Investigating rapid divergence of sensory systems between satellite populations of the Mexican tetra (*Astyanax mexicanus*)

A THESIS
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

Dr. Allen F. Mensinger, Advisor

January 2022

Acknowledgements

This work would have not been possible without the support and mentorship of Dr. Allen F. Mensinger. In particular, I would like to thank Dr. Mensinger not only for providing opportunities to further my scientific career, but also for teaching me to be a confident scientist. I would additionally like to thank my committee members Dr. Amanda Klein and Dr. Thomas Hrabik for their guidance throughout the writing process. I would like to thank Dr. Suzanne McGaugh for her collaboration on this exciting project. I would also like to share a special thanks to Dr. Ted Ozersky and his willingness to share statistical advice while on sabbatical.

There are many people I have met while completing my master's whom I want to extend my deepest gratitude for providing support and community. Thank you to Dr. Rosalyn Putland, Noland Michels, Emily Fleissner, Jackie Culotta and Lily Hall for being wonderful lab mates in the Mensinger lab. Special thanks to Amanda Kistner and Ti Tait for being my first undergraduate mentees and providing data for this thesis. Thank you to the IBS 2019 Cohort for unforgettable moments and memories, and thanks to Stormi Davis-Hagadone and Dr. Jennifer Liang for nurturing this graduate community.

I would like to acknowledge all the educators in my earlier education who supported my curiosity; I would not have gotten here without them. I want to acknowledge my wonderful partner, Derek Arrowood. This work would not have happened without his unwavering support and kindness. I would also like to acknowledge my brother Rae Enriquez for providing laughter and levity throughout the difficult parts of my degree progress. Last but certainly not least, I would like to thank the Enriquez Family – Ruben and Miriam Enriquez – for their love and fullhearted faith in me as I pursue my career as a scientist.

Dedication

This thesis is dedicated to the late Mary Martinez Enriquez and Sonia Medina Hurtado Vda. de Soto.

Abstract

The Mexican tetra (*Astyanax mexicanus*) has two primary ecotypes: cave fish and surface fish. Cave fish are characterized by troglomorphic phenotypes, such as vestigial eyes and reduced pigmentation. Studies have documented phenotypic differences in these ecotypes, which likely diverged between 0.2 to 1 million years ago. However, surface *A.mexicanus* fish were introduced relatively recently to the Edwards-Trinity aquifer in Texas in the early 1900s, and subsequent cave colonization by portions of this population show evidence of divergence through rapid phenotypic and behavioral evolution. The establishment of these satellite populations from cave and surface river invasions are a case study into the rapid evolution of traits within a new environment, allowing observation on how sensory systems may adapt in real time. Auditory evoked potentials (AEPs), particle acceleration (PAL) and electroretinography (ERG) assays were conducted to quantify sensory differences between satellite cave and surface populations. Honey Creek cave fish were found to be significantly more sensitive ($p < 0.05$) than Honey Creek surface fish to sound pressure levels between 0.5 kHz - 0.8 kHz, while some pairwise differences were found between San Antonio Zoo surface, Blue Hole cave and San Pedro Springs cave populations between 0.5 kHz - 0.7kHz ($p < 0.05$). Particle acceleration assays also showed significant differences between Honey Creek cave and surface ($p < 0.05$) as well as San Antonio Zoo surface, San Pedro Springs cave and Blue Hole cave ($p < 0.05$) within the same range of frequencies tested. Electroretinography data indicated that Honey Creek cave fish were significantly less sensitive ($p < 0.05$) to light than Honey Creek surface fish at 530 nm, while no differences were found between San Antonio Zoo surface, San Pedro Springs cave and Blue Hole cave. Collectively, these results indicate rapid divergence of *A.mexicanus* in cave habitats at the most sensitive ranges of their visual and auditory sensory systems, and future monitoring may demonstrate continual divergence of sensory systems in populations exposed to new environments.

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Chapter 1

Two Worlds, One Species: An Introduction to *Astyanax mexicanus*

Introduction

The purpose of this thesis is to provide background and describe research conducted on recently diverged populations of the Mexican tetra, *A.mexicanus*. While several studies have characterized differences between cave and surface ecotypes within their native range, this is the first characterization of sensory differences between a recently diverged population of this species within a non-native range. In addition to reporting study results, this work aims to place the findings within the context of rapid evolution, and how further monitoring of these populations may add to the corpus of rapid evolution in novel environments.

1.1 Natural History of *A.mexicanus*

Astyanax mexicanus, commonly known as the Mexican tetra, is a characiform fish native to central and southern Mexico, with a northern range extending to the lower Rio Grande and Nueces rivers (A. Gluesenkamp, pers. comm). The most notable characteristic of this fish is that there are two primary ecotypes and morphologies: cave fish and surface fish. The cave ecotype of this species, which is characterized by troglomorphic features, such as lack of pigmentation and vestigial eyes, was initially discovered in the late 1930s and incorrectly given the species distinction *Anoptichthys jordani* (C. L. Hubbs & Innes, 1936). Eventually, the cave ecotype was recognized as within the genus *Astyanax* after breeding experiments revealed cave forms could reproduce with surface forms, and crossed individuals could produce viable offspring (Şadoğlu, 1957). The exact taxonomy of the cave ecotype is highly disputed: studies have used a variety of names to describe the species, including *Astyanax fasciatus* (Gregson & Perera, 2007; Wilkens, 2010), *Astyanax hubbsi* (Teyke & Schaerer, 1994), and *Astyanax* *jordani* (Boudriot & Reutter, 2001; Strecker et al., 2012). Still others propose the separation of *A.mexicanus* into more than 15 separate species based on mitochondrial DNA differences (Ornelas-García et al., 2008). However, it is widely accepted that the cave ecotype of *A.mexicanus* is the same species as the surface ecotype.

The story of cavefish is both of evolution and geology. *Speleogenesis* is a term that describes the geological formation of cave systems (Engel, 2011). Understanding the process by which caves form can be valuable in determining the relative amount of time a population has been present in a given area, which is especially salient when describing the divergence and speciation of cave organisms (Avise & Selander, 1972; Fumey et al., 2018). While several invertebrate species are documented to exclusively inhabit caves, the only vertebrate groups that have successfully colonized cave systems are salamanders and fish (Soares & Niemiller, 2013). Cave fish are found on every continent except Antarctica (Behrmann-Godel et al., 2017; Soares & Niemiller, 2013), and their range typically coincides with the location of Karst cave systems (Soares & Niemiller, 2013). Karst cave systems form when acidified rain begins to dissolve soluble rock, such as limestone, creating cavernous networks underground (Niemiller & Soares, 2015). Over time, these networks can become vast, extending up to 100m into the ground, and typically include structures such as sinkholes, sinking streams, and caves (Engel, 2011). *A.mexicanus* cave colonization may have been aided by these geologic features, where ancestral fish could have fallen in and become trapped within the caves (Gross, 2012).

There are significant challenges that organisms living in subterranean environments face, for which the cave ecotype of *A.mexicanus* is well suited. Within caves, organisms must contend with factors such as the absence of light, low nutrient availability

(Niemiller & Soares, 2015; Soares & Niemiller, 2013; Wilson et al., 2021), and in some cases, anoxic conditions (Boggs & Gross, 2021). Nutrient availability is governed by both the lack of light, which significantly shapes cave food webs, as well as isolation, as a result of cave geography (Protas & Jeffery, 2012). Additionally, being that circadian rhythms are cued by light availability, cave *A.mexicanus* have gone through significant adaptation shifts in response to dark environments within caves (Beale et al., 2013; Mack et al., 2021).

Cave ecotype *A.mexicanus* have adapted to these environments through both constructive and regressive changes in phenotypic features (Jeffery, 2001; Porter & Crandall, 2003; Wilkens, 2010). Constructive changes refer to enhancements or novel adaptations in response to environmental pressures, while regressive changes refer to a diminishing of already present adaptations/structures in order to conserve energy in an environment where they are not needed. The most significant regressive changes include lack of pigmentation, the loss of aggressive behavior in comparison to surface ecotypes, the loss of functional eyes/visual systems, and reduction in circadian-mediated processes (Borowsky, 2016; Iwashita & Yoshizawa, 2021; Jeffery, 2001). Constructive changes, on the other hand, include increased development of sensory systems alternate to vision (i.e., olfactory, neuromast), increased response to vibrational stimuli, greater body fat content, and increased egg size (Borowsky, 2016; Jeffery, 2001; Porter & Crandall, 2003; Wilkens, 2010; Yoshizawa et al., 2015).

Within Mexico, there are 30 known populations of *A.mexicanus* found in different cave and surface river systems (Espinasa et al., 2018). The exact amount of time cave and surface populations have been isolated from each other remains unknown, but several genetic studies have attempted to narrow down the periods of migration of *A.mexicanus* into native cave systems. Microsatellite and mitochondrial DNA has been used to isolate three independent colonization events of surface-type fish from South America to the native range of *A.mexicanus* (Strecker et al, 2012; Gross et al, 2012), with each invasion leading to colonization of both surface and cave river systems. The first two invasions, commonly referred to as the "old stock" ancestral lineage, invaded the El Abra region first approximately 8 MYa, then again around 3 MYa (Gross, 2012). This first invasion is thought to have exclusively seeded the cave populations and gone extinct in surface environments, while the second invasion seeded both cave and surface populations (Bradic et al., 2013; Strecker et al., 2012). The third invasion, designated as "new stock" ancestral lineage, invaded the western Micos and northern Guatemala populations around 2 MYa (Gross, 2012).

In addition to these ancestral migrations, there is evidence of natural hybridization and gene flow between surface and cave populations. Some populations are significantly isolated from others due to geographic factors, such as Pachón Cave, which is perched above surface waterways with no direct access to other bodies of water (Dowling et al., 2002). In isolated caves, there are typically only highly troglomorphic cave fish present. However, other populations are known to receive an influx of surface fish from abiotic events, such as rainy seasons and floods, leading to population admixture. The Subterráneo cave system is known to receive large influxes of running water during the rainy season, carrying both nutrients as well as surface fish into the caves (Simon et al., 2017). Gene flow is confirmed by the presence of F2 hybrids, which exhibit phenotypic and genetic characteristics of both cave and surface ecotypes (Herman et al., 2018; Simon et al., 2017). Additionally, since geologic formations are dynamic and ever-changing, acidified rain may lead to collapsed structures, such as sinkholes, that allow further avenues of gene flow between populations.

1.2 *A.mexicanus* as a model organism

Thanks to their unique evolutionary adaptations, *A.mexicanus* is becoming more mainstream in its use as a model organism for answering a variety of physiological, behavioral, and genetic research questions. However, this is not the first time a cave organism has been recognized as a valuable tool for physiological research. Cave environments are consistent in their ecology across geographic regions, leading to the convergent evolution of similar constructive and regressive structures across taxa of cave organisms (Flórez et al., 2021; Liu et al., 2017; Niemiller & Soares, 2015; Porter & Crandall, 2003). This convergence of troglomorphic features becomes more evident when closely related or ancestral surface forms are readily available (Porter & Crandall, 2003). In the case of *A.mexicanus*, because surface and cave ecotypes are interspecific, it has become a powerful tool in determining the evolution of troglomorphic traits and comparing differences between cave and surface ecotypes.

Recent studies have been successful in genetically mapping several features in *A.mexicanus* owing to the discrete differences in phenotype between cave and surface forms. Genetic mapping not only consists of entire genome sequencing, as was done by Hinaux et al in 2013, but also identifying candidate genes that may explain certain traits, such as the candidate genes responsible for eye loss (McGaugh et al., 2014), the quantitative trait loci responsible for increased activity (Carlson et al., 2018), and the approximate timing of cave colonization events (Gross, 2012; Strecker et al., 2012). In addition to this work, contemporary studies have begun to use genome editing with tools such as CRISPR/Cas9 to better delineate how genetic regions pair onto phenotypes (Klaassen et al., 2018; Kowalko et al., 2016). Being that genetic architecture is incredibly complex for certain phenotypic modules, such as eye regression, genetics in *A.mexicanus* is a fast-growing field.

Some of the work conducted on *A.mexicanus* is foundational research, meant to better understand the natural history of this species. These works include research aimed at understanding the basis for troglomorphic evolution between cave and surface ecotypes, the genetic basis for pigment loss (Gross et al., 2009; Protas et al., 2006), and factors that led to the evolution of eye loss (Carlson et al., 2018; O'Quin et al., 2013; Yoshizawa et al., 2012). In addition to phenotypic features, cave ecotype fish exhibit differences in behavior when compared to surface ecotypes. Many of these behaviors are associated with adaptation to the cave environment. For example: cave ecotype fish exhibit a lack of schooling due to a lack of visual inputs (Kowalko et al., 2013), and also showed reduced aggression towards conspecifics since their feeding method has switched from attacking prey to peaceful grazing (Elipot et al., 2013).

