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A Five Million Year Old Experiment: The Evolutionary Dynamics of a Cave Environment

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A Five Million Year Old Experiment:
The Evolutionary Dynamics of a Cave Environment.

Tyler Bussian
2015

Sponsors: Joshua Gross, Jim Benedix
Committee: Tim Cope, Brian Carlson

OUTLINE

INTRODUCTION

Over the summer of 2014, I was given the opportunity to research at the University of Cincinnati to study the cavefish *Astyanax mexicanus*. Admittedly, I had never heard of the peculiar species of fish until that summer, but those pink-colored, eyeless fish were quite the specimen. Over my time there, I became more familiar with lab work and cave animals than I ever thought possible. The more papers I read and examined, the more I wondered about how these species came to lose their eyes and pigment.

The goal of this thesis is to satisfy my curiosity on the origins of cave organisms. It attempts to collect and summarize general trends about these animals and place them together to try and tell a story about their creation. By focusing on the cave environment, we can gain a sense of the setting that caused these species to evolve these cave-adapted traits. While some of these traits are constructive and easily explainable, other regressive traits require further examination for they continue to cause disagreement among scientists. While cave organisms provide an example of the effects of a very specific set of selective pressures, there are some negatives associated with research using them as model species.

My committee, Joshua Gross, Tim Cope, Jim Benedix, and Brian Carlson all deserve multiple pages of thanks for their continued help and support throughout this project. Additionally, I would like to thank the DePauw Honors Program for creating a program that has allowed me to not only research this fascinating topic, but also personally develop into a more learned person through four years of classes and curriculum. Finally, I would like to thank my parents for always pushing me in the right direction.

I GEOLOGY

In Greek mythology there are few characters with as interesting of a story as Zeus. The ruler of the gods, Zeus was a lightning-bolt-throwing, womanizing, ethereal warrior who was known for sometimes causing more harm than good. However mighty Zeus was, though, he would not have been anything without the help of his mother, Rhea. Zeus's father, Cronus, had a bad habit of eating his children after they were born, for he feared they would one day overthrow and kill him. After Cronus had eaten five of his and Rhea's kids, Demeter, Hestia, Hera, Hades and Poseidon, Rhea decided that she would flee her husband when the time came for Zeus to be born. She hid in the Diktaean Cave in Crete, and gave birth to Zeus in its darkness. As the story goes, Zeus grew strong in that cave and was able to eventually overthrow his father and release his brothers and sisters from Cronus's stomach. (Hesiod. and Athanassakis, 1983)

Just as Dikatean Cave provided Zeus protection from his father, caves allow many organisms protection from a variety of environmental elements. Progression of the seasons, which on the surface is accompanied by drastic shifts in weather and temperature patterns, result in only miniscule changes in the cave environment. Caves are such a stable ecosystem that some researchers have deemed them "nature's laboratories", for their protective nature causes organisms to develop troglobitic, cave-dwelling, traits that are found nowhere else on this planet (Culver, Kane and Fong, 1995). Fluctuations in temperature, water flow rates, and even oxygen concentration all can determine what type of organisms a cave system can support, if any at all.

As important as these abiotic selective features of a cave are, however, one needs to understand how these caves were created in the first place and what types of changes these systems are undergoing currently. Understanding the physical environment's advance through time helps to show what features may influence the evolution of the populations within these systems. To better understand the development of caves, the El Abra Limestone deposit in Mexico will be examined. This region, west of the Gulf Coast, is home to the blind cavefish, *Astyanax mexicanus*, which will be used in future sections to highlight some of the more complex aspects of cave evolution. Although these regions are very important for scientific research in both the fields of biology and geology, a general lack of information in some of these areas has left scientists to greatly infer how some of these organisms have come to be. Therefore, more research in this area will be required to truly understand the origin of these unique organisms.

