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Predator-Avoidance of Larval Black-bellied Salamanders (Desmognathus quadramaculatus) in Response to Cues from Native and Nonnative Salmonids

Brian L. Dempsey
East Tennessee State University

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In partial fulfillment

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Master of Science in Biology

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by

Brian L. Dempsey

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Dr. Joseph Bidwell, Chair

Dr. Thomas Jones

Dr. Thomas Laughlin

ABSTRACT

Predator-Avoidance of Larval Black-bellied Salamanders (*Desmognathus quadramaculatus)* in

Response to Cues from Native and Nonnative Salmonids

by

Brian Dempsey

In recent years, the stocking and encroachment of rainbow trout (Oncorhynchus mykiss)

into headwaters where the black-bellied salamander (Desmognathus quadramaculatus) and

brook trout (Salvelinus fontinalis) naturally coexist has raised concerns. This study aimed

to determine how co-occurrence with a predator influences black-bellied salamander

predator recognition behavior. To evaluate this, salamander activity metrics were recorded

before and after exposure to either native trout predator cue (brook), introduced trout

predator cue (rainbow), or conditioned tap water (control). Larvae were collected from

brook, rainbow, rainbow/brook, and no trout stream reaches. Our results show that larvae

that co-occur with trout reduced their activity when exposed to brook trout predator cue,

but their response to rainbow trout predator cue depended on their previous co-occurrence.

A follow-up test to determine the influence of alarm cue on predator response in larvae

indicated that the alarm cue enhanced the response to the rainbow trout predator.

DEDICATION

To my late Father, Donnell Dempsey and loving Mother, Christina Dempsey.

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CHAPTER 1. INTRODUCTION

Predator-Prey Interactions

Predator-prey interactions are known to have significant effects on the population dynamics of both participants. There are selective pressures on predators to develop ways of successfully catching prey and selective pressures on prey to develop ways to detect and avoid predators. At the proximate level of selection, predator-prey interactions are a contest of the sensory systems where predator and prey are each rewarded when they gain information over the other (Ferrari et al. 2010b). Early detection is the key in gaining that advantage. Predators that catch prey unaware are usually successful and prey with advanced knowledge of a predator's presence usually avoid predation by employing appropriate behavior, whether early flight, evading detection through crypsis or seeking shelter (Lima and Dill 1990; Barrett 2015). Prey exhibit a tradeoff between exposure to predation risk and energy intake through foraging, meaning that the risk of being preyed upon through extended foraging must be appropriately balanced with the risk of reduced energy reserves to meet metabolic requirements (Lima and Bednekoff 1999; Higginson et al. 2012). The cost associated with prey species avoiding predators is illustrated by the study of Kenison et al. 2016, who found that larval long-toed salamanders (*Ambystoma macrodactlyum*) exposed to both visual and chemical cues of an introduced predator had a 38% lower body weight, 24% shorter body length, and 29% shorter tail compared to salamanders that did not experience the predator. Ideally, prey would adjust their response between potential predators and non-predators to properly allocate energy towards either avoidance or foraging (Ferrari et al. 2010b). When larval hellbenders (*Cryptobranchus alleganiensis*) were exposed to chemical cues from both predatory and non-predatory fishes, it was found that the organisms only exhibited predator-avoidance (decreased activity) behavior

when exposed to predatory fishes, and individuals that were exposed to nonpredator fishes did not show any predator-avoidance behavior (Gall and Mathis 2010).

<p style="text-align:center;">Kairomones and Predator Detection</p>

An important way that prey recognize potential predators is through the detection of kairomones, or chemical cues released from one species and perceived by another, benefitting the species that received it (Ferrari et al. 2010b). In aquatic systems, prey particularly rely on chemical cues to detect predators and assess predation risk (Bronmark and Hansson 2012). The preference of chemical cues over visual cues is highlighted in Hickman et al. (2004). They tested predator-avoidance behavior to a number of fish predators by adult gray-belly salamanders (*Eurycea multiplicata griseogaster*) and found that even if there was high visual clarity between predator and prey (both within the stream and laboratory), the salamanders relied more on the chemical cues than visual cues. A key driver for this may be the variable turbidity that often characterizes freshwater systems (Ferrari et al. 2010b; Bronmark and Hansson 2012). Common chemical cues that aquatic prey species respond to have been reviewed by Ferrari et al. (2010) and include kairomones which are a predator's "signature odor" also known as a predator cue; disturbance cues which usually result from the release of ammonia or urea by distressed prey; alarm cues which are released by injured prey tissue; and dietary cues which derive from the predator's excretory products after consumption of prey.

The response to predatory kairomones by prey can be innate or influenced through learning and further mediated through dietary effects (Ferrari et al. 2010b). Innate anti-predator responses result from an evolutionary history between the predator and prey in stable habitats with consistent predatory regimes (Gall and Mathis, 2010). Innate predator recognition may pose a significant survival advantage if strong predation pressure exists for young age classes, if

predation is predictable with a stable predator community, or if the probability of surviving an encounter is low (Ferrari et al. 2007). Naïve prey lack an innate response or lack an evolutionary history with a predator, often exhibit weak or no predator-avoidance responses to novel predators (Sih et al. 2011). This is demonstrated in Martin (2014), who exposed both farm raised and wild caught red swamp crayfish (*Procambarus clarii*) to chemical cues from largemouth bass (*Micropterus salmoides*). The naïve farm raised *P. clarii,* in comparison to wild caught *P. clarii*, lacked the appropriate behavioral responses, both in terms of movement and use of structural refugia, when exposed to chemical cues from *M. salmoides*. Even though these prey would have little metabolic cost associated with antipredator behavior, they suffer heavy predation from the introduced predators (Sih et al. 2010).

Prey can learn to associate alarm cues from both injured conspecific or heterospecific individuals within their prey-guild (a group of prey that is readily preyed upon by a specific predator), in conjunction with visual or predator cues (Brown 2003). For learned recognition of a predator to occur, a predation event must pair both alarm and predator/visual cues (Smith 1992; Manassa et al. 2013). For example, Iberian green frog tadpoles (*Rana perezi*) exhibited an anti-predator response to a viperine snake (*Natrix maura*), a common predator of adult frogs, after a single exposure to paired alarm and predator cues (Gonzalo et al. 2007). Apart from learning, prey have been known to generalize predator recognition and display predator-avoidance behavior in the presence of both known predators and closely related novel predators. This is most likely because closely related species having similar chemical signals (Ferrari et al. 2007; 2008; 2009). Davis et al. (2012), found that predator-naïve San Marcos salamanders (*Eurycea nana*) displayed predator-avoidance behavior to both a nonnative fish predator (*Lepomis auritus*)

and native fish predator (*Lepomis cyanellus*), which they attributed to a generalized predator-avoidance response to the genus *Lepomis*.

Focal Species

Salamanders, like all amphibians, are ectotherms and they utilize all forms of development, with the majority possessing an aquatic larval and terrestrial life stage. They are predominantly carnivores, preying on invertebrates and in some cases, even smaller salamanders (Petranka 1998). The black-bellied salamander (*Desmognathus quadramaculatus*) is a stream-dwelling salamander that belongs to the family Plethodontidae. This family is characterized by being lungless, with respiration primarily occurring through the skin. The black-bellied salamander is the second-most aquatic, the largest, and possesses the longest larval stage (up to 4 years) within the genus *Desmognathus* (Peterman et al. 2008). In their adult stage, they inhabit refugia in stream banks and ambush their prey, while the larval and juvenile stages occupy smaller refugia and spend more time actively foraging (Camp and Lee 1996). The species is endemic to the Southern Appalachian Mountains, ranging from headwater tributaries to large streams, but occurs most commonly in headwaters (Camp and Lee 1996; Mills 1996). The southern Appalachian Mountains are a global biodiversity hotspot for salamanders, nearly all of which are members of the family Plethodontidae (Petranka 1998). Black-bellied salamander biomass can be substantial with numbers ranging from 78.83 to 99.30 kg ha^{-1} in low order headwater streams during summer and early fall. Additionally, when larval estimates are included, this biomass estimate may reach up to 130.0 kg ha^{-1} (Davic 1984; Peterman et al. 2008). Black-bellied salamanders are a keystone species of aquatic invertebrates and possibly indirectly of other stream salamanders (Davic 1984; Bruce 1996; Davic and Welsh 2004). To demonstrate their direct influence on macroinvertebrate communities Conor and Goforth (2013),

examined the effect of two abundant salamander species in the southern Appalachian Mountains, the black-bellied salamander and the blue ridge two-lined salamander (*Eurycea* wilderae). They observed that in-stream enclosures that contained both species of salamanders significantly altered macroinvertebrate community structure and reduced taxon richness by *c*. 57% compared to in-stream enclosures with only one species present or when both species were absent. Concluding that changes in salamander assemblages, especially that of the black-bellied and blue ridge salamander, may alter the effects of salamander communities on macroinvertebrates, which may then cascade through food webs to affect stream ecosystem function.

Nonnative Fish Introductions

Freshwater fish are reported as one of the most introduced groups of species throughout the world (Elvira and Almodóvar 2001), with at least 624 freshwater fish species being established in areas outside their natural range since the end of the twentieth century (Gozlan et al. 2010; Toussaint et al. 2016). A common method of dispersal is stocking for sport fishing (Fausch 1996; Rahel and Smith 2018), which may result in an above normal density of predator populations that can have a significant effect on prey abundance, survivorship, and foraging behavior (Kats and Ferrer 2003; Eby et al. 2006). Reduced survivorship and significant population decline may result from an inability to recognize and appropriately respond to novel predators (Sih et al. 2010) Fishes are one of the most dominant predators of amphibians and are commonly predators of eggs, larvae, and adults (Duellman and Trueb 1986; Petranka et al. 1987). Fish in the family Salmonidae are no exception, in that they have been widely introduced throughout the United States during the last century. Salmonids often prey upon amphibians and can have serious negative effects on an established amphibian population. For example, in a recent study, Miró et al. (2018), found that non-native trout negatively impacted four out of the

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six amphibian species they studied, with effects that included increased population fragmentation and decreased survivorship. Additionally, a review of the role introduced predators have played in amphibian declines by Kats and Ferrer (2003) found that 10 out of 19 different native amphibians were impacted negatively by introduced rainbow trout (*Oncorhynchus mykiss*). Some of the effects included decreased larval survivorship, reduced metamorphic size, and habitat use alteration.

Salmonids in Southern Appalachia

The Southern Appalachian brook trout (*Salvelinus fontinalis*) is the only salmonid native to the Southern Appalachian Mountains and has been identified as a genetically separate species from northern populations (Hayes et al. 1996; Galbreath et al. 2001). Logging in the early 1900s decimated brook trout populations by increasing habitat fragmentation and destroying crucial riparian zones, secondarily raising stream temperatures, and creating large silt deposits that inhibited the development of eggs (Kelly et al. 1980). To appeal to anglers since brook trout populations were so low, stocking of nonnative trout in headwaters (1st-3rd order streams) in the Southern Appalachian Mountains occurred, which led to the encroachment by brown trout (*Salmo trutta)*, and especially rainbow trout (*Oncorhynchus mykiss*), into natural brook trout habitat (Galbreath et al. 2001). The diet of both rainbow and brook trout do not differ greatly and primarily consists of aquatic macroinvertebrates, but both will prey upon vertebrates such as small fish and salamanders (Utz and Hartman 2006). It was originally hypothesized that rainbow trout possessed a competitive edge compared to brook trout and was a major contributing factor towards their decline in southern Appalachia (Rose 1986; Nagel and Deaton 1989). However, further studies indicated that this was not the case because after efforts that removed rainbow trout from brook trout habitat, the decline persisted, suggesting that habitat loss and degradation

contribute more towards the decline of brook trout than the nonnative rainbow trout (Hudy et al. 2008). Brook trout are now the subject of wide-spread reintroduction and rehabilitative efforts across the eastern United States in an attempt to halt the decline of their populations (Hudy et al. 2005). One notable restorative effort was the work done on the Left Prong Hampton Creek. In 1997, a log-crib rainbow trout barrier, which was later replaced by 3-meter waterfall in 2007, was placed to isolate brook trout populations in the headwater reaches of the stream from downstream rainbow trout (Habera et al. 2009). Later in 2008, after the waterfall barrier was constructed, efforts to remove all rainbow trout were successful. Since then, annual sampling of the stream suggests that Hampton Creek supports healthy populations of brook trout, with the mean biomass of brook trout in the headwaters being four times larger than the statewide average brook trout biomass in Tennessee (Habera et al. 2016).