While basic research is invaluable, much of the excitement around *A.mexicanus* has to do with its development as a model organism for studying human disease. For example, as mentioned previously, circadian rhythms are significantly altered in *A.mexicanus* due to the absence of light cues (Beale et al., 2013; Mack et al., 2021). This phenotype is not only relevant in the ways that it determines sleep duration, but also in the way that it regulates other genes. Research shows that the absence of light has led to modulation in circadian rhythms in order to tonically activate pathways necessary for life, such as DNA repair (Beale et al., 2013; Frøland Steindal et al., 2018). Being that sleep loss may be connected to food availability (Jaggard et al., 2018) and energy conservation (Moran et al., 2014), these dysregulations of circadian rhythms in *A.mexicanus* are being further explored in relation to how sleep could be linked to weight and obesity in humans (Mack et al., 2021; Reutrakul & Van Cauter, 2014; Warren et al., 2021).

In addition to circadian rhythms, the ways in which cave *A.mexicanus* manage nutrient-poor environments is also of special interest to the medical community. In comparison to surface fish, cave fish exhibit several features found in metabolic disorders, including increased fat stores (Xiong et al., 2018), hyperphagic feeding behaviors (Aspiras et al., 2015), and insulin resistance (Krishnan & Rohner, 2019). While these traits lead to deadly pathologies, these characteristics are advantageous in nutrient-limited cave environments. Genetic mapping of these traits has led to a better understanding of metabolic evolution as a response to environment (Riddle et al., 2021), and the use of *A.mexicanus* as a model for human metabolic disease. For instance, cave *A.mexicanus* is found to have differing levels of visceral adipose tissue accumulation between populations even when caloric consumption is controlled, suggesting there are factors beyond calorie consumption and energy expenditure that affect fat accumulation (Xiong et al., 2018). Such studies may lead to breakthroughs in the treatment of diseases like diabetes and obesity.

Researchers have also studied behavioral differences between surface and cave *A.mexicanus*, which has led to applied work looking at the underpinnings of atypical human behavioral differences. Many of the behaviors described coincide with other cave adaptations. As an example: vibrational attractive behavior (VAB) is stronger in cave ecotype *A.mexicanus* relative to surface (Espinasa et al., 2021; Lloyd et al., 2018). This is because sensory modalities besides vision, such as sensitivity to vibration, are enhanced in dark cave environments in order to better find prey in nutrient-deficient environments (Yoshizawa et al., 2010a). However, some of the behavioral characteristics found in *A.mexicanus* have been linked to symptoms found in psychiatric disorders. Cave ecotype fish of this species are known to avoid schooling (Kowalko et al., 2013) and exhibit asocial tendencies that are linked to the avoidant behaviors found in autism spectrum disorders (Iwashita & Yoshizawa, 2021; Yoshizawa et al., 2018). This species may provide an accessible model by which to understand the genetic factors associated with such behaviors.

1.3 Visual, Vibrational, and Auditory Sensory Ecology of *A.mexicanus*

All organisms use sensory systems to translate energy into useful information. Organismal sensory research has focused efforts on quantifying sensory phenomena, which is especially relevant to organisms that have adapted to extreme living conditions such as cave ecotype *A.mexicanus*. Within this species, the most novel detail about changing sensory ecology is its loss of functional eyes. Based on genetic studies, it appears eye loss evolved independently at least three times among different isolated cave populations (Borowsky, 2008; Moran et al., 2014). While extreme, this is not uncommon: many have suggested that the high metabolic cost of eyesight has allowed for convergent evolution of eye loss across a majority of cave organisms (Protas et al., 2006; Soares et al., 2004). The region of the brain responsible for interpreting visual information is significantly reduced in cave ecotype *A.mexicanus*, further emphasizing the extent to which the visual systems are unused in cave environments (Soares, 2014). However, the visual system is not entirely absent; studies exposing fish to different light photoperiods have found that the pineal gland, or "pineal eye" has retained the ability to detect light and shadow in lieu of functioning eyes (Yoshizawa & Jeffery, 2008). Interestingly, it only takes one generation of hybridization to bring back functionality of eyes to cave ecotype *A.mexicanus* (Borowsky, 2008), suggesting that while the pathways in vision are disused, they are not entirely eliminated from cavefish. Conversely, while so much is known about the regression of the visual system in cave ecotype *A.mexicanus*, the visual system has not been characterized in the sighted surface ecotype fish.

Other sensory modalities in cave ecotype *A.mexicanus* have evolved constructively in response to the regressive evolution of eyesight. Many of the feeding behaviors in this fish have developed in response to increased sensitivity of superficial neuromasts and the lateral line organ (Lloyd et al., 2018). The lateral line is a structure that allows fish to detect differences in pressure gradients and water flow, comprised of mechanoreceptive cells known as neuromasts (McHenry et al., 2008). While all fish possess a lateral line system (Mogdans & Bleckmann, 2012), the structure of this sensory organ and the density of superficial neuromasts vary greatly between species, and even between populations within species (Mogdans, 2019). Cave ecotype fish have neuromasts distributed on the cranium at a higher density than surface fish (Yoshizawa & Jeffery, 2011). This increased sensitivity explains the VAB as well as the larger strike distance during predation found in cave ecotype fish when compared to surface fish (Lloyd et al., 2018).

Hearing is an important sensory modality by which organisms incorporate external information for tasks such as navigation, feeding, and reproductive purposes (Cox et al., 2018; Hawkins, 2014; Popper & Hawkins, 2019). The foundational work on auditory ecology in *A.mexicanus* was done by Popper (1970) using a behavioral study paradigm to determine sound pressure sensitivity thresholds. The results of this study indicated that both cave and surface ecotype *A.mexicanus* were most sensitive around 1 kHz, with no significant difference in sensitivity at any of the frequencies tested. This was suggested to be in response to the lack of sounds in caves that go above this frequency level. Since this study was published, research groups have tackled topics related, but not directly, to auditory sensitivity in this cavefish. Hyacinthe et al (2019) documented the first known examples of interspecific communication between cave type fish, detailing three types of calls – clicks, clocs, and rumbling— and the types of social exchanges where these calls are exchanged. It is unclear whether the vocalizations produced are truly vocal calls, or simply a byproduct of certain types of movements (Mensinger, pers. comm.), similar to stridulation in catfish (Fine & Parmentier, 2015). Furthermore, Kibele et al (2019) examined the evoked potential of the *A.mexicanus* lateral line in response to a vibrating stimulus with and without chemical ablation of hair cells. The study found that detection of the stimulus was still present at low frequencies in fish with ablated hair cells, indicating that the inner ear – likely thanks to the connection to the swim bladder in otophysan fish – was contributing to motion sensitivity as well as sound pressure sensitivity.

1.4 Objective of Study

While much of the work on *A.mexicanus* focuses on the potential for applied research, there is still much we do not know about its natural history. This is especially notable when we consider that not all populations of *A.mexicanus* have been fully characterized. Since the landmark AEP study by Popper in 1970, studies that seek to determine the sensory ecology of *A.mexicanus* have been few. While recent work has determined the acoustics of interspecific communication in cave ecotype fish (Hyacinthe et al., 2019) and how the lateral line may complement inner ear sensitivity (Kibele et al., 2019), there have been no recent direct studies on the acoustic sensitivity of native cave or surface ecotype fish. Additionally, while several studies have thoroughly characterized eye development and degeneration in cave *A.mexicanus* (Carlson et al., 2018; Emam et al., 2020; O'Quin et al., 2013; Yoshizawa et al., 2012), none appear to define a baseline of vision function and sensitivity in native surface fish.

McGaugh et al. (2020) showed satellite populations of these fish exist outside of their native range. *A.mexicanus* was introduced to northern Texas less than 100 years ago, where the first documentation of this species was observed in 1953 in the Honey Creek tributary (Constable et al., 2010). The surface ecotype of this species likely migrated into the Edwards-Trinity aquifer system in central Texas through its use as a baitfish (C. Hubbs et al., 1991). There are a total of five current established populations of *A.mexicanus* within this watershed: Honey Creek cave, Honey Creek surface, San Antonio Zoo surface, Blue Hole cave, and San Pedro Springs cave. Of special note, *A.mexicanus* are found both in the Honey Creek surface tributary and in subterranean rivers of the Honey Creek Cave system, the longest cave system in Texas (Elliott, 1991; Veni, 1994).

With the discovery of these new populations, it is clear there is a knowledge gap describing the sensory ecology of both surface and cave *A.mexicanus* in their native and satellite ranges. The purpose of this thesis is to better define the natural history of satellite

A.mexicanus through electrophysiology studies aimed at providing benchmarks of sensitivity for auditory and visual modalities. In addition to defining the sensory ecology of these newly discovered populations, this study seeks to make comparisons between populations to quantify levels of divergence and determine if there is evidence for putative evolution in these new cave and surface satellite environments.

Chapter 2

Evidence for rapid divergence of sensory systems between satellite populations of the Mexican tetra (*Astyanax mexicanus*)

Abstract

The Mexican tetra (*Astyanax mexicanus*) has two primary ecotypes: cave fish and surface fish. Cave fish are characterized by troglomorphic phenotypes, such as vestigial eyes and reduced pigmentation. Studies have documented phenotypic differences in these ecotypes, which likely diverged between 0.2 to 1 million years ago. However, surface *A.mexicanus* fish were introduced relatively recently to the Edwards-Trinity aquifer, Texas in the early 1900s, and subsequent cave colonization by portions of this population show evidence of divergence through rapid phenotypic and behavioral evolution. The establishment of new satellite surface populations that recently colonized caves provide the potential of examining how new environments lead to sensory systems changes. Populations from Honey Creek (cave and surface), San Antonio Zoo (surface), Blue Hole (cave), and San Pedro Springs (cave) were compared using auditory evoked potentials (AEPs) and electroretinography (ERGs). Honey Creek cave fish were found to be significantly more sensitive to sound pressure levels between 0.5 - 0.8 kHz and particle acceleration levels between 0.4 - 0.8 kHz than Honey Creek surface fish. Additionally, Honey Creek cave fish were significantly less sensitive to light at 530 nm than Honey Creek surface fish. Pairwise differences were found between San Antonio Zoo surface and the San Pedro Springs cave and Blue Hole cave populations, with cave populations more sensitive to sound pressure levels between 0.5- 0.7 kHz. Electroretinography indicates that the San Pedro Springs cave population appears to be undergoing red-shifted divergence. Collectively, these results indicate rapid divergence of *A.mexicanus* in cave habitats at the most sensitive ranges of their visual and auditory sensory systems, and future monitoring may demonstrate continual divergence of sensory systems in populations exposed to new environments.

Introduction

Cave organisms offer a robust framework on which to study evolution. Due to the similarities of characteristics among cave systems, such as perpetual darkness, limited nutrient availability and the general absence of predators, there is a convergence of the phenotypic attributes observed in cave animals (Bradic et al., 2013; Herman et al., 2018; Jeffery, 2001). Around the globe, numerous animals have evolved to inhabit cave ecosystems in a process known as troglomorphic adaptation. Troglomorphy describes the phenotypic features associated with organisms that live in caves, and include lack of pigmentation, tolerance to low nutrient availability, vision loss, and the enhancement of non-visual sensory systems to navigate dark environments (Protas & Jeffery, 2012).While comparisons between cave and surface species have offered insight into how similar organisms adapt to extreme environments (Porter et al., 2007), little is known about how quickly these traits can evolve.

The Mexican tetra, *A.mexicanus*, provides a unique model for examining troglomorphic sensory adaptation, since it is found in two distinct environments: surface and underground waterways. *A.mexicanus* appears to have originated in South America and migrated to Central America approximately 8 to 3.3 million years ago (Gross, 2012). After establishment in Central America, this species colonized several cave systems at various timepoints, leading to the establishment of approximately 30 cave populations (Espinasa et al., 2018) with recent expansions to central Texas. The Mexican cave morphotype of this species has troglomorphic features, including lack of pigmentation and loss of eyes. However, they are able to interbreed with the surface morphotype fish and hybridize in the wild (Herman et al., 2018; Jeffery, 2020).

In the 1900s, Mexican surface *A.mexicanus* were introduced into the northernmost portion of the Nueces River in central Texas. Several populations were established in cave and surface waterways in the Edwards-Trinity Aquifer system, with surface and cave fish first observed in Honey Creek, a tributary of the Llanos river, in 1953 (Constable et al., 2010). Thus, the cave populations in central Texas likely diverged from Mexican surface populations within the last 100 years. Although Mexican *A.mexicanus* have acquired troglomorphic characteristics over millions of years (Porter et al., 2007), rapid divergence appears to be transpiring in populations found within the Edwards-Trinity Aquifer. A recent study has found evidence for changes in morphology and behavior between the two ecotypes in Honey Creek, suggesting rapid divergence in these satellite fish (McGaugh et al., 2019).