Limestone Caves and Abiotic Selective Pressures

A cave is a hollow opening that is found near the surface the surface of the Earth. While this definition seems straightforward, there are a variety of different types of these caves. Lava tubes are caves that are created as lava pours out of a volcano (Marshak, 2008). Oceans and seas erode caves into the sides of cliffs by repeatedly battering them with waves. Even glaciers can melt in certain ways to create underwater caverns. However, there is one type of cave more common than all of these on the planet, the limestone cave.

Limestone caves are formed by dissolution of calcium carbonate ($CaCO_3$). Limestone is made of calcite, a mineral form of calcium carbonate (Marshak, 2008). Limestone is formed through organic and inorganic precipitation of calcite from seawater. As these deposits of calcite accumulate over thousands and millions of years, burial and cementation create limestone. If limestone becomes exposed to meteoric water, either through erosion or fracturing of protective layers originally covering the deposit, the opportunity for weathering of the limestone emerges.

Meteoric water will not result in any changes unless it is somewhat acidic, though, since the calcium in the calcite will not readily leave the carbonate ions (CO_3) otherwise. Rain water can become slightly acidic by dissolving carbon dioxide (CO_2) in it to form carbonic acid. The calcite undergoes the process of dissolution, and the carbonic acid interacts with the calcite to form carbon dioxide and liquid water. The water then becomes saturated with the newly formed calcium atoms, which are held within the water until environmental conditions push the dissolution reaction in the reverse direction. This process is called precipitation, for the calcium rebinds with carbonate molecules and precipitates from the water as calcite (Marshak, 2008). Equation I.1 shows the chemical equation of calcite dissolution (left to right) and precipitation (right to left).

$$CaCO_3 + 2H^+ \leftrightarrow H_2O + CO_2 + Ca^{2+} \quad \text{(Equation I.1)}$$

This dissolution and precipitation can form many of the intricate and unique cave formations found in caves. For example, stalactites and stalagmites are created as this calcite solution drips from the roof of a cave. The water precipitates the calcite, which is deposited on the roof or floor of the cave. This repeated precipitation will accumulate over time to create cave rocks called dripstones. These accumulations, stalactites, stalagmites, and dripstone, are all referred to as speleothems.

Although limestone caves are not the only type of cave on the planet, they are the most common and largest. A general rule can be made about caves in that their environmental conditions vary relatively less than proximal surface environments (Culver, 1982). Even with a lessened amplitude, though, the slight fluctuations in these dark environments many times are enough to result in a reaction from the cave inhabitants.

The temperature of a cave usually mimics the average annual temperature of the surrounding surface location. Closer to the opening of the cave, the fluctuation in temperature is very similar to the temperature on the surface. This relationship diminishes the further you travel into the cave system, and temperature fluctuation sometimes becomes negligible. Crouau-Roy (1992) and his colleagues found that the population size of a troglobitic beetle, *Speonomus hydrophilus*, was correlated with the seasonal temperature fluctuations that were found within the Pyrenees caves in France. Between the months of June and July, temperature in the cave changed from approximately 11ºC to 14ºC, but after a month to respond, the population size increased seven fold by jumping from 100 beetles to 700 beetles from July to August (Crouau-Roy, Crouau and Ferre, 1992). For reference, the temperature fluctuation on the surface between June and July is about 15ºC to 23ºC (Crouau-Roy, Crouau and Ferre, 1992).

Another abiotic selective pressure that seems to dictate where cave organisms live is relative humidity. Cave species are extremely rare in caves with lower than 80% humidity, and most cave organisms live in or around open cave pools or streams (Culver, 1982). Although the mechanisms organisms use to sense and survive this high humidity environments will be discussed further in Section II, the benefits of sensing these changes allow insects to better position themselves to prevent dehydration or extreme saturation within the cave environment.