Goals of the Study

The aim of this study is to evaluate salmonid predator cue-based recognition and associated avoidance behavior in *D. quadramaculatus* larvae. In order to determine if an innate response plays a role in predator recognition, larvae from stream sections that having both allopatric and sympatric zones of brook and rainbow trout will be tested. Because brook trout are the only native salmonid in Southern Appalachia, it is expected that *D. quadramaculatus* larvae will exhibit an innate response to brook trout but be considered naïve (lack an innate response) to nonnative rainbow trout. Innate recognition of predator cues would be expected in environments that are evolutionarily stable and where interactions between the prey species and its predators have been consistent for many generations (Stephens 1993; Gall and Mathis 2010). Rainbow trout have only recently occurred (<100 years) in Southern Appalachian headwaters, suggesting that an innate response to rainbow trout in larval black-bellied salamanders is unlikely.

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Additionally, this study will evaluate if *D. quadramaculatus* larvae can learn to recognize, through the association of paired alarm and predator cues, a non-native salmonid as a novel predator. Despite lacking defenses during initial introductions to nonnative predators, naïve prey can adjust or acquire recognition of their predators throughout their life (Mathis et al. 1996; Ferrari and Chivers 2008; Ferrari et al. 2010b). This can be accomplished through generalization of predator recognition or through social or associative learning (Mathis et al. 1996; Polo-Cavia and Gomez-Mestre 2014). Larval black-bellied salamanders have the longest larval stage within the genus *Desmognathus* (Petranka 1998). In East Tennessee and Western North Carolina, larval life stages range anywhere from 2-3 years (Petranka 1998). This would likely increase the probability of an encounter with rainbow trout, especially in stream sections that have low numbers of or no brook trout. Additionally, larval and juvenile stages of black-bellied salamanders inhabit the middle sections of streams, likely for increased food availability and to avoid predation by conspecific adults that inhabit the stream edges (Petranka 1998). Also, it has been found that brook trout habitat use is altered in sympatric zones with rainbow trout (Lohr and West 1992). Rainbow trout will inhabit middle sections of the stream and force brook trout to the peripheries. Therefore, with a relatively long larval stage, the likely inability to recognize rainbow trout as a novel predator, and in the increased probability of encountering a rainbow trout; larvae may possess the ability to learn and identify rainbow trout as dangerous and alter their behavior in response to rainbow trout predator cues.

CHAPTER 2: PUBLICATION

PREDATOR-AVOIDANCE OF LARVAL BLACK-BELLIED SALAMANDERS (*DESMOGNATHUS QUADRAMACULATUS*) IN RESPONSE TO CUES FROM NATIVE AND NONNATIVE SALMONIDS

Brian L. Dempsey[a]*, John W. Roden[a], and Joseph R. Bidwell[a]

[a]Biological Sciences, East Tennessee State University, Johnson City, United States

Predator-Avoidance of Larval Black-bellied Salamanders (*Desmognathus quadramaculatus)* in Response to Cues from Native and Nonnative Salmonids

The introduction of nonnative salmonids into Southern Appalachia may pose a threat to resident salamander populations. In recent years, the stocking and encroachment of rainbow trout (Oncorhynchus mykiss) into headwaters where the black-bellied salamander (Desmognathus quadramaculatus) and brook trout (Salvelinus fontinalis) naturally coexist has raised concerns. In aquatic prey, predator-avoidance responses are primarily influenced through the detection of chemical cues released from predators. The objective of this study was to determine how co-occurrence with a predator influences black-bellied salamander predator recognition behavior. To evaluate this, salamander activity metrics (general activity, number of movements, and latency to move) were recorded before and after exposure to either native trout predator cue (brook), introduced trout predator cue (rainbow), or conditioned tap water (control). Larvae were collected from different streams based on their trout predator assemblage with larvae coming from brook, rainbow, rainbow/brook, and no trout stream reaches. Our results show that larvae that co-occur with trout reduced their activity when exposed to brook trout predator cue, but their response to rainbow trout predator cue depended on their previous co-occurrence. Larvae from areas with only brook trout exhibited a weak predator-avoidance when exposed to rainbow trout predator cue. A follow-up test to determine the influence of alarm cue on predator response in these larvae indicated that the alarm cue enhanced the response to the rainbow trout predator.

Keywords**:** *Desmognathus*; Predator-avoidance; predator cue; trout; nonnative predator

Introduction

Introduced fish species represent the most widespread alien predator of amphibians (Stebbins and Cohen 1995; Rahel and Smith 2018). In many cases, fish have been introduced into aquatic habitats to augment or establish a recreational fishery. Both brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*) are cold-water species that have been introduced into the tailwaters of many impounded rivers that provide suitable habitat due to the release of cool hypolimnetic water (Dibble et al. 2015). However, the introduction of these species has been associated with negative impacts on native fauna. A recent study by Miró et al. (2018), found that non-native trout negatively impacted four out of six amphibian species they studied, with effects that included increased population fragmentation and extirpation. Additionally, a review of the role alien predators has played in amphibian decline by Kats and Ferrer (2003) found that out of 19 different native amphibians, 10 of them were impacted

negatively by introduced rainbow trout. Some of the effects included decreased larval survivorship, reduced metamorphic size, and habitat use alteration. This reduced survivorship or significant population decline may result from an inability to recognize and appropriately respond to novel predators (Sih et al. 2010).

At the proximate level, predator-prey interactions are a battle of the senses where predator and prey each have an advantage when they gain information about the other (Ferrari et al. 2010b). For prey to persist with predators, they must be able to recognize and effectively respond to predatory threats (Epp and Gabor 2008), whether that is through avoiding predation through early detection of a predator or employing antipredator behavior to increase its chance of survival during an attack. Prey exhibit a trade-off between exposure to predation risk and energy intake through foraging. The risk of being preyed upon through extended foraging and the risk of reduced energy intake through insufficient foraging to meet metabolic requirements (Lima and Bednekoff 1999; Higginson et al. 2012).

Aquatic prey often detect predators through visual and olfactory cues that are released from potential predators (Ferrari et al. 2010b). Common chemical cues that aquatic prey species respond to have been reviewed by Ferrari et al. (2010b) and include kairomones such as predator cues or the "signature odor" of the predator; disturbance cues which usually result from the release of ammonia or urea by distressed prey; alarm cues which are released by injured prey tissue and provide no other context to prey except there is an actively foraging predator in the vicinity; and dietary cues which derive from the predator's excretory products after consumption of prey. Larval amphibians have been found to respond to kairomones more effectively than visual cues, even in areas with high water clarity (Hickman et al. 2004; Bronmark and Hansson 2012).

Responses to chemical cues can be influenced through learning, diet effects, or can be innate (Ferrari and Chivers 2009). Innate predator recognition by prey species is facilitated through identification of a predator's natural odor, or predator cue (Dixon et al. 2010). Innate predator recognition derives from consistent predator-prey regimes (Wisenden 2003; Gall and Mathis 2010). Naïve prey are those that lack innate predator recognition which makes them more susceptible to a predation event, and predator naivety is considered an important factor in the failure of native prey to respond to nonnative predators (Sih et al. 2010). This was demonstrated by Martin (2014), who exposed both farm-raised and wild-caught red swamp crayfish (*Procambarus clarii*) to chemical cues from largemouth bass (*Micropterus salmoides*). As compared to wild-caught individuals, the naïve farm-raised *P. clarii*, lacked the appropriate predator-avoidance responses, both in terms of movement and use of structural refuge, when exposed to chemical cues from bass. However, some naïve prey can detect nonnative predators. For example, Epp & Gabor (2008) found that captive-reared San Marcos salamanders (*Eurycea nana*) showed innate recognition of cues from the non-native redbreast sunfish (*Lepomis auratus*) and attributed this to the fish being closely related to the natural predator (*Lepomis cyanellus*) of the salamander since closely related species should share a similar chemical signal (Ferrari et al. 2007; 2008). This ability to display anti-predator responses upon a first encounter with a predator for which no innate response exists is known as generalization of predator recognition (Ferrari et al. 2009).

Prey can also learn to recognize an introduced predator through the association of alarm cues from both injured conspecific or heterospecific individuals within their prey-guild, paired with visual or predator cues (Brown 2003; Manassa et al. 2013). Iberian green frog tadpoles (*Rana perezi*) learn to associate a natricine snake (*Natrix maura*), a common predator of adult

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frogs, and a nonpredatory zebra danio fish (*Brachydanio rerio*) as dangerous after a single exposure to paired alarm and predator cues (Gonzalo et al. 2007).

Lastly, predator diet can also affect recognition of introduced and native predators by prey. Diet effects are closely related to alarm cues and are considered a post-digestion alarm cue (Wisenden and Chivers 2006). For example, a study by Nunes et al. (2013) found that 9 different species of larval anurans significantly decreased their activity when exposed to diet cues from a native predator (*Aeshma sp.*) and a nonnative (*Procambarus clarkii*) predator that had both been fed conspecifics.

The Southern Appalachian brook trout (*Salvelinus fontinalis*) is the only native salmonid in the Southern Appalachians. Logging in the early 1900s decimated brook trout populations and an urgency to re-establish recreational fishing led to the stocking of nonnative rainbow trout (Kelly et al. 1980). However, their competitive impact on native brook trout or how they would affect the native amphibian fauna does not appear to have been considered.

The black-bellied salamander (*Desmognathus quadramaculatus*) is a stream-dwelling endemic of the Southern Appalachian region and inhabits high order, cool, large cobble mountain streams anywhere from 375-1,725 m (Huheey 1966; Mills 1996; Petranka 1998). The black-bellied salamander possesses the longest larval stage in the genus *Desmognathus*, lasting up to 3 years in some instances (Petranka 1998). The introduction of rainbow trout into headwater mountain streams has likely increased the encounters between larval black-bellied salamanders and rainbow trout. The degree to which the salamander can recognize the non-native predator is unclear.

In this study, we aimed to determine if larval black-bellied salamanders can innately recognize and respond to rainbow trout predator cue. Also, with the movement of rainbow trout

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into brook trout areas, we aimed to test predator-avoidance responses from larvae inhabiting stream reaches with either one trout predator or both trout predators together. Lastly, we aimed to identify if larval black-bellied salamanders from a brook trout reach can enhance and maintain their response to rainbow trout when exposed to alarm cue and predator cue simultaneously.