The cave environment differs from the surface world in light availability, environmental sound, water temperature and lack of nutrient sources/prey availability (Niemiller & Soares, 2015). The aquatic soundscapes of caves often lack atmospheric input, such as wind and rain, that are major contributors to sound in marine (Parks et al., 2014) and freshwater surface environments (Putland & Mensinger, 2020) which may affect auditory sensitivity. Additionally, the lack of light has resulted in degradation of visual systems in native cave *A.mexicanus* (Soares & Niemiller, 2020; Wilkens, 1988) which is offset by increased hair cell density; this increase in density may lead to increased lateral line sensitivity (Lloyd et al., 2018; Yoshizawa et al., 2014). However, it is unclear if newly colonized cave populations, prior to undergoing visual degradation, may initially select for increased retinal sensitivity while inhabiting the twilight region between the cave mouth and perpetual dark recesses (Krishnan & Rohner, 2017).

Recent studies have documented rapid phenotypic evolution in organisms, detailing significant changes over time scales of a few generations. Several studies have demonstrated rapid evolution in fishes, including color pattern selection based on predator presence over 15 generations in Trinidadian guppies (J. A. Endler, 1980), morphometric changes in South American cichlids within decades following river damming (Gilbert et al., 2020), and ecotype divergence in three-spine stickleback within a single generation (Laurentino et al., 2020). The sensory systems of organisms evolve to match environmental conditions (Cummings & Endler, 2018; J. Endler, 1998), yet it remains unclear how quickly sensory systems adapt to changing conditions (Dunlop et al., 2018; Zakon, 2015).

Several studies have compared sensory systems between cave and surface ecotypes of *A.mexicanus* (Yoshizawa, 2016). No significant difference in auditory sensitivity between Mexican cave and surface populations was detected using classical conditioning (Popper, 1970). Behavioral studies indicate cave fish are less aggressive, (Elipot et al., 2013), exhibit increased wall following behavior (Sharma et al., 2009), and reduced schooling behaviors (Kowalko et al., 2013).

However, these studies have been confined to populations that may have diverged over millions of years. The relatively recent discovery of the Texas populations, in which cave and surface populations remain similar in external morphology, allows investigation into early divergence of sensory systems. The goal of this study is to compare auditory sensitivity using auditory evoked potentials (AEP) and particle acceleration (PAL) tests and compare visual sensitivity using electroretinography (ERG) between recently isolated cave and surface populations of *A.mexicanus* in Texas. We will be directly comparing both the previously documented Honey Creek cave and surface ecotype fish, as well as three additional recently discovered populations in transitional cave-surface habitats: San Antonio Zoo surface, San Pedro Springs cave and Blue Hole cave.

Methods

Population Sampling

A.mexicanus specimens were collected from five locations (Fig. 2.1). The Honey Creek populations were collected within the Guadalupe River Basin in Comal Country, Texas, with cave fish obtained 100 m into Honey Creek Cave June 2019 and February 2020, and surface fish obtained in the Honey Creek tributary approximately 1500 m away from the cave mouth February 2020. San Pedro Springs cave fish were collected June 2019 from springs emerging from a bedrock headwall at the San Pedro Springs Park, San Antonio, TX. Blue Hole cave fish were collected June 2019 from the Blue Hole Springs Well on the University of the Incarnate Word campus in San Antonio, TX. San Antonio Zoo surface fish were collected June 2019 from a section of the San Antonio Springs river running within the San Antonio Zoo. All five populations were transported to the University of Minnesota June 2020.

Animal husbandry

Honey Creek cave (*n* = 7, total length (TL): 90.3 ± 2.5 mm; mean ± standard error (SE)), San Pedro Springs cave (*n* = 7, 73.3 ± 2.6 mm), Blue Hole cave (*n* = 7, 78.9 ± 2.2 mm), Honey Creek surface (*n* = 10, 89.2 ± 3.4 mm), and San Antonio Zoo surface (*n* = 11, 82.9 ± 3.2 mm) fish were maintained indoors at the University of Minnesota Duluth. Each population was housed separately in 75.7 L glass tanks filled with buffered pond water (0.56 g KCl, 0.44 g NaCl, and 2.6 g $CaCl_2$ per 75.7 L, pH = 7.0) and equipped with mechanical, chemical, and biological filters. Water temperature was maintained between 20 - 22 °C. Illumination was provided by LED aquarium lights (LED Light Hood; Marineland; Blacksburg, VA) on top of each tank containing surface fish on a 9 hr:15 hr light: dark cycle. The cave tanks were not illuminated and separated from the surface tanks by opaque dividers but still received dim indirect light from the surface tanks. All populations were fed commercial fish flakes (Cichlid Flakes; Tetra; Melle, Germany) daily. Prior to each experiment, standard length (SL, mm), total length (TL, mm) and wet weight (M, g) were recorded. All experiments were conducted under protocol 2002-37827A and approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

Auditory evoked potentials

AEP testing was conducted in a 375 L cylindrical fiberglass tank (88 cm inner diameter, 62 cm height, 57 cm water depth) seated on a 1 cm thick rubber mat on cinderblocks (41 x 20 x 10 cm) to reduce vibrations. The experimental tank was housed within a galvanized angle iron frame (110 x 125 x 182 cm) surrounded on the top and three sides with FOAMULAR Insulation Sheathing (2.5 cm thick; Owens Corning; Toledo, OH) to reduce background sound and prevent the fish from seeing the experimenter.

Prior to electrode implantation, fish were anesthetized for 5 min using phosphate buffered tricaine methanesulfonate (0.005%; Western Chemical Inc.; Ferndale, WA) and then were suspended in a mesh sling using an adjustable arm boom stand (Omano Microscopes; China) within a smaller plastic anesthetic chamber (26.5 x 18.5 x 19 cm) containing the anesthetic solution to maintain quiescence throughout the experiment. The dorsal surface of the fish was maintained 4 cm below the surface of the water and 42 cm above an omnipole underwater speaker (Model UW-30; Electro-Voice; Burnsville, MN).

Stainless steel electrodes (Rochester Electro-Medical Inc; Tampa, FL) were insulated with acrylic paint to within 2 mm of the electrode tip and implanted subcutaneously. The recording electrode was positioned above the brainstem and placed medially on the dorsal surface of the head approximately 2 mm posterior to an imaginary line drawn between the anterior margins of the opercula. A reference electrode was placed medially between the nares. AEP signals were amplified using a headstage (gain = 10x) connected to an extracellular differential amplifier (Model EX1; gain = 100x; Dagan Corporation; Minneapolis, MN) with a 0.02 kHz low-pass filter and a 5.0 kHz high-pass filter. A Cambridge Electronic Design data acquisition system (Micro-3 1401; CED; Cambridge, UK) and custom Spike2 (Version 8; CED) scripts were used to set sound signal parameters, calibrate sound pressure level (SPL) attenuation, and digitize the received AEP signals. A programmable attenuator (Model 3505; CED) and amplifier (Model AS-35; Accusonic; Markham, Canada) controlled the SPL of the presented signals. The attenuator and amplifier were calibrated using a hydrophone (Model 8103; Brüel & Kjaer; Naerum, Denmark) placed in the position as the experimental fish. The hydrophone was connected to a Nexus Conditioning Amplifier (Model 2609-01s; Brüel & Kjaer). Pure tone signals were attenuated in 3 dB 1 μPa SPL_{rms} steps.

Auditory thresholds for fifteen frequencies between 0.1 and 4.0 kHz were tested. For stimulus presentation, pure tone bursts for each frequency were broadcast (50 ms; 500 repetitions; 3 ms delay) and responses were collected and averaged using a custom Spike 2 script. AEPs were verified by fast Fourier transform power spectrum analysis

(FFT, Hanning window = 1024). Visual AEPs (Fig. 2.2) with FFT peaks above the background noise (≥ 0.001 μV) at the second harmonic of the stimulation frequency were considered evoked potentials. The auditory threshold at each tested frequency was defined as the minimum SPL that elicited an observable AEP response and a FFT peak at the second harmonic of the stimulus frequency. Threshold measurements were conducted by gradually increasing SPL_{rms} until AEPs were detected or the maximum output of the speaker at a given frequency was reached.

Particle acceleration thresholds

Particle acceleration thresholds (PALs) were measured using a triaxial accelerometer (sensitivity, x = 10.47 mV/ms^{-2}, y = 10.35 $mV/\ ms^{-2}$, z = 10.29 $mV/\ ms^{-2}$, Model W356A12/NC; PCB Piezotronics Inc.; Depew, NY) modified to be neutrally buoyant and connected to a signal conditioner (482C15; Piezotronics Inc.) and positioned within the AEP experimental tank in the same position as the fish head. For each frequency, corresponding PAL measurements were made for each SPL throughout the attenuation range. The accelerometer was positioned with its x-axis in the rostral-caudal, the y-axis in the lateral, and the z-axis to the dorsal-ventral planes of the fish. To calculate the PAL, the V_{rms} was determined for each axis (x, y, and z) and then converted into individual magnitude vectors. The following equation was used to calculate PAL thresholds:

(1) $\text{dB re ms}^{-2}{}_{rms} = 20\log(\sqrt{x^2 + y^2 + z^2})$

Electroretinography

All ERG testing was conducted in a room illuminated by dim red light (15 W light bulbs with Kodak GBX-2 dark red safelight filter). Each fish was anesthetized with MS-222 solution between 0.0075% and 0.0085%, buffered with sodium bicarbonate to a pH of 7.0, for 5 min prior to placement in experimental chamber. The fish was then placed on a moist sponge in the acrylic experimental tank (13 x 18 x 8 cm) and covered with a wet Kimwipe (Kimberly-Clark Professional; Roswell, GA). The experimental tank was housed within an opaque metal faraday cage (77 x 67 x 97 cm) to prevent equipment light from reaching the fish. The buffered MS-222 solution was delivered to the fish via a gravity-fed tube placed in the buccal cavity of the fish to maintain the surgical plane of anesthesia.

A small incision through the limbus of the eye was made with a 0.3 mm 15° stab knife (Surgical Specialties; Westwood, MA). A 0.64 mm diameter silver-silver chloride electrode was inserted into the incision, with the reference electrode placed within the nostril of the ipsilateral side. ERG waveforms were amplified using a bioamplifier (DAM50; World Precision Inc.; 1000x gain; 1 Hz low pass, 3 kHz high pass; Sarasota, FL), filtered with a digital 60 Hz notch filter, and recorded with a Powerlab 4SP (AD Instruments; Castle Hill, Australia) using Lab Chart7 Software (AD Instruments) on a Dell laptop.

A 100 W quartz tungsten-halogen lamp (Model #6333; Newport; Stratford, CT) powered by a constant current power supply (Model #68938; Newport) produced the light stimulus. Stimulus duration (200 ms) was regulated with an electronic shutter (Model #76994; Newport) and controller (Model #76995; Newport). A dual filter wheel (Model #7736; Newport) containing neutral density filters from 0.1 to 3.0 regulated light intensity, with wavelength controlled by a monochromator (Model #77250; Newport). Light intensity was determined using a radiant power energy meter (Model #70260; Newport) and probe (Model # 70268; Newport). A fiber optic light pipe (Model #77632;

Newport) transmitted the light from the monochromator to the fish eye, completely illuminating the eye.

All fish were dark adapted for 30 to 60 min. Test flashes were initiated at the 30 min mark to determine if the retina was dark adapted, which was defined as the absence of the a-wave in the ERG. If the a-wave remained, the fish was allowed to dark adapt for an additional 10 min, and the process repeated until the a-wave was undetectable. Wavelengths between 425 and 700 nm were presented to the fish in random order with flash duration of 200 ms and interflash interval between 10 to 30 s. Experiments were conducted during the fish's light cycle to avoid circadian rhythm effects.

The response criterion was set as the b-wave amplitude (baseline to peak) at 425 nm and averaged approximately 30 mV. Other wavelengths were reduced in intensity using neutral density filters until the response equaled to the b-wave amplitude at 425 nm. Upon completion of the ERG, fish were revived by delivering buffered pondwater to the fish via a separate gravity-fed tube until gilling resumed, and fish were returned to home aquaria.

Statistics

Non-parametric statistical tests were used for analysis as the data was not normally distributed (Shapiro-Wilk test: SPL data [$W = 0.99$, $p <0.001$], PAL [$W = 0.98$, $p < 0.001$], ERG [$W = 0.81$, $p < 0.001$]). Honey Creek cave and surface population were directly compared using Welch's T-test due to their geographic proximity. Because less is known about the sampling environment for San Antonio Zoo surface, San Pedro Springs cave, and Blue Hole cave, these populations were compared separately using a one-way Kruskal-Wallis ANOVA. If significant differences ($p < 0.05$) were found between groups, post hoc pairwise Wilcoxon Rank-Sum tests with Bonferroni adjustment were implemented.

