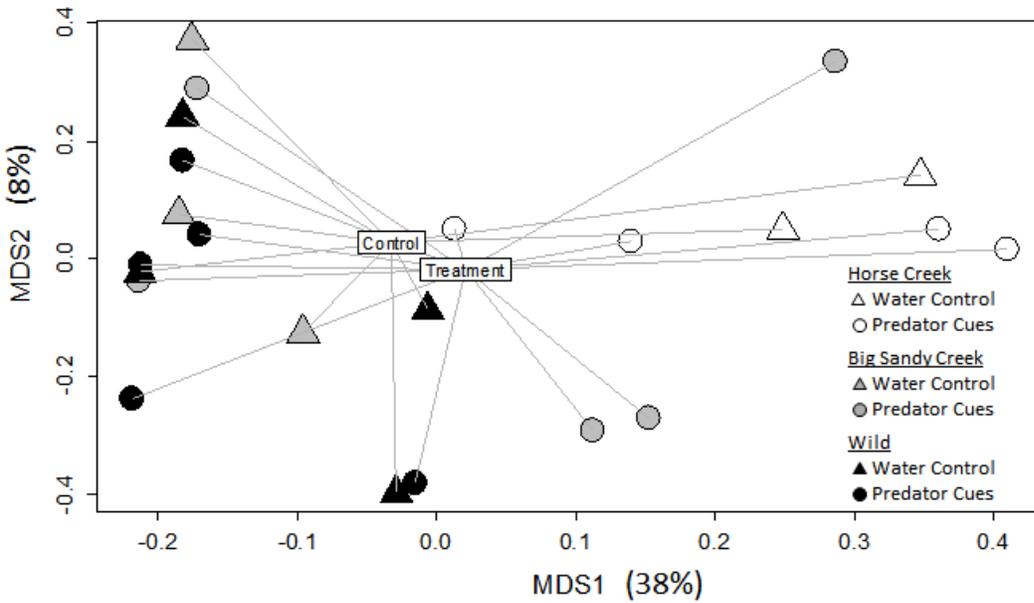
	Wild	Horse Creek		Big Sandy Creek	
		Abiotic Enrichment	Abiotic Control	Abiotic Enrichment	Abiotic Control
Predator Cues	5	5	4	0	5
Water Control	4	2	2	0	3
Total	9	13		8	



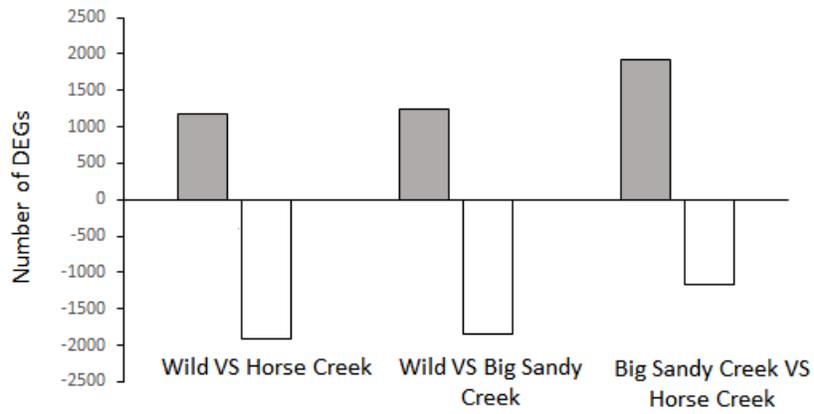
**Figure 3.2:** Multidimensional scaling plots showing the gene expression profiles for two populations (triangle = Wild, circle = Hatchery). The Horse Creek hatchery population was exposed to abiotic enrichment treatments (black = Abiotic Enrichment, white = Abiotic Control), whereas the wild population was not.



**Figure 3.3:** Bar chart showing the number of differentially expressed genes (DEGs) between Wild and abiotic enrichment treatments (Abiotic Control, Abiotic Enrichment). Bars show the number of DEGs that either increased (gray) or decreased (white) in expression for the second experimental group compared to the first.



**Figure 3.4:** Multidimensional scaling plot showing the gene expression profiles for three populations (black = Wild, gray = Big Sandy Creek, white = Horse Creek) that were exposed to biotic enrichment (predator training) treatments (circle = Predator Cues, triangle = Water Control).



**Figure 3.5:** Bar chart showing the number of differentially expressed genes among the three populations (Wild, Horse Creek, Big Sandy Creek). Bars show the number of DEGs that increased (gray) or decreased (white) in expression for the second population compared to the first.

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