Materials and Methods

Larval Collection & Maintenance

Larval collection, maintenance, and testing was carried out under permits from the Tennessee Wildlife Resource Agency (TWRA) (Permit #1947), Tennessee Department of Environment and Conservation (TDEC) (Permit #2019-031), and the East Tennessee State University IACUC (#P190102). Larval black-bellied salamanders were collected via dip-netting/hand-collection from both Hampton Creek Cove (HCC), Carter County, TN and Lamar Alexander Rocky Fork State Park (LARFSP), Unicoi County, TN. Trout population densities in Hampton Creek have been monitored by the TWRA for over two decades (Habera et al. 2016) and based on these data, three sites along the creek were selected to collect larval salamanders based on the type of trout predators present.; the middle sections of Left Prong of Hampton Creek (both rainbow and brook trout) (36.149993, -82.050581), the upper sections of Left Prong Hampton Creek (brook trout only, upstream movement of rainbow trout is inhibited by a 3-m high dam and rainbows were removed in 2008) (36.141499, -82.047049), and the lower sections of Hampton Creek (rainbow trout only) (36.153891, -82.056997). Larvae were also collected from the upper reaches of Long Branch Creek in LARFSP which has no trout present (36.061401, -82.571452). A total of 150 larvae were collected, 90 from HCC (30 from each site) and 30 from LARFSP during July-August 2019. Later, 30 larvae were collected from HCC (brook trout site) during March-April 2020. In the laboratory, larvae from each site were held

separately in 75.7-L tanks filled with moderately hard water (USEPA 2002) and fitted with aerators. The tanks were housed in a temperature-controlled environmental chamber at 15-16°C with a 12:12 Light:Dark cycle. Larvae from the same collection site were further grouped based on snout-vent-length (SVL), to the nearest mm, to avoid the risk of cannibalism. Individuals between 18-30 mm were used as the target size, since metamorphosis in streams of the Southern Appalachians can occur at SVLs between 35-42 mm (Petranka 1998). Larvae were fed a mixture of *Daphnia magna* and *Lumbriculus variegatus* (Carolina Biological Supply Company, Burlington, NC) four days per week and every 2 days 50% water changes occurred to control the accumulation of ammonia. Lastly, larvae were housed and allowed to acclimate to testing conditions for 10-14 days before behavioral testing.

Fish Collection

Wild rainbow and brook trout were obtained by electroshocking from Briar Creek, Washington County, TN (36.2290° N, 82.3820° W) with the assistance of fisheries biologists from the TWRA. Once collected, fish were transported back to the laboratory, separated by species, and monitored for 48-h for signs of injury, disease, or distress in a 378-L aquarium maintained at 16°C by using a Cyclone Drop-in Chiller (Aqua Logic Inc. San Diego, CA) and filled with dechlorinated tap water. Fish were then separated by species and placed in their own 94.6-L aquaria with an aerator. Water was maintained at 16°C using the chiller mentioned above. Fish acclimated for 5-6 days before cue collection and were fed blood worms and brine shrimp *ad libitum* to avoid diet effects. Ammonia, pH, and chlorine was monitored bi-weekly using the Hach Model HA-62 water test kit (Hach Inc. Loveland, CO) with 25% water changes every 10 days. Fish were all housed in a temperature controlled 12:12 Light:Dark room.

Cue Collection

After acclimation to laboratory conditions, 3 individuals of each fish species were weighed to the nearest tenth of a gram using a P-603D Precision Balance (Denver Instrument Company, Bohemia, NY) and placed into their own 5.67-L aquaria with an aerator and dechlorinated tap water at a ratio of 50 mL water per 1.0 g of fish for 24 h. The predator cue was then pooled by species and filtered through 1.5 µm polyester fiber filter (Acurel Filter Fiber, Cranbury, NJ) to remove solid particles and then separated into 30 mL aliquots. All stimuli were held at -6°C in Falcon 50mL Conical Centrifuge Tubes (Fisher Scientific, Waltham, MA) and used within 8 weeks. Control cue (dechlorinated tap water) was also generated using the same methods.

Alarm cue from *D. quadramaculatus* was prepared following Mirza et al. (2006) by sacrificing 3-5 larvae to get a concentration of roughly 1g of salamander mass per 100 mL of dechlorinated tap water. Larvae were chilled for 15 minutes to induce a hypothermic state and then dispatched by cranial compression (AVMA, 2013). Whole larvae were then macerated with a mortar and pestle using enough dechlorinated tap water to maintain a concentration of 1g of salamander mass per 100mL of dechlorinated tap water. Alarm cue mixture was then filtered to remove solid particles and then immediately frozen at -6°C in Falcon 15 mL Conical Centrifuge Tubes (Fisher Scientific, Waltham, MA).

Predator Cue Recognition

The experimental procedure used to evaluate larval recognition of predator cues was modified from Chapman (2017). The behavioral chamber was a glass 5.7-L aquarium (15.24 cm x 25.4 cm) containing 1.0-L of moderately hard water. The chamber was surrounded with black

plastic to minimize external visual influences. Airline tubing (0.5 cm diameter) served as the port

for introducing stimulus into the tank and was glued to the side and bottom of the tank in such

extended 10 cm along the bottom. All testing was done in complete darkness between 2100-0200

in a 16°C temperature-controlled room.

Prior to testing, larvae were randomly selected from their holding tanks and randomly

placed in the behavioural chamber for 10-min before the start of the experiment. After

acclimation, the pre-stimulus phase was initiated by drawing two 25 mL aliquots of test water

(via the airline tubing described above) from the behavioral chamber using two separate 30-mL

syringes. The first aliquot of water was discarded, while the syringe containing the second

aliquot was set aside. A third 30 mL syringe was used to inject 25 mL of dechlorinated tap water

into the behavior chamber, immediately after which the contents of the second syringe was

injected back into the chamber. After the pre-stimulus period was completed, the post-stimulus

phase was initiated by again removing two 25 mL aliquots of water from the behavioural

chamber as described above. However, in this phase 25 mL of a random treatment (dechlorinated

tap water (control), brook trout cue, or rainbow trout cue) was injected into the chamber,

immediately followed by injection of 25 mL of behavioural chamber water back into the tank to

disperse the treatment solution.

After both the pre and post-stimulus injection process, an additional 1-min acclimation

period was allowed for complete dispersion of stimulus. Larval behaviour during both 8-minute

phases was observed using 2 Defender 4K (8MP) Wired Security System infrared cameras

(Defender Phoenixm2, Niagara Falls, ON) with the recordings assessed after the experiment by

an observer who was blind to the treatment groups. Larval behaviors evaluated included: Time

spent active: the total time the larvae spent moving during the testing period, Latency to move:

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time spent motionless until first movement, and total number of movements: a movement was defined as a single period of activity, regardless how active the individual was. After completion of an individual behavior trial, the chamber and tubing were cleaned with hot tap water for 2 minutes, followed by rinsing with cold moderately hard water for 1 minute to flush out any residual cue. A total of 90 larvae from HCC (30 from each site) and 30 larvae from LARFSP were tested.

Conditioned Response

The objective of this experiment was to determine if larval black-bellied salamanders exhibited an enhanced response to rainbow trout cue when they had previous exposure to an alarm/predator cue combination. The experimental procedure closely followed that described above, including use of the same behavioral chambers, stimulus injection procedure, pre and post-stimulus periods, use of cameras to record behaviour and behaviour assessment. The key differences were that this assay was conducted over a 5-day period (3 days of testing separated by 1 day without testing) and larvae used in this experiment were obtained from the brook trout section of HCC and were separated into 'control' and 'experimental' groups with 15 larvae tested in each group.

The pre-stimulus cue was always 20 mL dechlorinated tap water, but the post-stimulus cue varied by treatment group and day. For the post-stimulus treatment on Day 1, individuals in both control and experimental groups were exposed to 20 mL dechlorinated tap water to establish baseline activity. On Day 3, individuals in the experimental group were exposed to 10 mL of predator cue and 10 mL of alarm cue, and the control group were exposed to 10 mL of predator cue and 10 mL of dechlorinated tap water. On Day 5, both control and experimental

groups were exposed 10 mL of predator cue and 10 mL of dechlorinated tap water in the post-stimulus phase. Cleaning of chambers to remove residual cue followed that described above.

Statistical Analysis

All behavioural data were evaluated using residual plots and Shapiro-Wilks' tests and were found to meet the assumptions of normality and homoscedasticity. For the study of predator cue recognition, a one-way analysis of variance (ANOVA) followed by a Tukey's HSD post hoc test was employed for each activity metric per location, with the post-stimulus period subtracted from the pre-stimulus period to control for individual animal behavior. The analysis was performed using SAS 9.4M6 (SAS Institute, Cary, NC, USA) software.

For the experiment examining the combination of rainbow trout and alarm cue, values for the post-stimulus behavioral metrics were again subtracted from the pre-stimulus values and a two-way repeated measures ANOVA followed by a Tukey's HSD post-hoc test was used to observe the change in the behaviour metrics between the control and experimental groups over the 3-day testing period. The analysis was performed using SigmaPlot 11. All statistical comparisons were performed at $\alpha=0.05$.

Results

Experiment 1: Determining predator cue recognition

In this experiment, we evaluated predator cue recognition and associated predator-avoidance behavior in larval *D. quadramaculatus* inhabiting stream reaches with different trout predators. Larvae from the stream reach with only brook trout exhibited significant differences between the cue treatments in time spent active ($F_{2,27} = 3.80$, p = 0.0352) and number of movements ($F_{2,27} = 5.80$, p = 0.0080), but not in latency to move ($F_{2,27} = 0.32$, P = 0.7272) (**Fig 1 a-c**). Both activity and number of movements were significantly reduced as compared to the