In addition to the statistical tests above, generalized additive models (GAM) were used to estimate variability in the sensitivity curves from AEP (SPL and PAL), and ERG assays, with frequency (AEP) or wavelength (ERG) as the predictor, and sensitivity thresholds as the response variable. An interaction model was fitted per the methodology described in Rose et al, 2012 using the following equation:

(2) $y_{ij} = \alpha 0 + \alpha_{1j}\text{Population}_{ij} + f_j(\text{Threshold}_i) + \varepsilon_i,\ \ \varepsilon_i \sim N(0,\sigma^2)$

where $\alpha 0$ is the model intercept (mean value of the response, y_{ij}, in the reference Population), $\alpha 1$ is the difference between the mean response for the *j*th population, $\alpha 0$ and f_j() are centered, smooth functions of a threshold variable and represent the trend in response for the *j*th population, and εi are the model residuals, assumed Gaussian distribution with mean 0 and variance σ^2. This model incorporates a separate trend for each population into a single model of threshold changes across five populations.

For each modelled distribution, a 95% confidence interval was fitted to each model to allow for comparisons between populations. In addition to the frequency sensitivity curves, these modelled distributions allowed for more detailed assessment of the sensitivity curves in spite of statistical power lost due to the dataset lacking a normal distribution. The fitted smooth functions, f_j, were then compared for selected pairs of populations via the use of a prediction matrix. These smooth functions were then subtracted from one another, and the resulting data was used to create a graph representing the difference in the two smooths for a pair of populations. Approximate 95% confidence intervals were then generated on the difference between pairs of smooths. Significant pairwise differences were defined where the 95% confidence interval band did not overlap with zero. Statistical analysis was completed using R Version 3.6.3 “Holding the Windsock” (R Core Team, 2017). Graphs were created in both R and SigmaPlot (Version 12.5). Data are reported as mean ± SE.

Results

Sound Pressure Thresholds

All populations responded to frequencies between 0.1 and 4.0 kHz, with the lowest auditory thresholds between 0.2 – 0.5 kHz. Honey Creek cave displayed significantly lower sound pressure thresholds (Welch’s T-test, $p < 0.05$) than surface conspecifics between 0.6 - 0.8 kHz (Fig. 2.3). Blue Hole and San Pedro Springs cave populations had significantly lower auditory thresholds (Pairwise Wilcox w/ Bonferroni Adj., $p < 0.05$) than San Antonio Zoo surface fish at 0.6 - 0.7 kHz and 0.7 kHz, respectfully (Fig. 2.4).

Particle Acceleration Thresholds

Honey Creek cave fish were significantly more sensitive (Welch’s T-test, $p < 0.05$) to particle acceleration than Honey Creek surface between 0.4 – 0.8 kHz, 1.2 – 1.5 kHz, and at 3.0 kHz (Fig. 2.5). Blue Hole cave and San Pedro Springs cave fish had significantly lower particle acceleration thresholds than San Antonio Zoo surface fish between 0.5 – 0.7 kHz and 1.2 kHz, and 0.6 – 0.7 kHz and 3.0 kHz, respectfully (Pairwise Wilcox w/ Bonferroni Adj., $p < 0.05$) (Fig. 2.6).

Electroretinography Thresholds

All populations were maximally sensitive to wavelengths between 500 – 600 nm. The Honey Creek cave population was significantly less sensitive to light at 530 nm than

Honey Creek surface (Welch's T-test, $p < 0.05$)(Fig. 2.7). No significant differences between the spectral sensitivity of San Antonio Zoo surface, San Pedro Springs cave, and Blue Hole cave populations were found (Fig. 2.8)

Generalized Additive Models

All populations exhibited differences in threshold values across generated smooth functions of AEP and PAL data (Fig. 2.9). Honey Creek cave had significantly different sound pressure levels when compared to Honey Creek surface between 0.5 – 0.7 kHz. Significant differences in sound pressure levels were found also between San Antonio Zoo surface compared to Blue Hole cave, and San Antonio Zoo surface compared to San Pedro Springs cave between 0.5 – 0.7 kHz, and above 2 kHz. Similarly, significant differences between particle acceleration sensitivity curves were found between Honey Creek surface and cave, San Antonio Zoo surface and Blue Hole cave, and San Antonio Zoo surface and San Pedro Springs cave at 0.5 – 0.7 kHz. San Antonio Zoo surface particle acceleration sensitivity was also significantly different from San Pedro Springs cave at 3 kHz. No significant differences were found between any populations in dark-adapted retinal sensitivity.

Discussion

The results indicate evidence of auditory sensory divergence. Honey Creek cave fish were significantly more sensitive to several frequencies of sound than surface conspecifics. The other two cave populations (San Pedro Springs and Blue Hole) were also more sensitive to certain frequencies than San Antonio Zoo surface fish. While most comparisons of retinal sensitivity were not statistically significant, qualitative examination of the ERG curves show two of the cave populations trending towards reduced sensitivity.

Sound Pressure and Particle Acceleration Sensitivity

The Mexican tetra is a member of the Characid family, in the superorder Ostariophysi (Gross, 2012; Nakatani et al., 2011), which possess Weberian ossicles that connect the swim bladder to the inner ear, allowing for greater detection of sound pressure (Schulz-Mirbach et al., 2020). Given ostariophysans extended hearing range compared to non-ostariophysans, we hypothesized that *A.mexicanus* would provide a good model for investigating any hearing changes within caves.

Minimal atmospheric and anthropogenic events combined with lower food web productivity can reduce ambient sound in terrestrial and aquatic cave environments compared to surface counterparts (Lee et al., 2012). In the karst cave systems inhabited by *A.mexicanus*, lower ambient sound is attributed to fewer abiotic and biotic sound sources, sound absorption by porous limestone, and dissipation by irregular surfaces within the caves (i.e. stalactites, stalagmites, concavities, tunnels) (Carvalho & Sousa, 2015; Iannace & Trematerra, 2014). Contributions to the cave aquatic soundscape include running and dripping water, and airflow/winds caused by temperature and pressure gradients between the cave and surface atmosphere (Badino & Chignola, 2019; Carvalho & Sousa, 2015). Ambient noise can vary within a single cave system depending on the aquatic environment, as standing pools are quieter than running streams (Niemiller et al., 2013).

Few studies have been conducted on auditory sensitivities of cavefishes (Soares et al, 2016), and previous investigations examining auditory sensitivity between cave and surface fish have not found many physiological differences. A behavioral assay indicated no significant differences in hearing sensitivities between cave and surface populations of Mexican *A.mexicanus* (Popper, 1970). Although cave and surface populations of the Atlantic Molly (*Poecilia mexicana*) showed differences in otolith morphology, AEP experiments were unable to detect any differences in auditory sensitivity (Schulz-Mirbach et al., 2010). Amblyopsid fish had similar hearing sensitivity up to 800 Hz, however only surface fish were able to hear frequencies > 800 Hz, suggesting cave amblyopsids may have lost higher frequency sensitivity in response to high frequency noise in caves (Niemiller et al., 2013). Cave and surface ecotypes of *A.mexicanus* display differences in vocal behavior, but produce similar vocalization ranging between 0.2 – 3 kHz (Hyacinthe et al., 2019).

The lateral line of fishes has evolved to detect hydrodynamic stimuli. Mexican *A.mexicanus* cave fish have increased density of hair cells in the lateral line (Yoshizawa et al., 2014) and increased reliance on the lateral line for prey detection (Lloyd et al., 2018). Sound waves have both a pressure and particle motion component, and it is the latter that stimulates the lateral line (Mogdans, 2019). Additionally, unlike sound pressure, particle acceleration is universally detected by the inner ear of fish (Radford et al., 2012).

Because all fish can detect the particle motion component of sound, it is imperative when conducting AEPs to measure both SPL and PAL. The current results show cave populations are even more sensitive to PAL than SPL compared to surface conspecifics. The only previous study which has measured cave fish particle motion sensitivity was done comparing cave and surface ecotypes of *P.mexicana*, and found no significant difference in particle motion sensitivity between the two ecotypes (Schulz-Mirbach et al., 2010). Our results may be indicative of the extent to which cave ecotype *A.mexicanus* utilize particle acceleration as opposed to sound pressure to navigate dark cave environments. Recent studies have shown that that the lateral line can contribute to hearing through the particle motion detection of low frequency stimuli (Higgs & Radford, 2013; Mensinger et al., 2018). However, in the absence of ablation studies, it is difficult to parse the relative contributions of the lateral line to the detection of auditory stimuli, since AEPs predominately measure neural activity from the eighth nerve fibers and brain stem in response to acoustic stimulation (Sisneros et al., 2016). Further studies are needed to better parse the relative contribution of the lateral line to particle motion detection and sensitivity, especially in understudied cave fish.

Cave organisms are characterized by constructive and regressive traits associated with adaptation to cave environments (Wilkens & Strecker, 2017). There are several theories debating how regressive traits arise, but constructive traits are generally accepted to be the result of selection acting on genetic variation to produce adaptive phenotypes for cave environments (Protas & Jeffery, 2012). Characteristics such as increased vibrational attraction behavior (VAB) (Yoshizawa et al., 2010b), increased number of taste buds (Varatharasan et al., 2009), and increased adipose tissue development (Xiong et al., 2018) are examples of constructive features found in Mexican cave *A.mexicanus*. The increased auditory sensitivity found in Honey Creek cave when compared to Honey Creek surface indicates that Honey Creek cave populations are in the process of acquiring constructive phenotypes for adaptation to cave life. Similarly, differences in auditory sensitivity between San Antonio Zoo surface fish and Blue Hole cave as well as San

Pedro Springs cave fish may also be indicative of constructive sensory phenotype development.

Visual Sensitivity Trends

The absence of functional eyes and reduction of the optic tectum is characteristic of Mexican *A.mexicanus* cave populations (Krishnan & Rohner, 2017; Protas et al., 2008), and this regression has been hypothesized as an evolutionary attempt to conserve energy due to the high metabolic demands of vision (Krishnan & Rohner, 2017). The eyeless morphology of Mexican cave *A.mexicanus* is suggested to be the result of multiple minute changes during eye development, such as retinal apoptosis, early lens development, and retina size, rather than one trait that codes for "eyes" (O'Quin & McGaugh, 2016). An alternative is the neutral mutation hypothesis, which suggests that eye degeneration is the product of random eye gene mutations that accumulate in the absence of selective pressures for vision (Jeffery et al., 2003). However, the rate at which visual regressive adaptations occur are unknown, and is it hypothesized that many generations over thousands or millions of years may have been necessary for the observed extreme phenotypic plasticity. Thus, both hypotheses make robust rapid visual sensory divergence within 100 years unlikely, as is reflected in our study results.

Newly diverged cave populations retain functional eyes. Whether this is due to ambient light impacting these populations, continual influx of surface fish into the cave population, or insufficient evolutionary time to diverge from surface populations is unknown. However, it does provide a transitional population to determine the initial effects on a cave environment to the visual system. Deep water fish have evolved elaborate retinal specialization to detect downwelling light (Collin & Partridge, 1996;

Warrant & Locket, 2004) and bioluminescent organisms (Locket, 1970). Thus, dim light, found in the transition zone between the cave mouth and perpetually dark recesses may select initially for increased scotopic vision. However, Honey Creek cave fish showed reduced sensitivity to light throughout the visual ranged with a significant decrease at 535 nm, suggesting that retinal degradation has already been selected for. While the Blue Hole cave and San Antonio Zoo surface fish have remarkably similar ERG curves, the San Pedro Springs populations has more sensitivity to red shifted light, and it is unclear if this is a constructive trait related to their specific cave environment or a difference in optical water clarity.

Environmental Factors of Sensory Divergence

While the results suggest that there are changes in both auditory and visual sensitivity between Honey Creek surface and cave populations, the results may be tempered by both the recent colonization of this environment and potential continual exchange between cave and surface populations. Honey Creek cave is the longest cave system in Texas (Veni, 2005), and is a long, horizontal cave system partially filled with water from a cavern spring system (Reddell, 1964). The cave remains only partially explored, as it is vast with many tunneled passages with irregular limestone structures. The Honey Creek tributary feeds into the cave and is characterized by riparian areas lined with grassland and Bald Cypress trees (*Taxodium distichum*) (Hensley, 2019). While the surface population was collected far from the cave mouth, the cave population was sampled within 100 m of the cave entrance. Although there is no physical barrier between the two populations, morphological changes and current physiological data (McGaugh et al., 2019) strongly infer that these two populations are independent. However, given that the stream flows into the cave, surface fish could be swept into the cave during high flow events; this gene inflow may temper the rate of regression. Further studies on the migration of cave and surface fish between environments need to be conducted.