Temperature and humidity are selective pressures related to the air within the caves, but many abiotic pressures originate from the water that has been used to form the limestone caverns in the first place. The rate of flow of water is a dynamic that many different organisms are sensitive to. Caves with a high level of variance in water flow possess a less diverse ecosystem than caves with a smaller amount of variance (Culver, 1982). This is likely due to increases of mortality of weaker organisms when flow rates increase, as is the case with the troglobitic beetle, *Gammarus minus* (Culver, Kane and Fong, 1995). Heartier animals are able to withstand the increased water force. Along with this trend, though, an increase in flow rate also is accompanied by an increase in food availability, since more nutrient-rich surface waste can be washed into the cave. Therefore some animals time their breeding patterns to coincide with seasonal flow-rate increases to capitalize on the increased nutrient availability (Culver, 1982).

Along with flow rate, oxygen availability is usually quite high within cave waters (Culver, 1982). This is likely due to the water's ability to hold more dissolved oxygen since it is at a colder temperatures[1] (Pray, Schweickert and Minnich, 1952). However, some deep cave pools that do not have connecting access to a surface water source often have very low concentrations of oxygen. This is due to the microorganisms in the water undergoing respiration in the pools and consuming the free oxygen (Howard Perlman, 2015). These oxygen-void pools sometimes still contain life. Some pools in Banner's Corner Cave in Virginia have an oxygen concentration of 3 mg/L[2] but still contained a population of arthropods and planarians (Holsinger, 1966).

The final abiotic selective pressure, which may seem somewhat trite, is the lack of light. As we will examine in future sections, the presence of light in cave environments has a major effect on the rate of evolution of the animals within the cavern. The Sótano del Caballo Moro is a cave that has developed at the bottom of a doline or sinkhole (Espinasa and Borowsky, 2000). The cave has a large pool at its opening, which contains a population of both surface and cave *Astyanax mexicanus*. Figure I.1 shows a diagram of Sótano del Caballo Moro. The presence of the surface fish is somewhat confusing since there is no surface stream feeding into the cave, and

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[1] The relationship between oxygen concentration and water temperature is a trend that is only true under normal atmospheric conditions (Pray, Schweickert and Minnich, 1952).

[2] Normal concentrations for this type of pool should be between 7 and 12 mg/L (Holsinger, 1966)

the closest population of surface fish is almost 4 km away in the Río Boquillas (Mitchell, Russell and Elliott, 1977). The entrance of this cave is called a karst window, since sunlight is able to reach about halfway across the pond during the day. Although both fish are present, they seem to segregate themselves in the pond based on the presence or absence of light (Espinasa and Borowsky, 2000). While their presence is not completely understood to this day, it is generally agreed that the cave and surface fish developed from the same ancestral stock. It may be that the division has always been present in the cave, and the cave and surface forms interbreed regularly, or that the entire cave population originally developed cave traits, and the geological formation of the karst window has allowed reacquisition of eyes and pigmentation (Espinasa and Borowsky, 2000). In either situation, the presence of light is a major selective pressure since its presence is the primary variable in both hypotheses.

All of these different selective pressures within a cave have an effect on the organisms living there. In Section II, we will examine the results of these and other biotic pressures in the physical traits that troglobitic organisms develop to thrive in the cave environment.

Development of Sierra de El Abra Karst System

Although understanding the various abiotic selective pressures of a cave environment is important for future understanding of the evolutionary pressures exerted on organisms that reside in caves, to truly understand these systems requires us to look back even further to understand how these environments have formed these unique pressures. The Sierra de El Abra is a limestone ridge found to the west of the Gulf of Mexico in central Mexico where one of the most important research organisms, *Astyanax mexicanus* – the blind Mexican cavefish, originates

(Mitchell, Russell and Elliott, 1977). The unique evolutionary dynamics of this region would not have taken place if certain geological events had not happened millions of years earlier. (See Figure I.2)

The story of the Sierra de El Abra begins almost 150 million years ago (Ma) during the Late Jurassic period (Fish, 1977). The waters began to cover parts of what would be known as the Mesozoic Basin in Mexico, and with this covering a thin layer of red bed deposits was placed on the basin (Fish, 1977). Red bed deposits form sandstone and shale over time, and these sedimentary rocks began to accumulate on the margins of the platform. This deposit helped to create the Valles-Sán Luis Platform, which would serve as the base for the Sierra de El Abra Formation. At the beginning of the Cretaceous period, approximately 10 million years later, the majority of eastern Mexico had become covered in water, and reef growth began on the Valles-Sán Luis Platform (Fish, 1977).