control in larvae exposed to the brook trout cue. These metrics were also reduced in larvae exposed to the rainbow trout cue, but the effect was not significantly different from the control, indicating there was a stronger response to the brook trout cue.
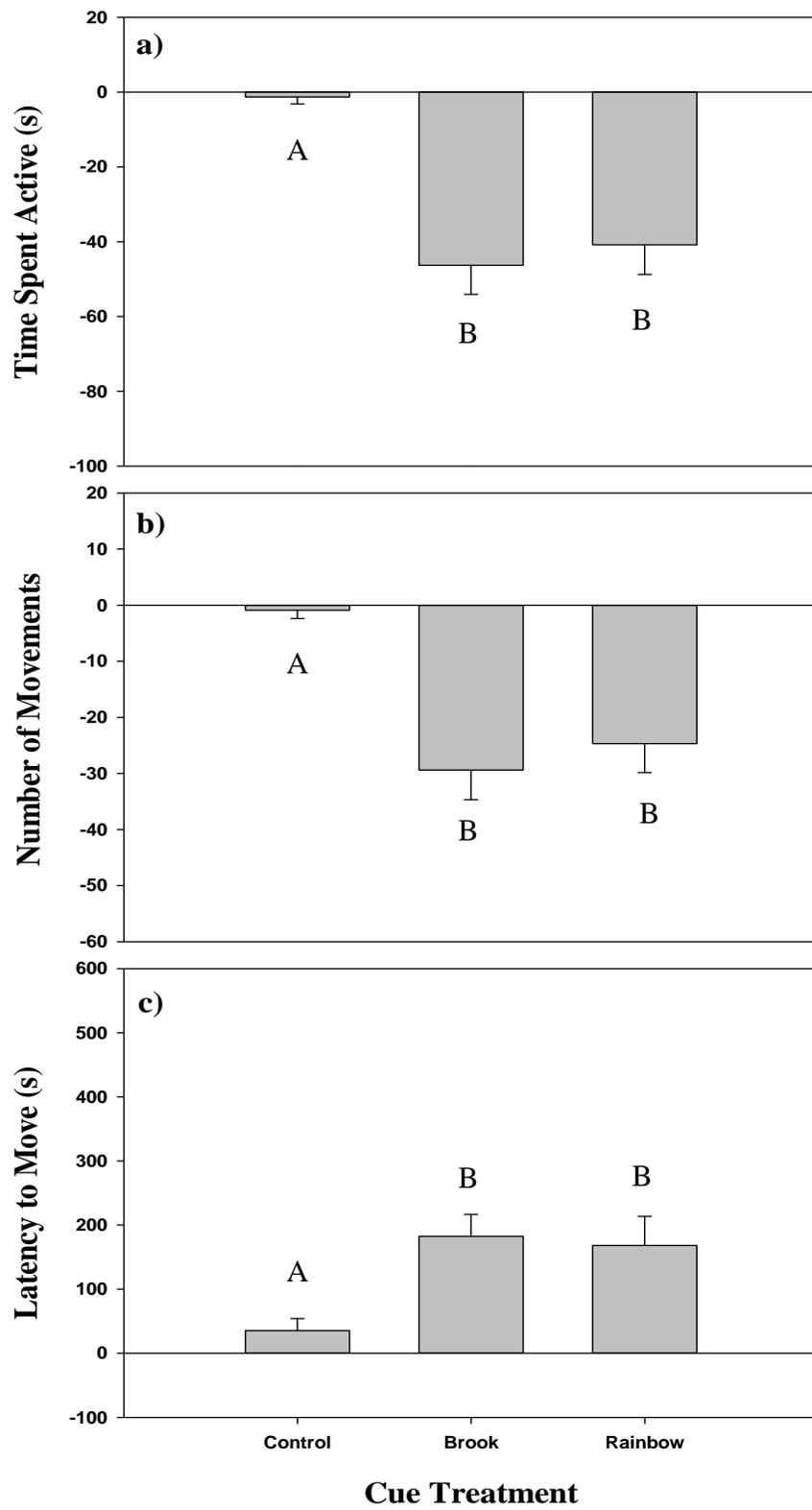
Figure 1
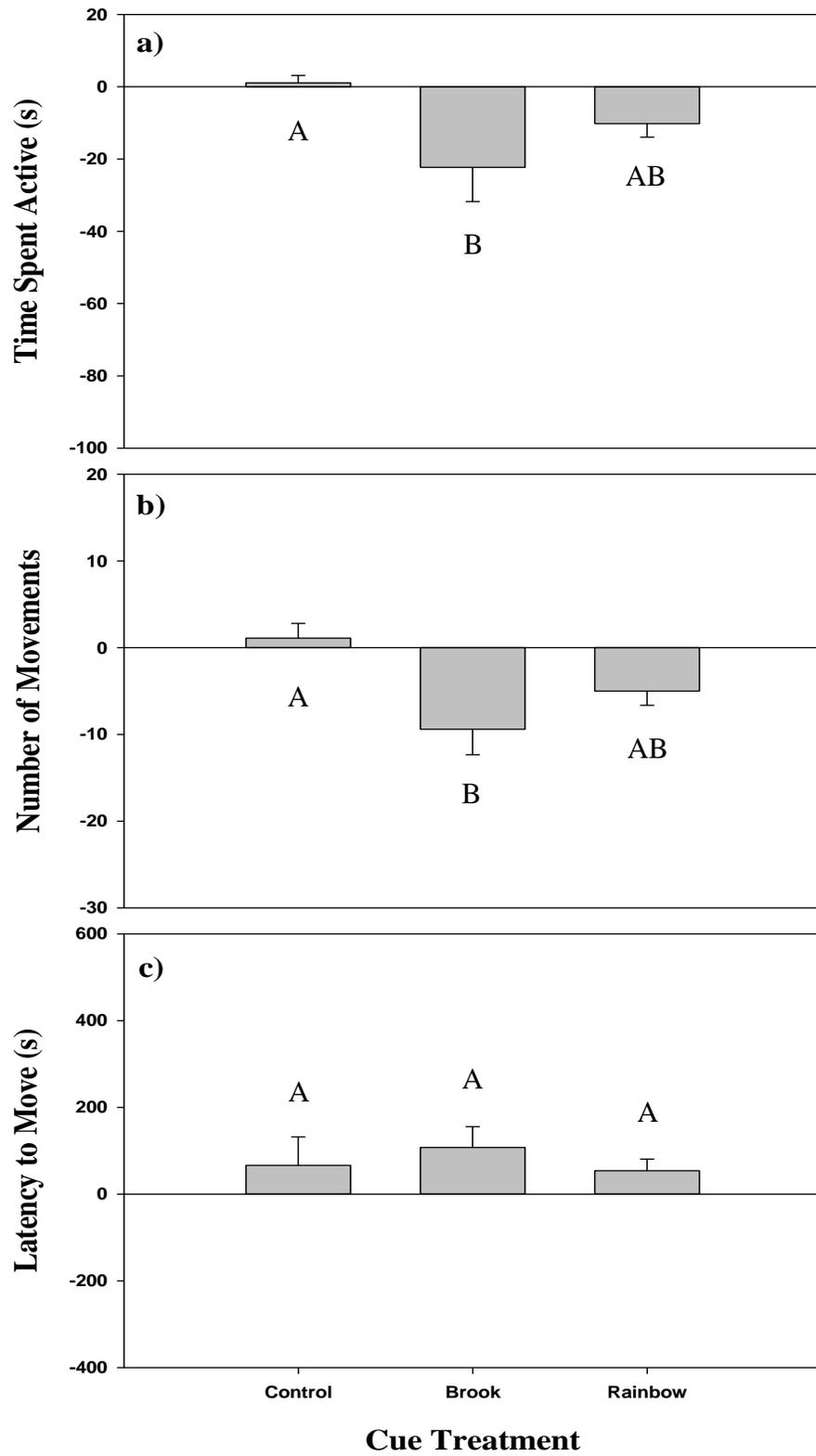
For the rainbow trout reach, larvae exhibited significant differences between the cue treatments in time spent active ($F_{2,27} = 8.66$, p = 0.0012), number of movements ($F_{2,27} = 11.35$, p = 0.0003), and latency to move ($F_{2,27} = 4.82$, p = 0.0162) (**Fig 2 a-c**). Both activity and number of movements were significantly reduced as compared to the control in larvae exposed to both rainbow trout cue and brook trout cue, indicating there was an equal predator-avoidance response to both brook trout and rainbow trout cue. Latency to move was also reduced in larvae exposed to the rainbow trout cue, but the effect did not lead to a significant difference from the control or brook trout cue.

Figure 2

Figure 2
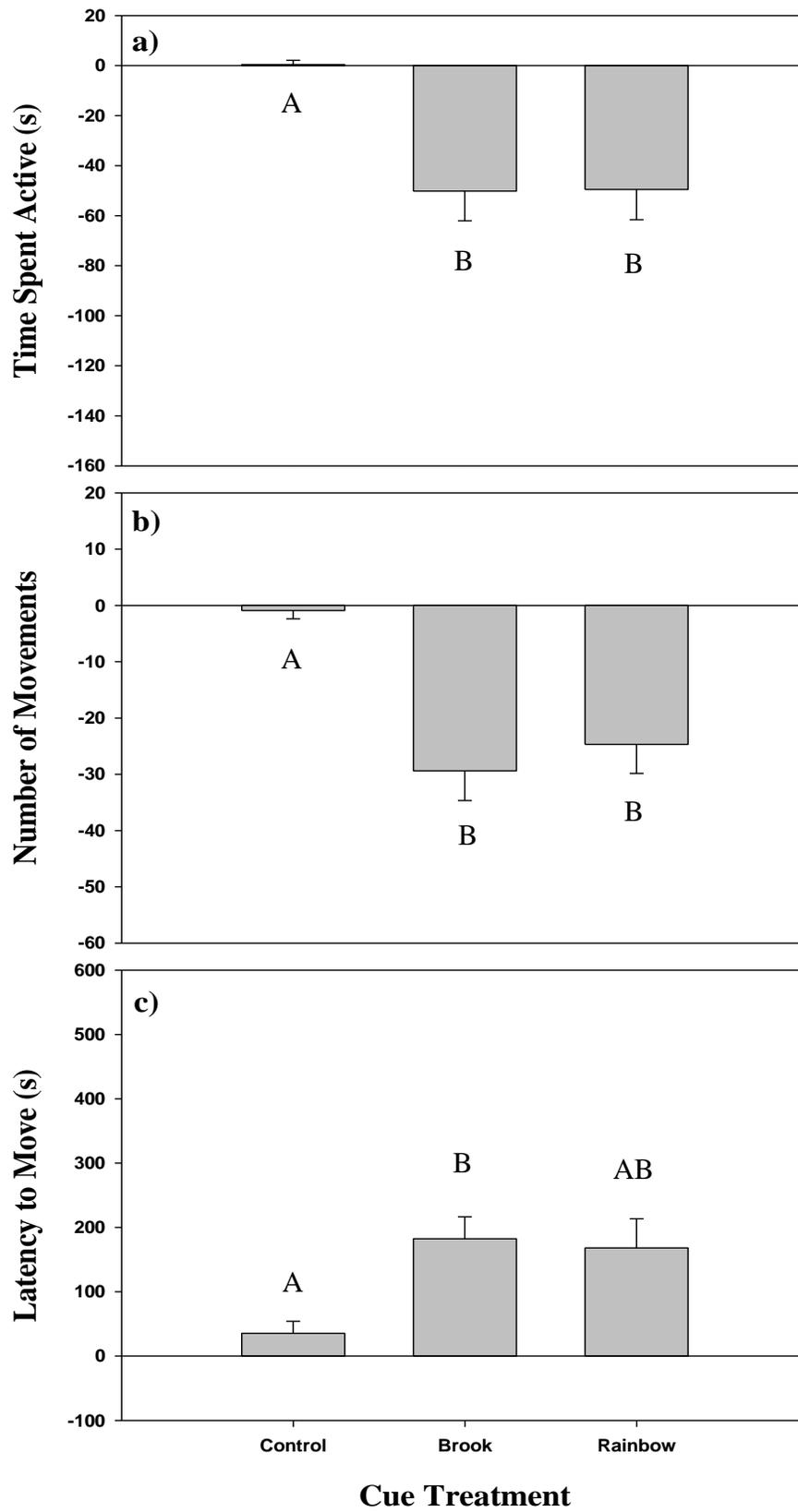
For the rainbow and brook trout reach, larvae exhibited significant differences between the cue treatments in time spent active ($F_{2,27} = 14.34$, $p < 0.0001$), number of movements ($F_{2,27} = 12.40$, $p = 0.0002$), and latency to move ($F_{2,27} = 5.52$, $p = 0.0098$) (**Fig 3 a-c**). All activity metrics were significantly reduced as compared to the control in larvae exposed to both rainbow trout cue and brook trout cue, indication there was a strong equal predator-avoidance response to both brook trout and rainbow trout cue.