While the evidence suggests that Honey Creek cave and surface populations may be distinct, the other three sites examined offered more intermediate populations. The Headwaters Sanctuary at University of the Incarnate Word houses the headwaters of the San Antonio River, with the Blue Hole spring well serving as the centerpiece of the sanctuary. The spring is ephemeral: during the dry season, the majority of the population retreats to the subterranean portion of the spring. However, the population is believed to have access to the San Antonio River surface habitats (S. McGaugh, pers. comm) and at least a portion of this population surfaces in the well during the wet season. San Pedro Spring fish were collected from a spring originating in the San Pedro Springs Park, which is a public recreational area with springs feeding into an outdoor pool. Therefore, it likely that the cave fish in these populations may have access surface waters at least during a portion of the year. The San Antonio Zoo surface population does not have a contiguous cave population and was used as a proxy for surface fish to compare against the San Pedro Springs cave and Blue Hole cave populations. Given the potential seasonal access to surface environments, it was not surprising that these two cave populations showed intermediate hearing levels between Honey Creek cave and San Antonio surface fish. Whether this is due to individuals moving between both environments or the influx of surface light into these subterranean habitats remains to be determined.

Thanks to the recent invasion of *A.mexicanus* in a novel environment, we have been able to observe a quantifiable adaptation to the environment that differs from traits found in Mexican populations of these fish. Our data suggests that satellite populations of *A.mexicanus* are exhibiting evidence of rapid sensory divergence in response to novel environmental pressures, with divergence much more strongly observed in auditory sensitivity rather than vision. Additional investigations should examine the both the soundscapes as well as the light levels of these cave environments to better characterize the environmental pressure in question. Likewise, continual monitoring of *A.mexicanus* in these new satellite environments over a larger timescale may allow us to better quantify how populations diverge through phenotypic and genetic traits.

TABLE 2.1: Statistics of auditory sensitivity thresholds between populations.

		Honey Creek Cave vs Honey Creek Surface	**San Antonio Zoo vs Blue Hole**	**San Antonio Zoo vs San Pedro Springs**
Frequency (kHz)	**0.1**	ns	ns	ns
	0.2	ns	ns	ns
	0.3	ns	ns	ns
	0.4	ns	ns	ns
	0.5	ns	ns	ns
	0.6	P = 0.023	P = 0.026	P = 0.016
	0.7	P = 0.017	P = 0.012	ns
	0.8	P = 0.027	ns	ns
	0.9	ns	ns	ns
	1.0	ns	ns	ns
	1.2	ns	ns	ns
	1.5	ns	ns	ns
	2.0	ns	ns	ns
	3.0	ns	ns	ns
	4.0	ns	ns	ns

TABLE 2.2: Statistics of particle acceleration sensitivity thresholds between populations.

		Honey Creek Cave vs Honey Creek Surface	**San Antonio Zoo vs Blue Hole**	**San Antonio Zoo vs San Pedro Springs**
Frequency (kHz)	**0.1**	ns	ns	ns
	0.2	ns	ns	ns
	0.3	ns	ns	ns
	0.4	P = 0.003	ns	ns
	0.5	p = 0.002	P = 0.041	ns
	0.6	P = 0.036	P = 0.049	P = 0.024
	0.7	P = 0.009	P = 0.006	P = 0.044
	0.8	P = 0.002	ns	ns
	0.9	ns	ns	ns
	1.0	ns	ns	ns
	1.2	P = 0.048	P = 0.006	ns
	1.5	P = 0.041	ns	ns
	2.0	ns	ns	ns
	3.0	P = 0.025	ns	P = 0.004
	4.0	ns	ns	ns

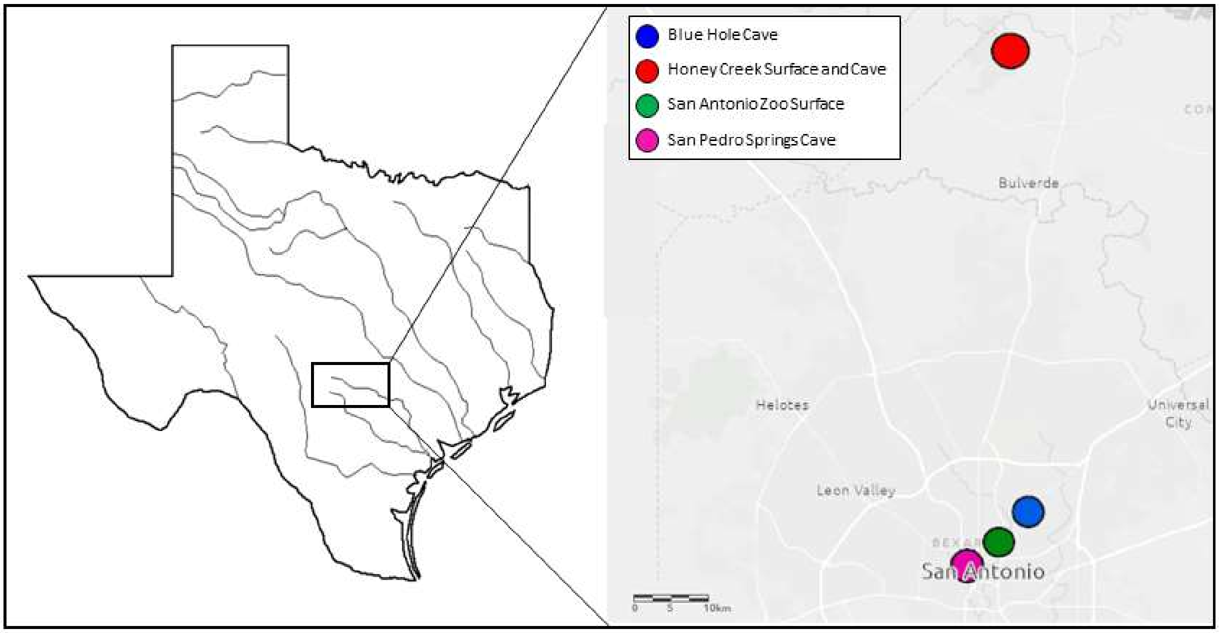

FIGURE 2.1: Geographic locations of satellite population sampling. Five satellite populations of *A.mexicanus* found in the Edwards-Trinity Aquifer system were sampled for this study.

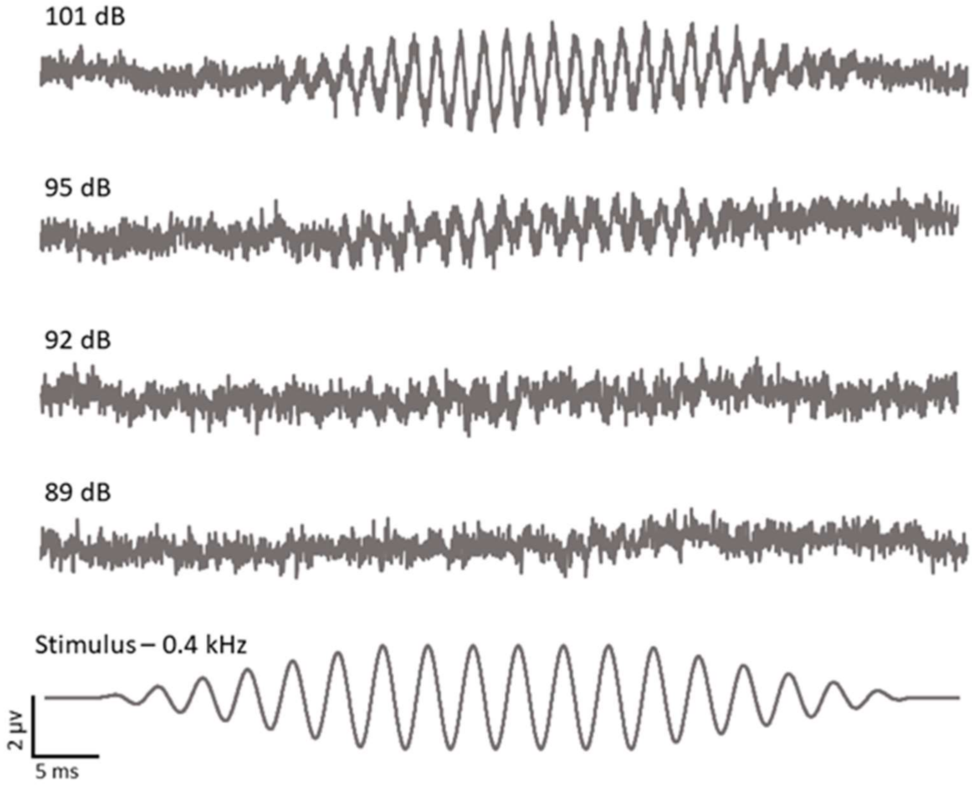

FIGURE 2.2: Auditory Evoked Potential Waveform. Example of an auditory evoked potential at 0.4 kHz recorded on a Blue Hole population fish at four different sound pressure levels, with a robust response at 101 dB and a threshold response at 95 dB.

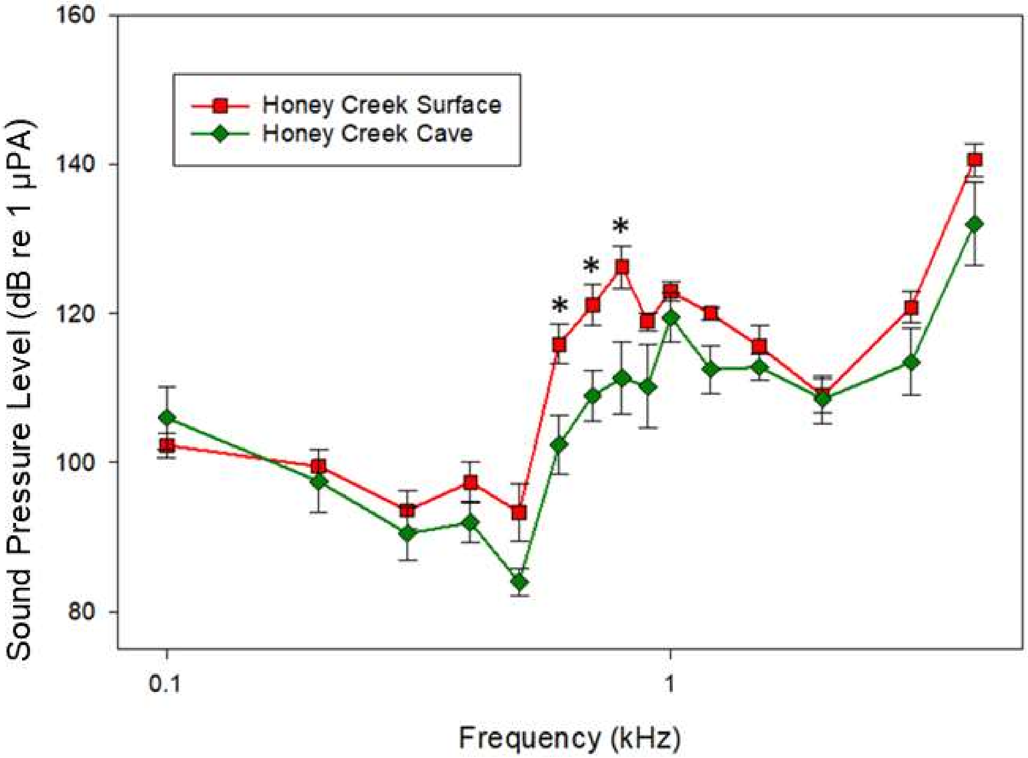

FIGURE 2.3: Honey Creek Cave and Surface AEP threshold levels. (A) Mean auditory sound pressure level (± SE) between Honey Creek Cave (green) and Honey Creek Surface (red). Asterisks (*) indicate a significant difference (Welch's T test, $P < 0.05$) between populations.