As the Valles-Sán Luis Platform continued to deposit outward, the reef growth followed the expansion east. The original reef was about 8 kilometers wide, but over a span of 5 million years, from 99 Ma to 94 Ma, the platform and reef expanded out hundreds of kilometers (Mitchell, Russell and Elliott, 1977). The reef was a rudist reef, which was populated by large numbers of rudist clams that built the structural framework of the reef (Scott L. Cross, Robin G. Lighty, 1986). The Valles-Sán Luis Platform was very important to the formation of the Sierra de El Abra because it allowed the reef system to grow exceptionally well in the light-filled shallow waters that covered it. At about 93 Ma, the center of Mexico began to become uplifted relative to sea level, and erosion of this uplift generated terrigenous sediments (sediment that has been created from rocks on land), which covered the great reef (Fish, 1977, Mitchell, Russell and

Elliott, 1977). This ended the formation and growth of the reef system and hid the remnants of the great marine population from the surface of the Earth for millions of years.

At around 70 Ma, after the Del Abra Reef had been covered in silt and mud, the Laramide Orogeny began. The Laramide Orogeny is a regional geological event that happened over the span of approximately 30 million years (80 Ma– 55 Ma) where the Kula and Farallon plates, which at the time floored the Pacific Ocean, slid underneath the North American plate (English and Johnston, 2004). This event caused the formation of the Rocky Mountains in what is now the United States, and the Sierra Madre Oriental mountains in Mexico. While these mountain ranges were being formed, the Sierra de El Abra also was being uplifted. The terrigenous sediment overlying the reef system had compacted into a layer of impermeable shale, protecting the limestone of the Del Abra Reef from the elements. Uplift and erosion of this covering strata due to the Laramide Orogeny allowed the Del Abra limestone to be exposed to the elements again (Mitchell, Russell and Elliott, 1977).

The importance of the Laramide Orogeny to the current formation of the Sierra de El Abra limestone formation cannot be stated enough. Besides exposing the limestone to the elements, the exhuming of this formation has guided the development of the current fluvio-karst, the landscape and subterranean hydrology that has developed as a consequence of continued chemical and mechanical erosion by moving water (Ford and Williams, 2007). The Laramide Orogeny caused fracturing of the limestone deposit as it was being exhumed. These fractures are the cracks that meteoric water has followed to create the caves within the region. As time passes, dissolution occurs first along these crack lines in the limestone and expands the fissures into karstic formations. These initial cracks allow for complex cave systems to occur throughout the Sierra de El Abra limestone. Without these cracks, erosion would have occurred on the surface or by enlarging the initial porosity of the limestone, meaning the karstic landscape would evolve very differently without the Laramide Orogeny. The types of caves that have allowed for the isolation and development of cave animals, specifically the cavefish *Astyanax mexicanus*, are due to these fissure-guided karstic formations.

The evolution of the karstic landscape based on this fissure variation has caused a variety of different surface and subterranean features to develop. On the surface, these limestone formations can fall into one of three water flow roles: input, throughput, or output (Ford and Williams, 2007). Input sites, where surface water is directed underground, usually are the most common. The Sierra de El Abra has two distinct input types, stream captured sótanos, which are large eroded vertical shafts in the limestone bed, and swallets[3], sinkholes that form as erosion underneath the surface removes support from the limestone above (Ford and Williams, 2007).