Figure 3
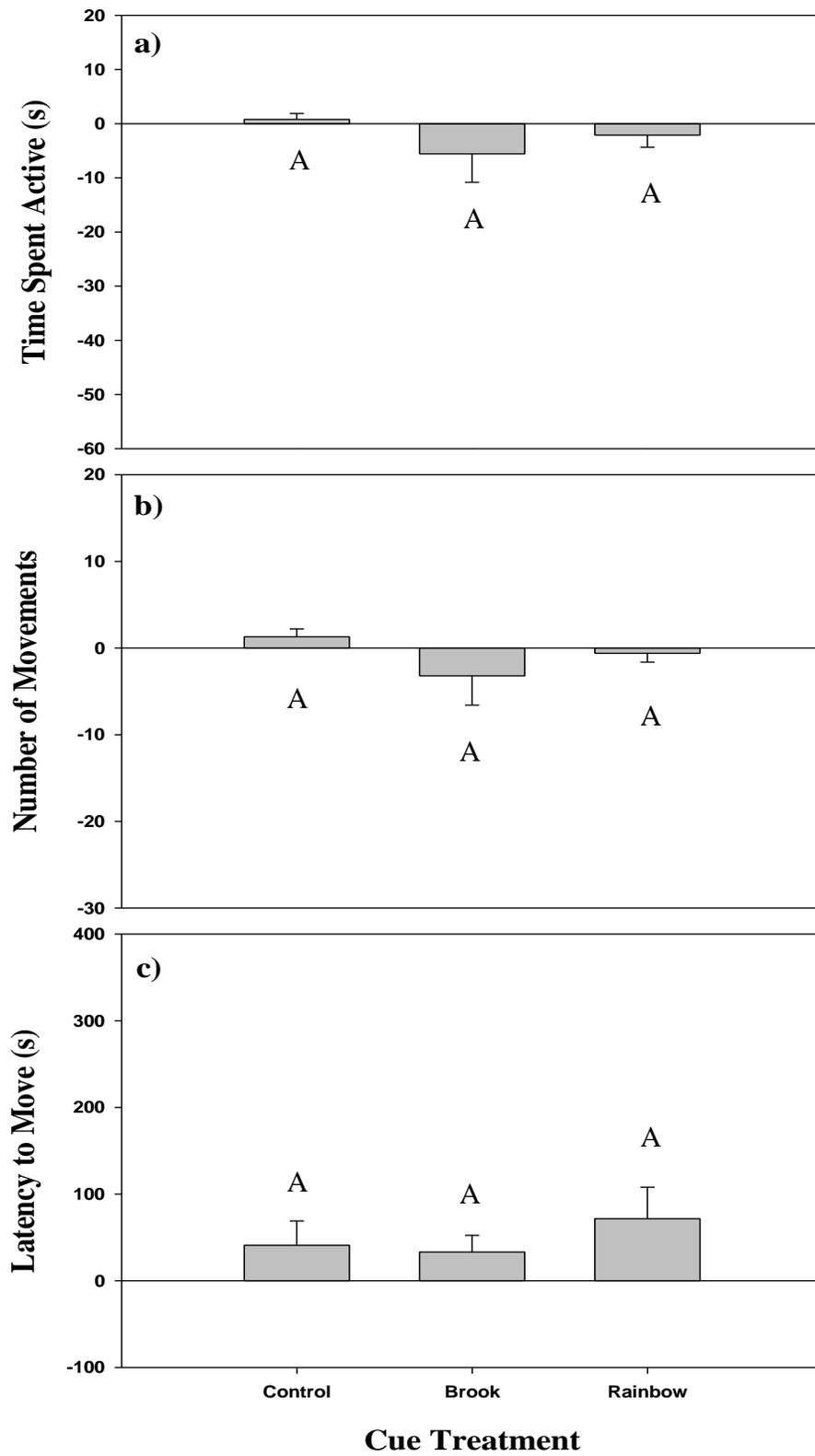
For the no trout reach, larvae exhibited no significant differences between the cue treatments in time spent ($F_{2,27} = 1.19$, $p = 0.3184$), number of movements ($F_{2,27} = 1.14$, $p = 0.3334$), and latency to move ($F_{2,27} = 0.50$, $p = 0.6116$) (**Fig 4 a-c**).

Figure 4

Experiment 2: Determining Conditioned Response

The objective of this experiment was to determine if pairing alarm cue and predator cue from rainbow trout enhances the response of naïve larval *D. quadramaculatus* from brook trout reaches to the latter.

Over the 3 days of testing in both the control and experimental treatment groups, there was a significant interaction between the effects of the treatment and the day for time spent active ($F_{2,27} = 5.985$, p = 0.004) and for number of movements ($F_{2,27} = 5.307$, p = 0.008). For latency to move, there was no significant interaction between the effects of the treatment and the day; although, there were still significant differences within the treatment groups and days. On day 1, for all activity metrics, there was no significant difference within or between the treatment groups (**Fig. 5**). This was expected since all larvae were exposed to only control cue. On day 3, for all activity metrics, there was a significant difference within and between the treatments groups as well a significant difference in relation to day 1. Larvae in the experimental group exhibited a significantly stronger response than those in the control group (**Fig. 5**), indicating that the paired alarm and predator cue stimulus elicits a stronger predator-avoidance response than predator cue alone. On day 5 there was no significant difference between treatment groups, but for within treatment groups larvae in both groups exhibited a significant reduction in relation to day 1 (**Fig. 5**). However, larvae in the control group did not vary in any activity metric between day 3 and day 5 (**Fig. 5**). For the experimental group, both time spent moving and number of movements were significantly different from what was recorded for day 3, with latency to move not changing.

Figure 5
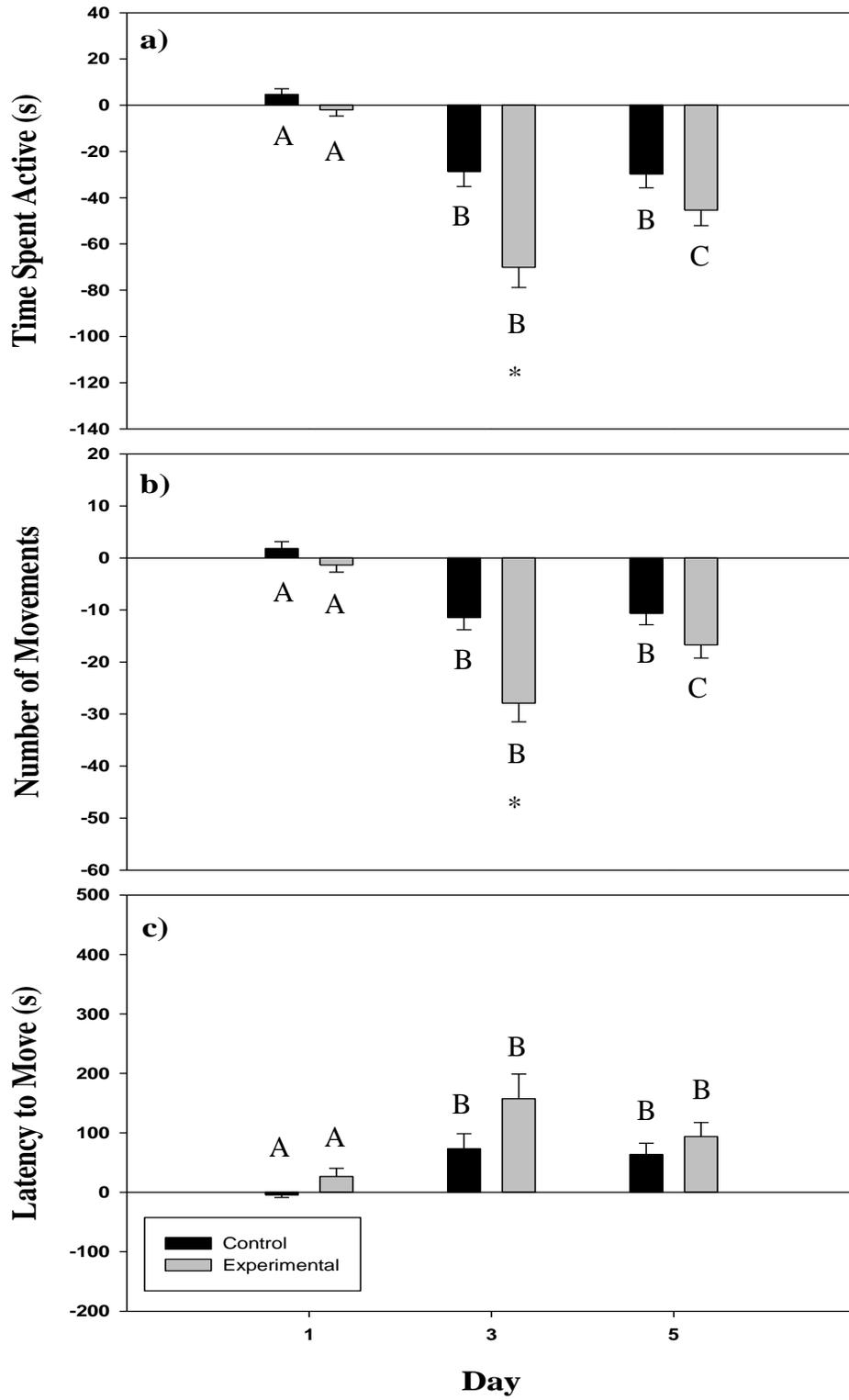
Discussion

The objective of the first experiment was to determine if a difference exists in the ability of larval black-bellied salamanders to recognize predator cue from native brook trout versus nonnative rainbow trout. The hypothesis was that larval black-bellied salamanders would lack predator recognition to the nonnative rainbow trout due to their extremely short period of evolutionary time as predator and prey. The results from the first experiment weakly supported the hypothesis. Larvae from the brook trout reach did not significantly differ in their response to rainbow trout when compared to the control. However, it appears they are slightly responsive to the cue and that there might be a partial learning component, but also with some support for a predator generalization response.

This weak response by brook trout reach larvae to nonnative rainbow trout (**Fig. 1**) could be the result of the relatively limited evolutionary time the salamanders and rainbow trout have co-occurred. Behavioral responses to predation risk are strong where predator-prey interactions have co-evolved (Ferrari et al. 2010b, Watkins 1996), and attacks by novel predators result in either reduced or ineffective responses by native prey due to the lack of prior experience (Schlaepfer et al. 2005). Like the present study, Gall and Mathis (2010) found that larval eastern hellbenders (*Cryptobranchus alleganiensis*) responded to cues from nonnative salmonids when compared to a blank control but not nearly as strongly as they responded to native predatory fish. They attributed this weak response to the relatively small amount of co-occurrence that is required for innate recognition. Alternatively, the difference in trout predator response observed in this experiment could be a result of prey experiencing a low-risk predator environment. Studies have shown that inexperienced fish and larval amphibians in low-risk environments do not respond to novel kairomones but those in high-risk environments do (Brown et al. 2013,

Brown et al. 2014, Chivers et al. 2014). The encroachment of rainbow trout into brook trout habitat of Hampton Creek has only recently occurred and rainbow trout were removed via electroshocking in 2008 (Habera et al. 2016). As such, larvae in this reach of the stream may not encounter rainbow trout regularly and so identify them as a low-risk predator.

Larvae from reaches with rainbow trout and reaches with both trout species strongly reduced their activity to trout predators (**Fig. 2 and Fig. 3**). Larval Iberian frogs (*Rana iberica*) that coexist with native and nonnative salmonids have also been observed to exhibit decreased activity to cue from both salmonid predators (Polo-Cavia et al. 2020). Like black-bellied salamanders, these larval Iberian frogs lacked a strong evolutionary relationship with their nonnative salmonid predator but still possessed the innate ability to detect and avoid a novel predator. Since these larvae were collected from streams and not captive-reared, it is expected they would have some level of risk assessment experience that could influence their perception of predators. It has been found that innate responses can be fine-tuned by predator experience. Epp and Gabor (2008) found that wild caught (experienced) *Eurycea nana* exhibited diminished responses to a nonnative predator when compared to naïve *E. nana.* However, it was suggested that the wild caught individuals may have refined responses to predators given their experience. Therefore, it is possible that larval black-bellied salamanders that occur in reaches with rainbow trout are affected by their experience with the native predator and generalize their response to the nonnative predator. Predator cue generalization can occur when a novel predator is a member of the same genus as the native predator and this has been found to diminish with increasing phylogenetic difference between the two (Ferrari et al. 2016). For example, fathead minnows (*Pimephales promelas*) generalize predator cue when exposed to brown trout and novel rainbow trout, both in the family Salmonidae (Ferrari et al. 2010a), and *E. nana* responded to predator cue

from a novel non-centrarchid cichlid (*Herichthys cyanoguttatum*) that is a member of the same family as its native fish predator (Davis et al. 2012). Additionally, woodfrog tadpoles (*Lithobates sylvaticus*), respond to rainbow trout (learned predator) and brown trout (native) more strongly than the distantly related brook trout (Davis et al. 2012). Because rainbow trout are phylogenetically closer to brown trout (Stearley and Smith 1993) it is likely that larval black-bellied salamanders are generalizing rainbow trout with the native brook trout.