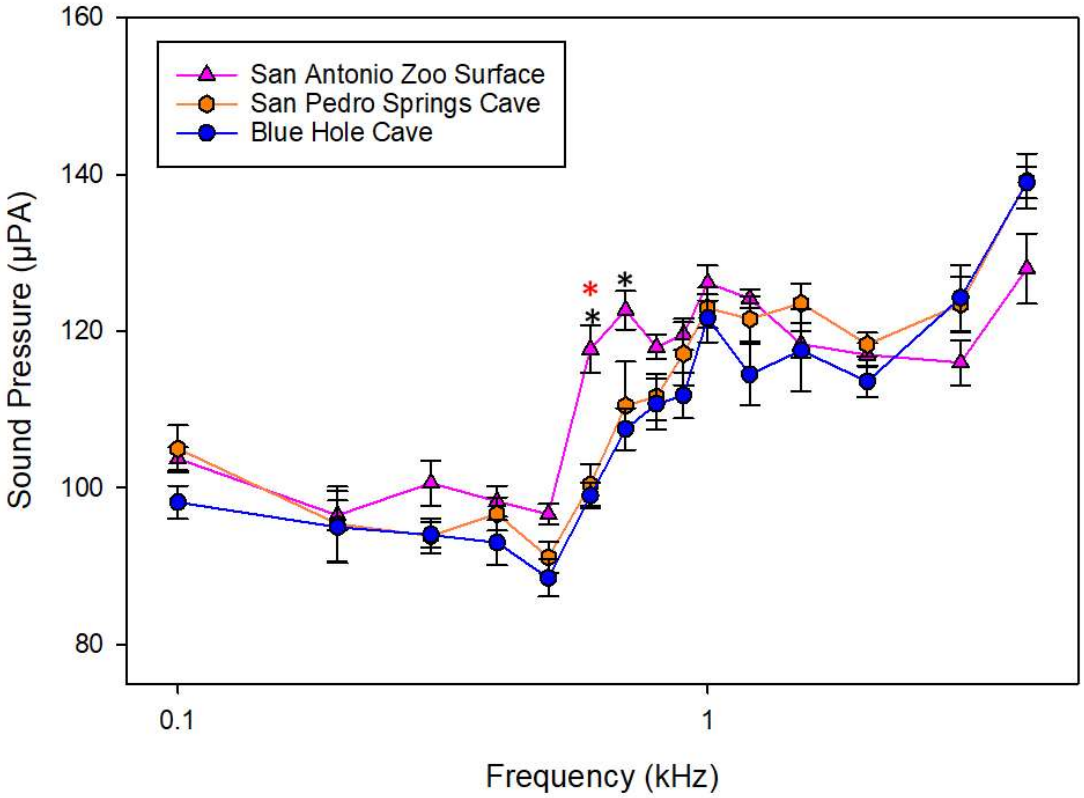

FIGURE 2.4: San Antonio Zoo Surface, San Pedro Springs Cave, and Blue Hole Cave AEP threshold levels. Mean auditory sound pressure level (± SE) between San Antonio Zoo Surface (pink), San Pedro Springs Cave (orange) and Blue Hole Cave (blue). Black asterisks (*) indicate a significant (Kruskal-Wallis with post-hoc pairwise Wilcox w/ Bonferroni correction, $p < 0.05$) difference between San Antonio Zoo Surface and Blue Hole Cave. Red asterisks (*) indicate a significant difference between San Antonio Zoo Surface and San Pedro Springs Cave.

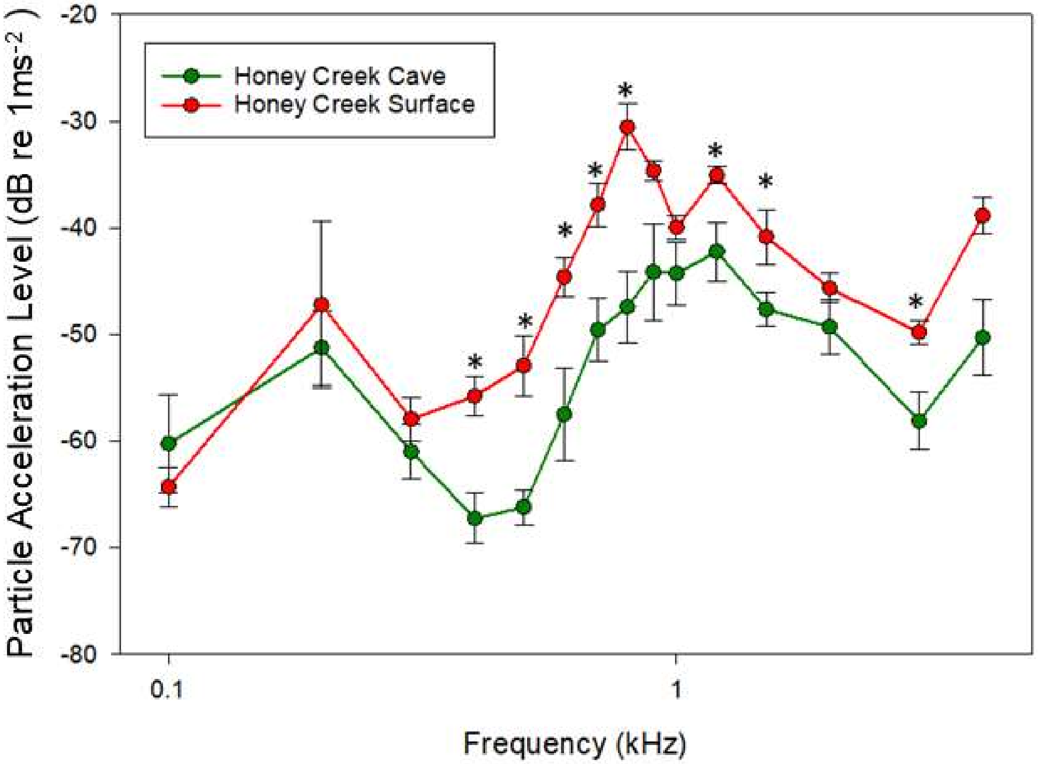

FIGURE 2.5: Honey Creek Cave and Surface PAL threshold levels. Mean particle acceleration level (± SE) between Honey Creek Cave (green) and Honey Creek Surface (red). Asterisks (*) indicate a significant difference (Welch's T test, $P < 0.05$) between populations.

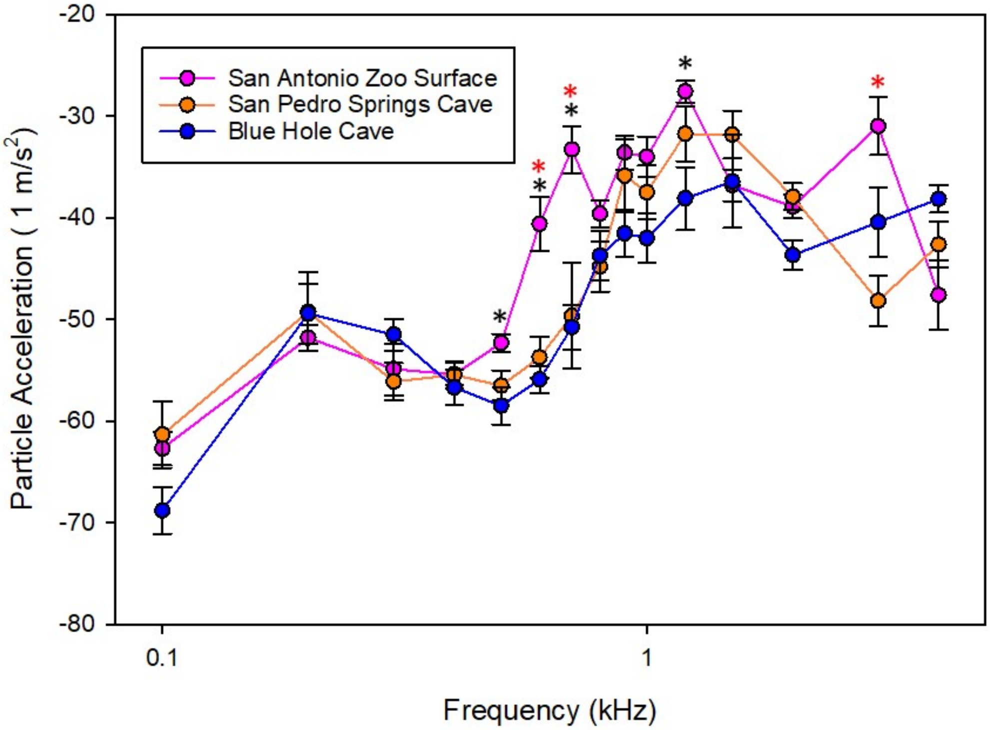

FIGURE 2.6: San Antonio Zoo Surface, San Pedro Springs Cave, and Blue Hole Cave PAL threshold levels. Mean particle acceleration level (± SE) between San Antonio Zoo Surface (pink), San Pedro Springs Cave (orange) and Blue Hole Cave (blue). Black asterisks (*) indicate a significant (Kruskal-Wallis with post-hoc pairwise Wilcox w/ Bonferroni correction, $p < 0.05$) difference between San Antonio Zoo Surface and Blue Hole Cave. Red asterisks (*) indicate a significant difference between San Antonio Zoo Surface and San Pedro Springs Cave.

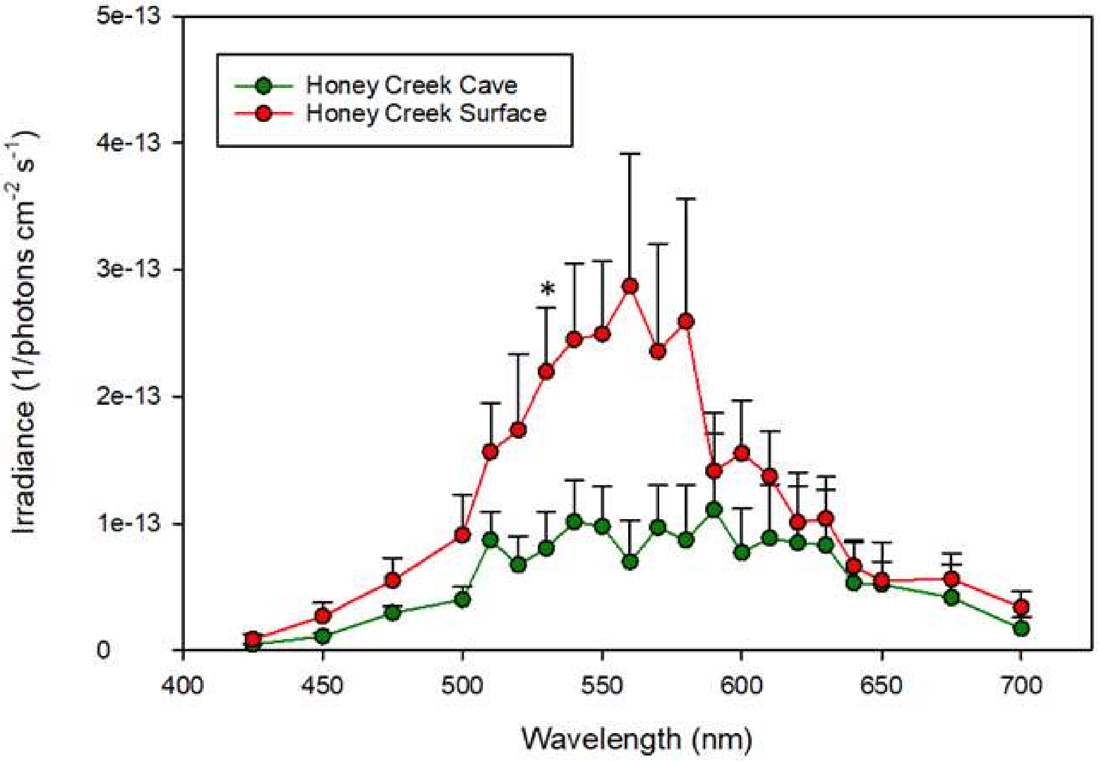

FIGURE 2.7: Honey Creek Cave and Surface ERG threshold levels. Mean irradiance threshold levels (± SE) between Honey Creek Cave (green) and Honey Creek Surface (red). Asterisks (*) indicate a significant difference (Welch's T test, $P < 0.05$) between populations.

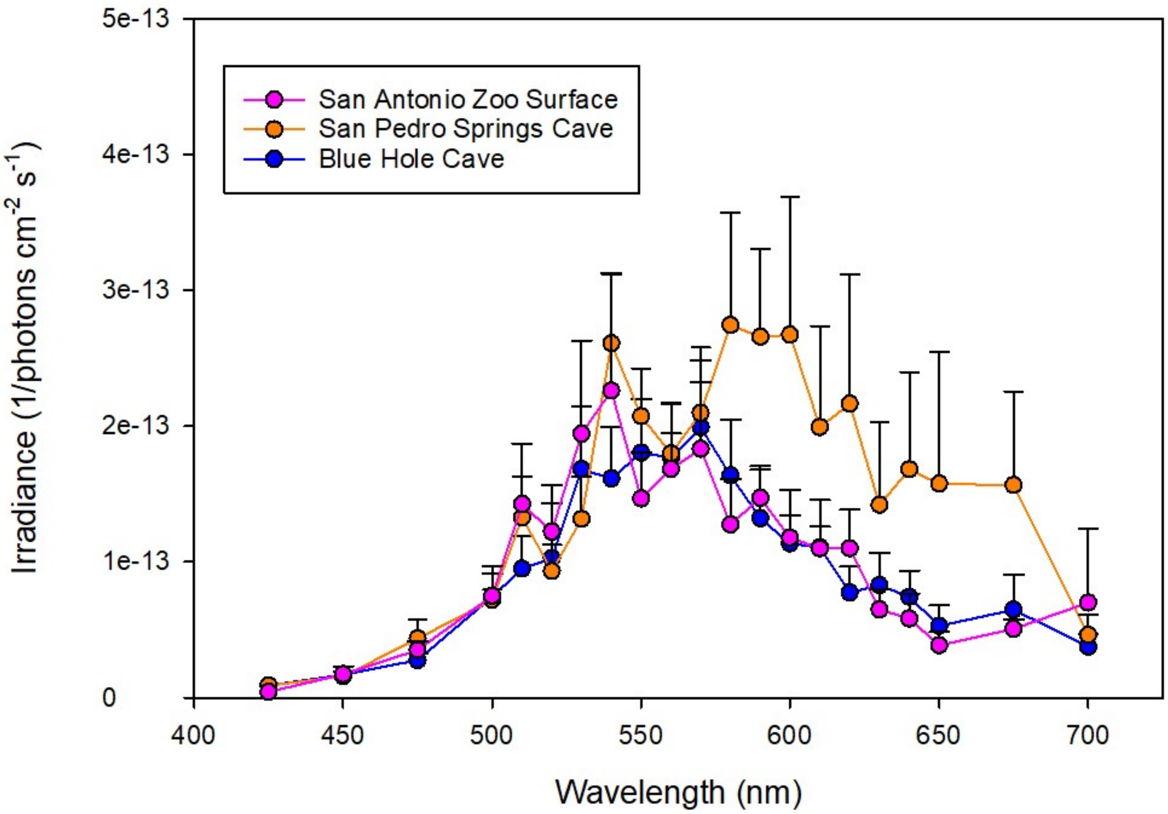

FIGURE 2.8: San Antonio Zoo, San Pedro Springs, and Blue Hole ERG threshold levels. Mean irradiance threshold levels (± SE) between San Antonio Zoo Surface (pink), San Pedro Springs Cave (orange) and Blue Hole Cave (blue). Black asterisks (*) indicate a significant (Kruskal-Wallis with post-hoc pairwise Wilcox w/ Bonferroni correction, $p < 0.05$) difference between San Antonio Zoo Surface and Blue Hole Cave. Red asterisks (*) indicate a significant difference between San Antonio Zoo Surface and San Pedro Springs Cave.

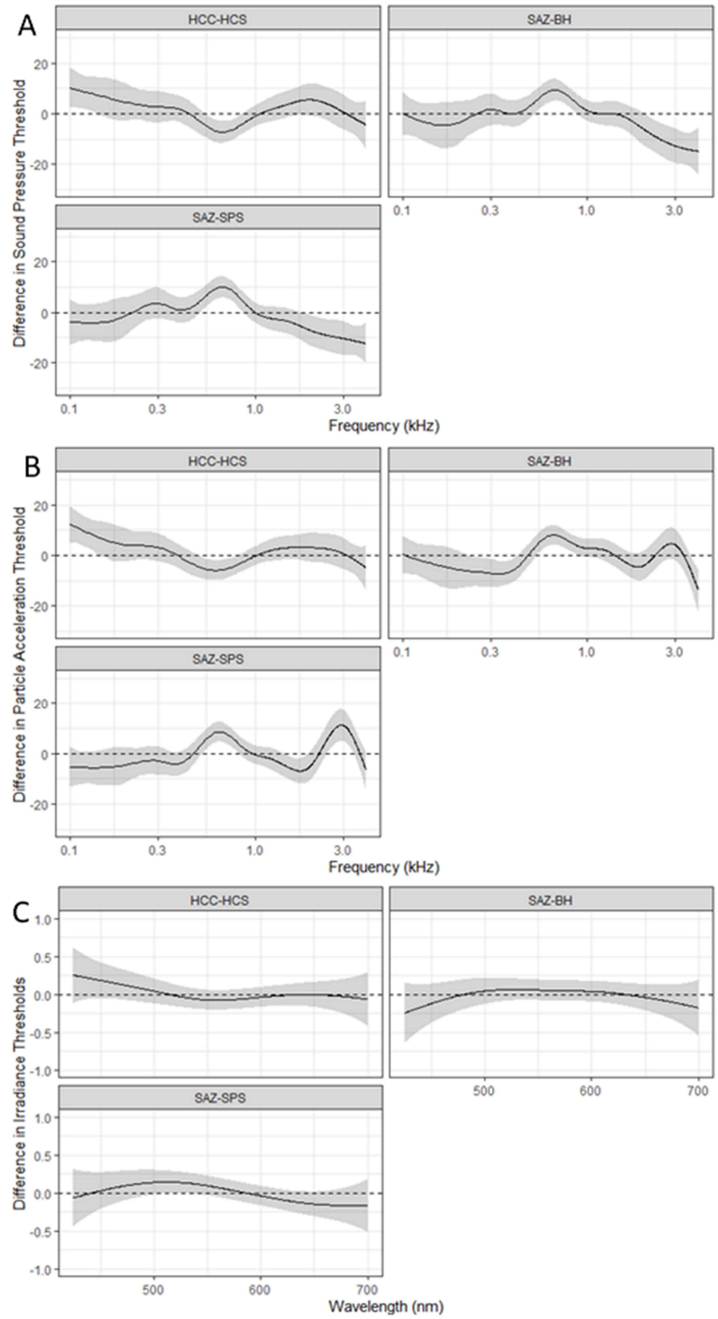

FIGURE 2.9: Comparisons using Generalized Additive Models (GAMS). Graphs showing the calculated difference between population smooth functions generated via generalized additive models. Significant pairwise differences are indicated where the 95% confidence interval band does not overlap with zero. Graph shows AEP (A), PAL (B), and ERG (C) threshold data of Honey Creek Cave (HCC) to Honey Creek Surface (HCS), San Antonio Zoo Surface (SAZ) to Blue Hole Cave (BH) and San Pedro Springs Cave (SPS).

Chapter 3

Contextualizing Rapid Divergence in *A.mexicanus*

Conclusions

The purpose of this research has been to describe the auditory and visual sensory capabilities of recently diverged populations of the Mexican Tetra, *A.mexicanus*. We hypothesized that although the Texas populations have diverged within less than 100 years, satellite *A.mexicanus* found in cave environments would have significantly lower sound pressure and particle motion thresholds than satellite *A.mexicanus* surface fish in an adaptive attempt to better exploit environments with fewer visual cues. We also predicted that Texas cave satellite *A.mexicanus* populations would have significantly higher dark-adapted spectral sensitivity when compared to surface satellite populations in order to better perceive what little light did come into the cave environment.

Our study has revealed significant differences in auditory sensitivity and particle acceleration between Honey Creek cave and surface populations, with some differences found between San Antonio Zoo surface and the presumed cave environments of San Pedro Springs and Blue Hole. While a significant difference was found between Honey Creek cave and surface dark-adapted spectral sensitivity at 530 nm, no significant overall trends were found between populations in relation to dark-adapted visual sensitivity. This chapter will serve to contextualize the results of our study and provide future directions for work with Texas satellite *A.mexicanus*.

3.1 The Acoustics of Cave Life

Underwater soundscapes have several qualities that differentiate it from terrestrial soundscapes. Underwater sound travels about five times faster than sound above water, which in turn results in soundwaves being carried farther thanks to the lack of absorption by the environment (Putland et al., 2019). Sound propagation in water is also dependent on pressure, temperature, salinity, and pH. Shallow water may impede some frequency propagation because the shallow depth cuts off the sound wavelength, acting as a high-pass filter (Ladich & Winkler, 2017) and potentially encouraging hearing sensitivity at higher frequencies (Amoser & Ladich, 2005). There are two main sources of sound in cave underwater environments: abiotic sources (flowing water, wind, or water dripping from stalactites) and biotic sources (other organisms).

Caves are isolated environments that require further study and characterization, especially pertaining to biologically relevant acoustic soundscapes. Several previous works on cave acoustics are concerned with anthropogenic archeology, such as the acoustics of prehistoric painted caves (Fazenda et al., 2017) and the use of cave environments for art and recreation based on their resonant qualities (Iannace & Trematerra, 2014). Few studies have documented the soundscape within caves, partly because of the difficulty of reaching subterranean environments (Christman et al., 2016; Culver et al., 2004).

However, cave environments offer an ideal case study into evolution, being that caves are documented to be consistent habitats with little change in temperature, humidity and light availability (Poulson & White, 1969). Convergent evolution of troglomorphic traits in cave animals can be found across various geographic locations due to this similarity of cave systems (Protas & Jeffery, 2012). Cave systems not only rarely have environmental fluctuation, but also are characterized by reduced or absent biotic and abiotic cues, such as the presence of day/night cycles, (Friedrich, 2013), few trophic levels (Espinasa et al., 2017), and nutrient availability (Riddle et al., 2018). Because of the absence of these cues, cave organisms tend to increase their sensitivity in senses other than vision to better exploit the information available in these static environments (Protas & Jeffery, 2012).

Particle Acceleration Thresholds

The bodies of fish have a similar density to that of the surrounding water, such that the whole body vibrates when a sound wave passes through the water (Nedelec et al., 2016). Fish auditory systems have adapted to exploit this source of environmental information through structures like the lateral line that detect particle motion. This has been confirmed with particle motion studies (De Vries, 1950), although some of the research has been restricted due to limited developments of equipment to quantify particle motion (Popper & Hawkins, 2018).

Previous studies with *A.mexicanus* have heavily documented what is known as vibrational attraction behavior (VAB) in troglomorphic cavefish. Studies carried out both within an artificial lab environment and natural Mexican cave systems containing cave *A.mexicanus* have shown an increase in response to vibrational stimuli in cavefish when compared to surface fish (Yoshizawa et al., 2014; Yoshizawa & Jeffery, 2011). It is suggested that VAB assists in finding food (Yoshizawa & Jeffery, 2011), although recent studies have suggested that this trait is highly plastic and dependent on the amount of organic debris in cave pools (Espinasa et al., 2021). While it is uncertain whether VAB is related to the detection of particle motion from sound, we hypothesized that satellite cave *A.mexicanus* would have more sensitivity to particle acceleration that satellite surface *A.mexicanus* in an evolutionary attempt to better sense dark cave environments. This hypothesis was supported, with the results showing that Honey Creek cave fish were more sensitive to particle acceleration that Honey Creek surface fish. Additionally, significant differences were found between San Antonio Zoo surface and the Blue Hole and San Pedro Springs cave populations, with cave fish being more sensitive to particle acceleration. Our results suggest that it has taken less than 100 years for divergence in auditory sensitivity to occur, specifically particle motion sensitivity.

Auditory Evoked Potentials

All fish have inner ear structures which convey information about gravity, acceleration, and sound (Hawkins, 2014). Ray-finned fishes (*Actinopterygii*) appeared approximately 450 million years ago and comprise 95% of all living fish species, as well as half of all known vertebrate species (Near et al., 2012; Volff, 2005). Approximately 99.8% of Actinopterygians fall into the group of *Teleostei*, or bony fishes, which have additionally radiated into almost 30,000 species (Volff, 2005). Ostariophysans, which possess additional auditory structures that detect sound pressure, form a subset of this grouping, and comprise 64% of freshwater fish species (Chen et al., 2013).

While all fish can detect particle motion, only ostariophysans are proposed to have the ability to detect sound pressure thanks to structures such as the Weberian Ossicles and other accessory connections between the swim bladder and inner ear (Lechner & Ladich, 2008). There are several theories proposing how different evolutionary pressures may have led to certain structural patterns and hearing sensitivities within ostariophysan fish, which include *A.mexicanus*.

The first AEP study in fish, also referred to as the auditory brainstem response, was published in 1998 (Kenyon et al., 1998) and led to research of auditory sensitivity across more than 100 species using this technique (Ladich & Fay, 2013). This electrophysiological technique was popularized thanks to the noninvasive and rapid nature of the assay, as well as its successful application in diverse animal models (Xiao & Braun, 2008). While it is a standard methodology in animal sensory studies, AEP research also comes with certain drawbacks. One drawback is the fact that measures of neural activity at sites of interest do not necessarily correspond to biologically relevant sensory thresholds. Physiological responses do not necessarily equate to active perception (Ladich & Fay, 2013), and critics of this methodology argue that thresholds found in AEP studies are usually much higher than those found in behavioral studies (Popper et al., 2019). However, proponents of electrophysiological techniques contend that AEP measurements are independent to behavioral methodologies and offer a different perspective to the same question (Ladich & Fay, 2013).