The lack of predator avoidance response to both brook and rainbow trout in larval salamanders in the reach with no trout predators was expected. Anti-predator behavior is only expected to be maintained if it provides net fitness benefits. Otherwise it is costly in terms of time and access to resources (Sih et al. 2010, Anson and Dickman 2013). The loss or removal of a strong selective force imposed by a predator may therefore lead to the relaxation of predator recognition and associated anti-predator behavior in prey (Lahti et al. 2009, Wertheim et al. 2015). The history of salmonids in this reach is unknown. However, larvae that inhabit this stream do not currently encounter trout predators. If salmonid predators previously occurred here it would be disadvantageous to possess antipredator behaviors towards a predator that does not occur in the system. Antipredator behavior may vary based on predator-prey overlap, with predation pressure not being consistent along species distributional ranges, resulting in variation in antipredator behavior (Foster 1999). For example, Garcia et al. (2012) found that American bullfrog (*Lithobates catesbeianus*) tadpoles varied in their response to a historical predator, largemouth bass (*Micropterus salmoides*). They found that tadpoles from populations that co-occur with bass exhibited antipredator behavior (increased refuge use) while individuals that did not co-occur failed to exhibit any antipredator behavior towards chemical cues from the bass or chemical cues with diet cues (largemouth bass fed bullfrog tadpoles).

For the present study, a reduction in activity was used to determine if individuals are displaying predator-avoidance behavior when exposed to kairomones. Freezing or decreasing activity is a common predator-avoidance mechanism that is used in several amphibian species, especially salamanders (Petranka 1998; Davis and Gabor 2015). Therefore, any individuals that decreased their activity when exposed to kairomones were considered to be displaying predator-avoidance behavior. Both time spent moving and number of movements complimented each other and produced almost identical results for each reach. However, latency to move produced high variation, particularly in the brook trout reach. It was initially thought that the variation in latency to move was the result of an underlying response or behavior. However, upon further inspection, the variation in response to latency to move could be the result of an experimental artifact related to the way the cue was introduced into the behavioral chamber. During the observational period, the stimulus tube would sometimes release air bubbles into the chamber. This disturbance elicited different responses from the larvae in that they either oriented themselves towards it or it caused them to flee. This occurred in every trial at least once but occurred the most frequently in experiments with the brook trout reach larvae. Therefore, this experimental artifact likely influenced the latency to move activity metric and should not be as strongly considered as the other two.

The objective of the second experiment was to determine if larval black-bellied salamanders from the brook trout reach would exhibit an enhanced response to rainbow trout after they had been exposed to rainbow trout predator cue combined with alarm cue. The hypothesis was that larvae would decrease their activity when exposed to alarm cue and predator cue as compared to exposure to predator cue alone and would continue that response once alarm cue was removed. The results from this experiment did not support the hypothesis entirely since

the addition of alarm cue did reduce activity in larvae, but that heightened response was not maintained once alarm cue was removed.

On day 1, both treatment groups were exposed to control cue and did not significantly differentiate in activity from one another. For day 3, the addition of alarm cue caused a significant decrease in activity in the experimental group compared to the control group. This was not surprising because alarm cues should invoke stronger behavioral responses to avoid predation since damaged epidermal tissues are released in no other context and advertise the presence of an actively foraging predator (Ferrari et al. 2010b). This has been demonstrated by Kenison et al. (2018) in studies exposing rusty crayfish (*Orconectes rusticus*) to salamander and fish predator cues and conspecific alarm cues. They found that antipredator responses were magnified when crayfish were exposed to predator cues in combination with conspecific alarm cues compared to just predator cue. On day 5, both treatment groups were subjected to rainbow trout predator cue and there was no significant difference between them. Thus, while alarm cue may have strengthened the response on day 3, this response was not maintained on day 5, indicating the larvae did not "learn" from the single exposure event on day 3 and still depended on the presence of alarm cue to exhibit an increased predator avoidance response. It is possible that a single conditioning event was not enough to induce a learned response. Prey must continually update their perception of risk and adjust their behavior in response to predation risk because it can fluctuate widely across space and time (Lucon-Xiccato et al. 2016). Exposure to a single predation event does not always inform prey about the risk of being attacked in the future because predation risk may be assessed based on the frequency of events over longer periods of time (background risk) (Lucon-Xiccato et al. 2016). As a result, it seems background risk often has a large effect in determining future antipredator decisions (Lima et al. 1999, Brown et al.

2014). Lucon-Xiccato et al. (2016) found that wood frog tadpoles (*Lithobates sylvaticus*) exposed to alarm cues from conspecifics raised in a high-risk environment exhibited a stronger antipredator response and an enhanced learned response to novel predators as compared to tadpoles exposed to alarm cues from conspecifics raised in a low-risk environment. Black-bellied salamanders in rainbow/brook trout and rainbow trout reaches are experiencing a nonnative predator more frequently than larvae from the brook trout reach. This difference in predator frequency and level of risk may be the reason why larvae showed a weak predator-avoidance response in the first experiment and did not maintain an enhanced response to rainbow trout predator cue once alarm cue was removed in the second. It could also be that more than one exposure event may be required for larvae to maintain a heightened response to nonnative rainbow trout. For example, Vilhunen (2006) found that conditioning Arctic char (*Salvelinus alpinus*) with predator cue and alarm cue increased their chances of survival, with Arctic char that had experienced multiple conditioning events having a higher chance of survival as compared to Arctic char that had only received one conditioning event. For black-bellied salamanders, further work studying alarm cue and predator cue response in reaches with rainbow trout may be needed to verify this.

Conclusion

The results suggest that larvae are employing predator generalization of nonnative rainbow trout with native brook trout and their response is likely influenced by predator background risk and experience. However, the possibility of a learned response to rainbow trout predators in larval *D. quadramaculatus* is not completely excluded. The differences observed in predator-avoidance responses to rainbow trout predator cue and the associated trout predator background risk and experience compliments the idea that prey have tuned their predator

generalization responses to rainbow trout. However, further work studying the possibility of learned predator recognition in truly naïve individuals and predator conditioning through exposure to multiple predation events may be needed to rule out the possibility of learned predator recognition and confirm predator generalization.

Acknowledgements

Declaration of Interest

The Authors declare that there is no conflict of interest.

References

Anson JR, Dickman CR. 2013. Behavioral responses of native prey to disparate predators: naiveté and predator recognition. *Oecologia*, *171*(2), pp.367-377.

AVMA. (2013). American Veterinary Medical Association (AVMA) guidelines on euthanasia. https://www.avma.org/KB/Policies/Documents/euthanasia.pdf.

Brönmark C, Hansson LA, eds., 2012. *Chemical ecology in aquatic systems*. Oxford

    University Press.

Brown, GE. 2003. Learning about danger: chemical alarm cues and local risk assessment in

    prey fishes. *Fish and Fisheries*, *4*(3), pp.227-234.

Brown GE, Ferrari MC, Elvidge CK, Ramnarine I, Chivers DP. 2013. Phenotypically plastic

    neophobia: a response to variable predation risk. *Proceedings of the Royal Society*

    B: Biological Sciences, *280*(1756), p.20122712.

Brown GE, Elvidge CK, Ramnarine I, Ferrari MC, Chivers DP. 2015. Background risk and

    recent experience influences retention of neophobic responses to predators. *Behavioral*

    Ecology and Sociobiology, *69*(5), pp.737-745.

Chapman TL, Spivey KL, Lundergan JM, Schmitz AL, Bast DL, Sehr EK, Gall BG. 2017. Only

    fear the fatal foe: predation risk assessment by eastern newts (*Notophthalmus*

    viridescens) in response to common snapping turtles and other potential

    predators. *Ethology Ecology & Evolution*, *29*(3), pp.218-228.

Chivers DP, McCormick MI, Mitchell MD, Ramasamy RA, Ferrari MC. 2014.

    Background level of risk determines how prey categorize predators and non-

    predators. *Proceedings of the Royal Society B: Biological Sciences*, *281*(1787),

    p.20140355.

Davis DR, Epp KJ, Gabor CR. 2012. Predator Generalization Decreases the Effect of

    Introduced Predators in the San Marcos Salamander, *Eurycea nana*. *Ethology*, *118*(12),

    pp.1191-1197.

Davis DR, Gabor CR. 2015. Behavioral and physiological antipredator responses of the San

    Marcos salamander, *Eurycea nana*. *Physiology & Behavior*, *139*, pp.145-149.

Dibble KL, Yackulic CB, Kennedy TA, Budy P. 2015. Flow management and fish density

    regulate salmonid recruitment and adult size in tailwaters across western North

    America. *Ecological Applications*, *25*(8), pp.2168-2179.

Dixson DL, Munday PL, Jones GP. 2010. Ocean acidification disrupts the innate ability of fish

    to detect predator olfactory cues. *Ecology letters*, *13*(1), pp.68-75.

Epp KJ, Gabor CR. 2008. Innate and learned predator recognition mediated by chemical signals

    in Eurycea nana. *Ethology*, *114*(6), pp.607-615.

Ferrari MC, Gonzalo A, Messier F, Chivers DP. 2007. Generalization of learned predator

    recognition: an experimental test and framework for future studies. *Proceedings of the*

    Royal Society B: Biological Sciences, *274*(1620), pp.1853-1859.

Ferrari MC, Messier F, Chivers DP. 2008. Can prey exhibit threat-sensitive generalization

    of predator recognition? Extending the predator recognition continuum

    hypothesis. *Proceedings of the Royal Society B: Biological Sciences*, *275*(1644),

    pp.1811-1816.

Ferrari MC, Chivers DP. 2009. Temporal variability, threat sensitivity and conflicting

    information about the nature of risk: understanding the dynamics of tadpole antipredator

    behavior. *Animal Behaviour*, *78*(1), pp.11-16.

Ferrari MC, Lysak KR, Chivers DP. 2010a. Turbidity as an ecological constraint on learned

    predator recognition and generalization in a prey fish. *Animal Behaviour*, *79*(2),

    pp.515-519.

Ferrari MC, Wisenden BD, Chivers DP. 2010b. Chemical ecology of predator–prey

    interactions in aquatic ecosystems: a review and prospectus. *Canadian Journal of*

    Zoology, *88*(7), pp.698-724.

Ferrari MC, Crane AL, Chivers DP. 2016. Certainty and the cognitive ecology of generalization of predator recognition. *Animal Behaviour*, *111*, pp.207-211.

Foster, S.A. and Endler, J.A. eds., 1999. *Geographic variation in behavior: perspectives on evolutionary mechanisms*. Oxford University Press on Demand.

Garcia TS, Thurman LL, Rowe JC, Selego SM. 2012. Antipredator behavior of American bullfrogs (*Lithobates catesbeianus*) in a novel environment. *Ethology*, *118*(9), pp.867-875.

Gall BG, Mathis A. 2010. Innate predator recognition and the problem of introduced trout. *Ethology*, *116*(1), pp.47-58.

Gonzalo A, López P, Martín J. 2007. Iberian green frog tadpoles may learn to recognize novel predators from chemical alarm cues of conspecifics. *Animal Behaviour*, *74*(3), pp.447-453.

Habera JW, Bivens RD, Carter BD, Williams CE. 2009. Region IV Trout Fisheries Report No. 09-01. Tennessee Wildlife Resources Agency. Nashville, Tennessee.

Habera JW, Bivens RD, Carter BD, Williams CE. 2016. Region IV Trout Fisheries Report No. 17-02. Tennessee Wildlife Resources Agency. Nashville, Tennessee.

Hickman CR, Stone MD, Mathis A. 2004. Priority use of chemical over visual cues for detection of predators by graybelly salamanders, Eurycea multiplicata griseogaster. *Herpetologica*, *60*(2), pp.203-210.

Higginson AD, Fawcett TW, Trimmer PC, McNamara JM, Houston AI. 2012. Generalized optimal risk allocation: foraging and antipredator behavior in a fluctuating environment. *The American Naturalist*, *180*(5), pp.589-603.

Huheey, JE. 1966. The desmognathine salamanders of the Great Smoky Mountains National

   Park. *Journal of the Ohio Herpetological Society*, pp.63-72.

Kats LB. Ferrer RP. 2003. Alien predators and amphibian declines: review of two decades of

   science and the transition to conservation. *Diversity and distributions*, *9*(2), pp.99-110.

Kelly GA, Griffith JS, Jones RD. 1980. *Changes in distribution of trout in Great Smoky*

   Mountains National Park, 1900-1977 (Vol. 102). US Department of the Interior, Fish and

   Wildlife Service.

Kenison EK, Weldy PY, Williams RN. 2018. There must be something in the water:

   assessing the behavioral responses of rusty crayfish (Orconectes rusticus) to fish and

   amphibian predator kairomones. *Journal of ethology*, *36*(1), pp.77-84.

Lahti DC, Johnson NA, Ajie BC, Otto SP, Hendry AP, Blumstein DT, Coss RG, Donohue K,

   Foster SA. 2009. Relaxed selection in the wild. *Trends in ecology & evolution*, *24*(9),

   pp.487-496.

Lima SL, Bednekoff PA. 1999. Temporal variation in danger drives antipredator behavior: the

   predation risk allocation hypothesis. *The American Naturalist*, *153*(6), pp.649-659.

Lucon-Xiccato T, Chivers DP, Mitchell MD, Ferrari MC. 2016. Making the dead talk: alarm

   cue-mediated antipredator behavior and learning are enhanced when injured conspecifics

   experience high predation risk. *Biology letters*, *12*(8), p.20160560.

Manassa RP, Dixson DL, McCormick MI, Chivers DP. 2013. Coral reef fish incorporate

   multiple sources of visual and chemical information to mediate predation risk. *Animal*

   Behaviour, 86, p.717–722.

Martin, CW. 2014. Naïve prey exhibit reduced antipredator behavior and

   survivorship. *PeerJ*, *2*, p.e665.

Mills, GR. 1996. A study on the life history and seasonal foraging habits of the salamander

      Desmognathus quadramaculatus Holbrook, in WV.

Miró A, Sabás I, Ventura M. 2018. Large negative effect of non-native trout and minnows on

      Pyrenean lake amphibians. *Biological Conservation*, *218*, pp.144-153.

Nunes AL, Richter-Boix A, Laurila A, Rebelo R. 2013. Do anuran larvae respond behaviorally

      to chemical cues from an invasive crayfish predator? A community-wide

      study. *Oecologia*, *171*(1), pp.115-127.

Petranka, JW. 1998. Salamanders of the United States and Canada Smithsonian. *Washington,*

      DC.

Polo-Cavia N, Boyero L, Martín-Beyer B, Navazo T, Bosch J. 2020. Effects of coexistence and

      predator experience on antipredatory responses of montane amphibian larvae towards

      native and introduced salmonids. *Biological Invasions*, *22*(2), pp.379-390.

Sih A, Bolnick DI, Luttbeg B, Orrock JL, Peacor SD, Pintor LM, Preisser E, Rehage JS, Vonesh

      JR. 2010. Predator–prey naïveté, antipredator behavior, and the ecology of predator

      invasions. *Oikos*, *119*(4), pp.610-621.

Stearley RF, Smith GR. 1993. Phylogeny of the Pacific trouts and salmons

      (*Oncorhynchus*) and genera of the family Salmonidae. *Transactions of the American*

      Fisheries Society, *122*(1), pp.1-33.

Stebbins RC, Cohen NW. 1995. Declining amphibians. *A Natural history of amphibians*,

      pp.210-251.

Schlaepfer MA, Sherman PW, Blossey B, Runge MC. 2005. Introduced species as

      evolutionary traps. *Ecology Letters*, *8*(3), pp.241-246.

Smith, RJF. 1992. Alarm signals in fishes. *Reviews in Fish Biology and Fisheries*, *2*(1), pp.33-63.

United States. Environmental Protection Agency, 2002. *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms* 5th ed., Washington, DC.

Vilhunen, S. 2006. Repeated antipredator conditioning: a pathway to habituation or to better avoidance?. *Journal of Fish Biology*, *68*(1), pp.25-43.

Watkins, TB. 1996. Predator-mediated selection on burst swimming performance in tadpoles of the Pacific tree frog, *Pseudacris regilla*. *Physiological Zoology*, *69*(1), pp.154-167.

Wertheim JO, Murrell B, Smith MD, Kosakovsky-Pond SL, Scheffler K. 2015. RELAX: detecting relaxed selection in a phylogenetic framework. *Molecular biology and evolution*, *32*(3), pp.820-832.

Wisenden, BD. 2003. Chemically mediated strategies to counter predation. In *Sensory processing in aquatic environments* (pp. 236-251). Springer, New York, NY.

Wisenden BD, Chivers DP. 2006. The role of public chemical information in antipredator behavior. *Communication in fishes*, *1*, pp.259-278.

Figure legends

1. **Figure 1.** Mean ± SE a) time spent active (s), b) number of movements, and c) Latency to move (s) for larval *D. quadramaculatus* from a brook trout reach exposed to control cue, brook cue, and rainbow cue. Responses not connected by the same letter are significantly different (α = 0.05).

2. **Figure 2.** Mean ± SE a) time spent active (s), b) number of movements, and c) Latency to move (s) for larval *D. quadramaculatus* from a rainbow trout reach exposed to control cue, brook cue, and rainbow cue. Responses not connected by the same letter are significantly different (α = 0.05).

3. **Figure 3.** Mean ± SE a) time spent active (s), b) number of movements, and c) Latency to move (s) for larval *D. quadramaculatus* from a brook + rainbow trout reach exposed to control cue, brook cue, and rainbow cue. Responses not connected by the same letter are significantly different ($\alpha = 0.05$).

4. **Figure 4.** Mean ± SE a) time spent active (s), b) number of movements, and c) Latency to move (s) for larval *D. quadramaculatus* from no trout reach exposed to control cue, brook cue, and rainbow cue. Responses not connected by the same letter are significantly different ($\alpha = 0.05$).

5. **Figure 5.** Mean ± SE a) time spent active (s), b) number of movements, and c) Latency to move (s) for larval *D. quadramaculatus* from a brook trout reach. Black is the control group and grey is the experimental group. Responses not connected by the same letter are significantly different within treatment groups ($\alpha = 0.05$) and responses with separated by an asterisk (*) are significantly different between groups ($\alpha = 0.05$).

REFERENCES

Anson JR, Dickman CR. 2013. Behavioral responses of native prey to disparate predators:

naiveté and predator recognition. *Oecologia*, *171*(2), pp.367-377.

AVMA. (2013). American Veterinary Medical Association (AVMA) guidelines on euthanasia.

https://www.avma.org/KB/Policies/Documents/euthanasia.pdf.

Barrett, HC. 2015. Adaptations to predators and prey. *The handbook of evolutionary

psychology*, pp.1-18.

Brönmark C, Hansson LA, eds., 2012. *Chemical ecology in aquatic systems*. Oxford

University Press.

Brown, GE. 2003. Learning about danger: chemical alarm cues and local risk assessment in

prey fishes. *Fish and Fisheries*, *4*(3), pp.227-234.

Brown GE, Ferrari MC, Elvidge CK, Ramnarine I, Chivers DP. 2013. Phenotypically plastic

neophobia: a response to variable predation risk. *Proceedings of the Royal Society

B: Biological Sciences*, *280*(1756), p.20122712.

Brown GE, Elvidge CK, Ramnarine I, Ferrari MC, Chivers DP. 2015. Background risk and

recent experience influences retention of neophobic responses to predators. *Behavioral

Ecology and Sociobiology*, *69*(5), pp.737-745.

Bruce, RC. 1996. Life-history perspective of adaptive radiation in desmognathine

salamanders. *Copeia*, pp.783-790.

Camp CD, Lee TP. 1996. Intraspecific spacing and interaction within a population of

Desmognathus quadramaculatus. *Copeia*, pp.78-84.

Chapman TL, Spivey KL, Lundergan JM, Schmitz AL, Bast DL, Sehr EK, Gall BG. 2017. Only

fear the fatal foe: predation risk assessment by eastern newts (*Notophthalmus

viridescens) in response to common snapping turtles and other potential predators. *Ethology Ecology & Evolution*, *29*(3), pp.218-228.

Chivers DP, McCormick MI, Mitchell MD, Ramasamy RA, Ferrari MC. 2014. Background level of risk determines how prey categorize predators and non-predators. *Proceedings of the Royal Society B: Biological Sciences*, *281*(1787), p.20140355.

Conor Keitzer S, Goforth RR. 2013. Salamander diversity alters stream macroinvertebrate community structure. *Freshwater Biology*, *58*(10), pp.2114-2125.

Davic, RD. 1984. An investigation of salamander guild predation in a North Carolina stream: an experimental approach.

Davic RD, Welsh Jr HH. 2004. On the ecological roles of salamanders. *Annu. Rev. Ecol. Evol. Syst.*, *35*, pp.405-434.

Davis DR, Epp KJ, Gabor CR. 2012. Predator Generalization Decreases the Effect of Introduced Predators in the San Marcos Salamander, *Eurycea nana*. *Ethology*, *118*(12), pp.1191-1197.

Davis DR, Gabor CR. 2015. Behavioral and physiological antipredator responses of the San Marcos salamander, *Eurycea nana*. *Physiology & Behavior*, *139*, pp.145-149.

Dibble KL, Yackulic CB, Kennedy TA, Budy P. 2015. Flow management and fish density regulate salmonid recruitment and adult size in tailwaters across western North America. *Ecological Applications*, *25*(8), pp.2168-2179.

Dixson DL, Munday PL, Jones GP. 2010. Ocean acidification disrupts the innate ability of fish to detect predator olfactory cues. *Ecology letters*, *13*(1), pp.68-75.

Duellman WE, Trueb L. 1986. Biology of Amphibians McGraw-Hill. *New York*.

Eby LA, Roach WJ, Crowder LB, Stanford JA. 2006. Effects of stocking-up freshwater food webs. *Trends in ecology & evolution*, *21*(10), pp.576-584.

Elvira B, Almodóvar A. 2001. Freshwater fish introductions in Spain: facts and figures at the beginning of the 21st century. *Journal of fish Biology*, *59*, pp.323-331.

Epp KJ, Gabor CR. 2008. Innate and learned predator recognition mediated by chemical signals in Eurycea nana. *Ethology*, *114*(6), pp.607-615.

Ferrari MC, Gonzalo A, Messier F, Chivers DP. 2007. Generalization of learned predator recognition: an experimental test and framework for future studies. *Proceedings of the Royal Society B: Biological Sciences*, *274*(1620), pp.1853-1859.

Ferrari MC, Messier F, Chivers DP. 2008. Can prey exhibit threat-sensitive generalization of predator recognition? Extending the predator recognition continuum hypothesis. *Proceedings of the Royal Society B: Biological Sciences*, *275*(1644), pp.1811-1816.

Ferrari MC, Chivers DP. 2009. Temporal variability, threat sensitivity and conflicting information about the nature of risk: understanding the dynamics of tadpole antipredator behavior. *Animal Behaviour*, *78*(1), pp.11-16.