Electrophysiological experiments of auditory sensitivity have been successfully conducted in several families of ostariophysans, including closely related serrasalmids (Mélotte et al, 2018) and cypriniforms (Nissen et al, 2019), suggesting that the auditory evoked potential (AEP) methodology would be appropriate for *A.mexicanus*. Being that there is no standard hearing sensitivity across species of fish, or even fish within the same family, this study sought to compare relative differences in hearing between fish across newly colonized environments. While there are certain benchmarks established by previous studies regarding hearing sensitivity in *A.mexicanus* (Popper, 1970), there have been no previous studies working with these specific satellite populations from Texas. Additionally, while original landmark experiments with this species of fish have been cited several times, no new studies have sought to characterize hearing sensitivity in *A.mexicanus* using updated electrophysiological techniques.

We hypothesized that although previous work with *A.mexicanus* showed no significant difference in hearing sensitivity between cave and surface morphotypes (Popper, 1970), these recently diverged populations may show auditory divergence, with satellite cave fish having more sensitivity than satellite surface fish. Our study revealed that across all five satellite populations, the highest auditory sensitivity of *A.mexicanus* was in the lower frequency range between 0.1 – 0.5 kHz, with the least sensitivity shown above 1 kHz. Additionally, the AEP sensitivity curves of all populations had the highest sensitivity at 0.5 kHz. These results line up with previous study on Characid fish hearing thresholds that show the highest hearing sensitivity between 0.05-0.9 kHz (Mélotte et al., 2018).

We noted that while comparisons between satellite cave and surface particle acceleration sensitivity revealed significant differences across several frequencies, there were fewer frequencies where the sound detection sensitivity was significantly different between populations. While ostariophysans are commonly referred to as “hearing specialists” due to their connection between the swim bladder and inner ear, many researchers have attempted to dispute this notion, being that it has not been experimentally shown that ostariophysans possess greater hearing capacities than non-ostariophysan fishes (Popper & Fay, 2011). Our results seem to suggest that divergence is occurring mostly in particle acceleration detection, which could indicate to the extent to which the detection of particle acceleration is biologically relevant to survival in cave environments.

3.2 Visual Adaptation in Dark Environments

Adaptation to dark environments is observed not only in cave ecosystems, but also in deep sea communities. The deep sea, similar to caves, provides a case study in organismal adaptation to extreme environments previously thought uninhabitable (Paulus, 2021). Countless studies have recorded unique adaptations to the abiotic challenges of deep sea environments, including high hydrostatic pressure, darkness, cold, and scarce food sources (Robison, 2004). Similar to cave environments, some fishes exhibit evidence of convergent evolution, such as the convergence of cellular mechanisms necessary for survival at such depths (Shen et al., 2019; Weber et al., 2020).

One trait heavily studied among deep sea fish is the evolution of retinal structure and sensitivity (E. Warrant et al., 2003). Unlike troglomorphic organisms, who are found to consistently lose visual sensitivity and function over evolutionary time (Stern & Crandall, 2018), deep sea fish typically exploit the little light available in deep water environments through the evolution of unique retinal adaptations suited to little light availability (Collin & Partridge, 1996). Visual adaptation in the deep sea is dominated by two major factors: bioluminescence, and the change in visual environments over different depts (E. Warrant et al., 2003). Bioluminescence is a biotic factor that has many different ecological functions for different organisms (Rees et al., 1998), but the way light filters through the ocean is an abiotic factor that applies evolutionary pressure on all organisms occupying different oceanic depths (E. Warrant, 2004).

While deep sea organisms appear to be a suitable case study to compare visual evolution in cave systems, there is the added complexity of variation in freshwater visual environments. Light passes through water differently depending on a variety of environmental factors, such as salinity and temperature (Pegau et al., 1997). The scattering of light allows for certain wavelengths to be filtered and others absorbed: longer wavelengths of light (red, orange) are absorbed at depths of 10-30 m, while shorter wavelengths of light (blue, green) reach farther depths of more than 200 m (Webb, 2017) (Figure 3.1). This means that while the ocean is heavily "blue-shifted", or allows for the penetration of shorter wavelengths of light, freshwater environments are typically "red-shifted" thanks in part to water turbidity and suspended organic material that favorably absorbs wavelengths of light above 500 nm (Enright et al., 2015; Toyama et al., 2008).

While a direct comparison between fresh and saltwater environments is not possible due to the factors stated above, we used evidence from the study of deep-sea organisms to hypothesize that cave *A.mexicanus* would initially exhibit higher sensitivity to light prior to the degradation of the visual system. Although no case study has been done to determine how quickly visual structures regress in troglomorphic organisms, ancestral *A.mexicanus* may have adapted their visual system initially towards high sensitivity in order to take advantage of what little light availability could be found in caves, similar to the development of highly sensitive retinas in deep sea organisms. However, our results showed the opposite: Honey Creek surface fish were significantly more sensitive to light than Honey Creek cave fish at 530 nm. No significant differences were found between the two populations at any of the other 20 wavelengths of light tested. Additionally, no significant differences were found in visual sensitivity between San Antonio Zoo surface, San Pedro Springs cave, or Blue Hole cave.

While it is surprising that auditory divergence is being observed prior to visual divergence, this may be the result of several factors, one being the time it takes to evolve certain phenotypic features due to the genetic structure of such traits. Phenotypic differences among individuals in a population are attributed to both genetic and environmental sources (Willmore et al., 2007). Complex traits are characterized by the fact that they are controlled by many genes and environmental factors (Goddard et al., 2016). Eye degeneration in *A.mexicanus* has been identified as a complex trait thanks in part to experimental genetic crosses done between cave and surface ecotype fish (Ma et al., 2020; Strickler et al., 2007). Because the genetics of eye structure and function in *A.mexicanus* have compounding factors affecting development, we may not be able to see divergence yet simply because not enough time has passed to allow for these changes to precipitate.

Another factor that may explain lack of difference in visual sensitivity between satellite populations is the availability of light in different cave zones. Subterranean habitats are zoned into different categories based on environmental characteristics, such as light availability, moisture, and air flow (Howarth & Moldovan, 2018). The three main zones in a cave are the entrance, twilight, and dark zones, with the dark zone additionally subdivided into transition, deep, and stagnant air zones (Poulson & White, 1969). The environmental factors found at each zone have influenced the structure of the microbial and animal communities that inhabit these cave areas (Manenti et al., 2015; Mazina et al., 2021; Simões et al., 2015). The majority of sampled satellite cave fish were collected from the entrance and twilight zones of the three caves sampled. We cannot exclude the possibility that sampled fish were exposed to more light than fish residing in darker cave zones. Being that visual regression is a complex trait, it is possible that the populations sampled receive enough light to prevent regressive changes from occurring in the visual system.

Several factors can affect the penetration depth of light in aquatic environments, such as phytoplankton or dissolved organic matter, which reduce the intensity and wavelength of light transmitted through the water (E. J. Warrant & Johnsen, 2013). This may explain one of the trends found in the visual sensitivity curve graphs. Although no significant differences were found between San Antonio Zoo surface, San Pedro Springs cave and Blue Hole cave, the San Pedro Springs cave visual sensitivity curve showed increased sensitivity between 575 and 675 nm when compared to the two other populations. This is indicative of "red-shifted" light sensitivity and may be the result of unique environmental factors only found in the San Pedro Springs cave population area.

While there are no robust differences between populations, the differences seen in the sensitivity curvatures between Honey Creek cave and surface may indicate the beginning of visual divergence and necessitates further monitoring of these populations. Additionally, while San Pedro Springs, Blue Hole Cave and San Antonio Zoo surface show no significant differences in visual sensitivity, the red-shifted curvature seen in San Pedro Springs cave fish merits further examination of the sampled environment in comparison to the two other populations.

3.3 Future Directions

Previous studies have documented "rapid" physiological adaptation in fish occurring between the span of 15,000 years (Peichel et al., 2001) to 50 years (Divino et al., 2016; Dunlop et al., 2018), and even within single generations (Laurentino et al., 2020). To this extent, finding sensory divergence in 100 years or less is within the realm of possibility. However, several factors identified in our study necessitate further examination to better contextualize the rapid divergence observed within these five satellite populations.

The most pertinent work to be done following our study is a comparison between these satellite populations and native Mexican populations. While native hearing thresholds have been previously established via behavioral methodologies (Popper, 1970, 1971), a reexamination of these thresholds using the methodology described in this study would allow for direct comparison. While the most exigent comparisons would be between Mexican cave *A.mexicanus* and satellite cave *A.mexicanus* to determine if similar particle motion and sound pressure thresholds are observed, comparisons between surface *A.mexicanus* and satellite surface fish could also reveal the extent to which surface fish characteristics are preserved in different environments.

In order to better compare native and satellite populations of *A.mexicanus*, further work needs to be done in characterizing the sampling environments. Several studies have recorded and examined cave sampling sites in Mexico, characterizing features such as cave temperature (Tabin et al., 2018), trophic ecology (Wilson et al., 2021), and oxygen level (Boggs & Gross, 2021). However, due to the recent discovery of these Texas satellite populations, no such documentations have been made about these environments. Being that our questions concern visual and auditory sensitivity, our results would benefit from data collection on light availability and acoustic soundscape of these cave environments. Passive recording via remote loggers have been successfully used in previous cave studies (Hyacinthe et al., 2019; Revilla Martín et al., 2020; Sugai et al., 2019), and light loggers can be programed to record data at regular intervals (Baker, 2014).

Phenotypic plasticity is an additional concern regarding the fish sampled in this study. While all fish assayed were wild caught, they had also been housed in laboratory environments for one year prior to testing. The unique plasticity of behavior and phenotype are not only an evolutionary adaptation to environmental variation (Sommer, 2020), but also a confounding factor in animal experimental research. Several studies have identified sources of environmental bias in animal research (Lewejohann et al., 2006). While methodology standardization helps to curb the confounding effects of plasticity in animal behavior (Chesler et al., 2002), further testing is always preferred for robust conclusions in behavioral animal research. Thanks to the diligence of the McGaugh lab, we are fortunate to have the recent opportunity to test Blue Hole cave fish that were spawned and reared in the lab. If testing finds similar thresholds in lab-reared fish to those sampled in the wild, we can exclude phenotypic plasticity as a source of error in our results.

The cave and surface ecotypes of *A.mexicanus* offer a unique example of successful adaptation to extreme environments. Furthermore, as evidenced by our study, the ecotype divergence of this species allows for exploration of sensory plasticity in response to environmental differences. The satellite populations of *A.mexicanus* in central Texas allow for the observation of trait divergence in real time and showcase the incredible adaptation capabilities of this fish within a documentable timeframe. We foresee further work expanding the knowledge established by this study and look forward to the continuous monitoring of this resilient organism.

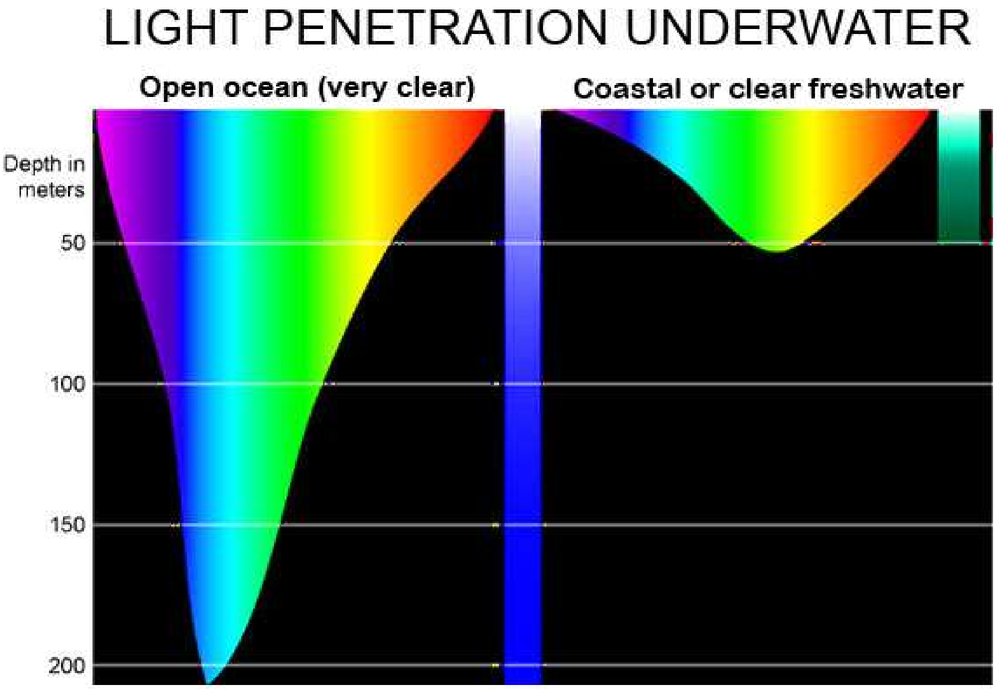

Figure 3.1. **Underwater penetration of different wavelengths of light**. How different wavelengths of light penetrate oceanic vs freshwater environments. Image courtesy of the National Oceanic and Atmospheric Administration

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