Ferrari MC, Lysak KR, Chivers DP. 2010a. Turbidity as an ecological constraint on learned predator recognition and generalization in a prey fish. *Animal Behaviour*, *79*(2), pp.515-519.

Ferrari MC, Wisenden BD, Chivers DP. 2010b. Chemical ecology of predator–prey interactions in aquatic ecosystems: a review and prospectus. *Canadian Journal of Zoology*, *88*(7), pp.698-724.

Ferrari MC, Crane AL, Chivers DP. 2016. Certainty and the cognitive ecology of generalization of predator recognition. *Animal Behaviour*, *111*, pp.207-211.

Foster, S.A. and Endler, J.A. eds., 1999. *Geographic variation in behavior: perspectives on evolutionary mechanisms*. Oxford University Press on Demand.

Garcia TS, Thurman LL, Rowe JC, Selego SM. 2012. Antipredator behavior of American bullfrogs (*Lithobates catesbeianus*) in a novel environment. *Ethology*, *118*(9), pp.867-875.

Galbreath PF, Adams ND, Guffey SZ, Moore CJ, West JL. 2001. Persistence of native southern Appalachian brook trout populations in the Pigeon River system, North Carolina. *North American Journal of Fisheries Management*, *21*(4), pp.927-934.

Gall BG, Mathis A. 2010. Innate predator recognition and the problem of introduced trout. *Ethology*, *116*(1), pp.47-58.

Gozlan RE, Britton JR, Cowx I, Copp GH. 2010. Current knowledge on non-native freshwater fish introductions. *Journal of fish biology*, *76*(4), pp.751-786.

Gonzalo A, López P, Martín J. 2007. Iberian green frog tadpoles may learn to recognize novel predators from chemical alarm cues of conspecifics. *Animal Behaviour*, *74*(3), pp.447-453.

Habera JW, Bivens RD, Carter BD, Williams CE. 2009. Region IV Trout Fisheries Report No. 09-01. Tennessee Wildlife Resources Agency. Nashville, Tennessee.

Habera JW, Bivens RD, Carter BD, Williams CE. 2016. Region IV Trout Fisheries Report No. 17-02. Tennessee Wildlife Resources Agency. Nashville, Tennessee.

Hayes JP, Guffey SZ, Kriegler FJ, McCracken GF, Parker CR. 1996. The genetic diversity of native, stocked, and hybrid populations of brook trout in the southern Appalachians. *Conservation Biology*, *10*(5), pp.1403-1412.

Hickman CR, Stone MD, Mathis A. 2004. Priority use of chemical over visual cues for detection of predators by graybelly salamanders, Eurycea multiplicata griseogaster. *Herpetologica*, *60*(2), pp.203-210.

Higginson AD, Fawcett TW, Trimmer PC, McNamara JM, Houston AI. 2012. Generalized optimal risk allocation: foraging and antipredator behavior in a fluctuating environment. *The American Naturalist*, *180*(5), pp.589-603.

Hudy M, Thieling TM, Gillespie N, Smith EP. 2005 Distribution, status, and threats to brook trout within the eastern United States. Trout Unlimited for the Eastern Brook Trout Joint Venture. 1-37.

Hudy M, Thieling TM, Gillespie N, Smith EP. 2008. Distribution, status, and land use characteristics of subwatersheds within the native range of brook trout in the eastern United States. North American Journal of Fisheries Management. 28: 1069-1085.

Huheey, JE. 1966. The desmognathine salamanders of the Great Smoky Mountains National Park. *Journal of the Ohio Herpetological Society*, pp.63-72.

Kats LB. Ferrer RP. 2003. Alien predators and amphibian declines: review of two decades of science and the transition to conservation. *Diversity and distributions*, *9*(2), pp.99-110.

Kelly GA, Griffith JS, Jones RD. 1980. *Changes in distribution of trout in Great Smoky Mountains National Park, 1900-1977* (Vol. 102). US Department of the Interior, Fish and Wildlife Service.

Kenison EK, Litt AR, Pilliod DS, McMahon TE. 2016. Larval long-toed salamanders incur

nonconsumptive effects in the presence of nonnative trout. *Ecosphere*, *7*(5), p.e01258.

Kenison EK, Weldy PY, Williams RN. 2018. There must be something in the water:

assessing the behavioral responses of rusty crayfish (Orconectes rusticus) to fish and

amphibian predator kairomones. *Journal of ethology*, *36*(1), pp.77-84.

Lahti DC, Johnson NA, Ajie BC, Otto SP, Hendry AP, Blumstein DT, Coss RG, Donohue K,

Foster SA. 2009. Relaxed selection in the wild. *Trends in ecology & evolution*, *24*(9),

pp.487-496.

Lohr SC, West JL. 1992. Microhabitat selection by brook and rainbow trout in a southern

Appalachian stream. *Transactions of the American Fisheries Society*, *121*(6), pp.729-736.

Lima SL, Dill LM. 1990. Behavioral decisions made under the risk of predation: a review and

prospectus. *Canadian journal of zoology*, *68*(4), pp.619-640.

Lima SL, Bednekoff PA. 1999. Temporal variation in danger drives antipredator behavior: the

predation risk allocation hypothesis. *The American Naturalist*, *153*(6), pp.649-659.

Lucon-Xiccato T, Chivers DP, Mitchell MD, Ferrari MC. 2016. Making the dead talk: alarm

cue-mediated antipredator behavior and learning are enhanced when injured conspecifics

experience high predation risk. *Biology letters*, *12*(8), p.20160560.

Manassa RP, Dixson DL, McCormick MI, Chivers DP. 2013. Coral reef fish incorporate

multiple sources of visual and chemical information to mediate predation risk. *Animal

Behaviour, 86*, p.717–722.

Martin, CW. 2014. Naïve prey exhibit reduced antipredator behavior and

survivorship. *PeerJ*, *2*, p.e665.

Mathis A, Chivers DP, Smith RJF. 1996. Cultural transmission of predator recognition in fishes: intraspecific and interspecific learning. *Animal Behaviour*, *51*(1), pp.185-201.

Mills, GR. 1996. A study on the life history and seasonal foraging habits of the salamander Desmognathus quadramaculatus Holbrook, in WV.

Miró A, Sabás I, Ventura M. 2018. Large negative effect of non-native trout and minnows on Pyrenean lake amphibians. *Biological Conservation*, *218*, pp.144-153.

Mirza RS, Ferrari MC, Kiesecker JM, Chivers DP. 2006. Responses of American toad tadpoles to predation cues: behavioral response thresholds, threat-sensitivity and acquired predation recognition. *Behaviour*, *143*(7), pp.877-889.

Nagel JW, Deaton JE. 1989. Growth and longevity of rainbow trout in two headwater streams in northeastern Tennessee. *Journal of the Tennessee Academy of Science*, *64*(1), pp.9-12.

Nunes AL, Richter-Boix A, Laurila A, Rebelo R. 2013. Do anuran larvae respond behaviorally to chemical cues from an invasive crayfish predator? A community-wide study. *Oecologia*, *171*(1), pp.115-127.

Peterman WE, Crawford JA, Semlitsch RD. 2008. Productivity and significance of headwater streams: population structure and biomass of the black-bellied salamander (*Desmognathus quadramaculatus*). *Freshwater Biology*, *53*(2), pp.347-357.

Petranka JW, Kats LB, Sih A. 1987. Predator-prey interactions among fish and larval amphibians: use of chemical cues to detect predatory fish. *Animal Behaviour*, *35*(2), pp.420-425.

Petranka, JW. 1998. Salamanders of the United States and Canada Smithsonian. *Washington, DC*.

Polo-Cavia N, Gomez-Mestre I. 2014. Learned recognition of introduced predators determines survival of tadpole prey. *Functional Ecology*, *28*(2), pp.432-439.

Polo-Cavia N, Boyero L, Martín-Beyer B, Navazo T, Bosch J. 2020. Effects of coexistence and predator experience on antipredatory responses of montane amphibian larvae towards native and introduced salmonids. *Biological Invasions*, *22*(2), pp.379-390.

Rahel FJ, Smith MA. 2018. Pathways of unauthorized fish introductions and types of management responses. *Hydrobiologia*, *817*(1), pp.41-56.

Rose, GA. 1986. Growth decline in sub-yearling brook trout (Salvelinus fontinalis) after emergence of rainbow trout (Salmo gairdneri). *Canadian Journal of Fisheries and Aquatic Sciences*, *43*(1), pp.187-193.

Schlaepfer MA, Sherman PW, Blossey B, Runge MC. 2005. Introduced species as evolutionary traps. *Ecology Letters*, *8*(3), pp.241-246.

Sih A, Bolnick DI, Luttbeg B, Orrock JL, Peacor SD, Pintor LM, Preisser E, Rehage JS, Vonesh JR. 2010. Predator–prey naïveté, antipredator behavior, and the ecology of predator invasions. *Oikos*, *119*(4), pp.610-621.

Sih A, Ferrari MC, Harris DJ. 2011. Evolution and behavioral responses to human-induced rapid environmental change. *Evolutionary applications*, *4*(2), pp.367-387.

Smith, RJF. 1992. Alarm signals in fishes. *Reviews in Fish Biology and Fisheries*, *2*(1), pp.33-63.

Stearley RF, Smith GR. 1993. Phylogeny of the Pacific trouts and salmons (*Oncorhynchus*) and genera of the family Salmonidae. *Transactions of the American Fisheries Society*, *122*(1), pp.1-33.

Stebbins RC, Cohen NW. 1995. Declining amphibians. *A Natural history of amphibians*, pp.210-251.

Stephens, DW. 1993. Learning and behavioral ecology: incomplete information and environmental predictability. In *Insect learning* (pp. 195-218). Springer, Boston, MA.

Toussaint A, Beauchard O, Oberdorff T, Brosse S, Villéger S. 2016. Worldwide freshwater fish homogenization is driven by a few widespread non-native species. *Biological Invasions*, *18*(5), pp.1295-1304.

United States. Environmental Protection Agency, 2002. *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms* 5th ed., Washington, DC.

Utz RM, Hartman KJ. 2006. Temporal and spatial variation in the energy intake of a brook trout (Salvelinus fontinalis) population in an Appalachian watershed. *Canadian Journal of Fisheries and Aquatic Sciences*, *63*(12), pp.2675-2686.

Vilhunen, S. 2006. Repeated antipredator conditioning: a pathway to habituation or to better avoidance?. *Journal of Fish Biology*, *68*(1), pp.25-43.

Watkins, TB. 1996. Predator-mediated selection on burst swimming performance in tadpoles of the Pacific tree frog, *Pseudacris regilla*. *Physiological Zoology*, *69*(1), pp.154-167.

Wertheim JO, Murrell B, Smith MD, Kosakovsky-Pond SL, Scheffler K. 2015. RELAX: detecting relaxed selection in a phylogenetic framework. *Molecular biology and evolution*, *32*(3), pp.820-832.

Wisenden, BD. 2003. Chemically mediated strategies to counter predation. In *Sensory processing in aquatic environments* (pp. 236-251). Springer, New York, NY.

Wisenden BD, Chivers DP. 2006. The role of public chemical information in antipredator

behavior. *Communication in fishes*, *1*, pp.259-278

BRIAN DEMPSEY

Education:	B.S. Biology, East Tennessee State University, Johnson City, Tennessee, 2018
	M.S. Biology, East Tennessee State University, Johnson City, Tennessee, 2020
Professional Experience:	Research Facilitator, Ronald E. McNair Program East Tennessee State University; Johnson City, Tennessee, 2020
	Graduate Assistant, East Tennessee State University, College of Arts and Sciences, 2018-2020
Honors and Awards:	Graduate Studies Research Grant
	School of Graduate Studies, East Tennessee State University, 2020