Stream capture is the process of a surface stream or river cutting down and entering a cave with a completely vertical entrance (Mitchell, Russell and Elliott, 1977). The process begins with a stream eroding the protective layer above the limestone formation. The river slowly cuts through the rock to the softer limestone, and will continue to cut down evenly unless it hits a fissure or crack in the soluble rock. Powered by gravity, water will follow the fissure network until it finds a way to exit the porous limestone. Over time, the entrance fissure will widen, and more of the river will be diverted underground until the opening is wide enough that all of the water enters the opening. When this occurs, the river becomes a waterfall that flows underground. Headward erosion will continue on the side of the opening that the river is tumbling over, and eventually the surface river will cut down to the level of the underground

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[3] Swallets/sinkholes can also be referred to as dolines (Kohl, 2001)

river, which is held stable by a lower level of impermeable rock (Mitchell, Russell and Elliott, 1977). (Figure I.3)

Swallets or sinkholes are another input surface formation that results in a large shaft in the karstic landscape. Unlike stream capture, which is created with dissolution caused by water running in a vertical direction, sinkholes are created as the limestone underneath the surface is slowly dissolved away[4]. As this dissolution begins to create a void underneath the surface, the structural integrity of the surface landscape becomes weakened and eventually a collapse occurs. Sinkholes can vary in initial size from a few centimeters to a few kilometers, but this initial size will increase due to weathering of the newly exposed limestone (Zhou and Beck, 2007). Unlike sótanos, which develop an entrance to a cave, sinkholes collapse above areas with cave formations beneath. This means that a subterranean pool harboring cave animals may become exposed to the elements, causing a change in the selective pressures working in this system.

The majority of the surface formations in karstic landscapes serve as input landforms, but throughput and output forms also have an effect on the evolution of the surface topography. Throughput features, called poljes, usually are found at the bottom of valleys and flat-floored depressions (Ford and Williams, 2007). These areas serve as a closed collecting basin for many subsurface water systems, and guide the waters across the karst landscape and back underground. Poljes can function as broad erosion planes and create large gorges in the landscape, or they can serve as an environment for precipitation of the calcite solution to occur on the surface (Ford and Williams, 2007). These transitional waters between subsurface flows also are a major location for nutrient deposit. Poljes send plant detritus or even deceased animal carcasses beneath the

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[4] This method of sinkhole formation is specific to geological features in dissolvable rock such as limestone. Other types of sinkhole formation can occur based on the material undergoing modification (Zhou and Beck, 2007)

surface, meaning they serve as a collection for nutrient sources for the cave animals found under the ground.

Output karst forms are areas where subsurface water systems expulse. They are commonly referred to as springs (Ford and Williams, 2007). Discharge occurs at springs if the spring is at a lower elevation than the level of the water table elsewhere in the karst network. Meteoric water travels downwards until it reaches impermeable rocks or until it reaches standing water in the subsurface (the water table). If the subsurface water finds a pathway back to the surface, it will travel upwards due to the pressure caused by the higher elevation of the water table where it was collected (Fish, 1977). Additionally, if the water travels deep enough, the collected meteoric water may mix with warmer groundwater creating hot springs (Ford, 2000).

Surface components of the karst landscape are not permanent features. As dissolution and weathering occur, output springs may transform into input caves, or throughput poljes may capture streams and instead function as an input feature. The important aspect of limestone karsts is that they are dynamic systems. A general trend following this evolution is the change from overall surface drainage to spring drainage east of the Sierra de El Abra. The Valle de Antiguo Morelos east of the Sierra de El Abra, found between the Sierra de El Abra and the Sierra de Nicholás Perez (Figure I.2), at one time had a surface river that served as a large drainage system. The stream cut across the Sierra de El Abra limestone formation and ran east towards the Gulf of Mexico. North of this location, however, where the western Río Boquillas crosses the Sierra de El Abra and forms the Río Commandante, waters began to flow in the northern direction. This flow caused headward erosion of the surface rock in the region, and deepened the valley from the north to the south. As the elevation of the valley dropped the surface drainage changed directions and traveled along rivers northward (Fish, 1977; Personal Contact).

This northward shift had a variety of effects on the Valle de Antiguo Morelos, but one of the most significant was the lowering of the local water table in the region. Springs that originally served as resurgence areas and output formations for collected water became relict karsts, which are limestone formations that become removed from the fluid system as the water table lowers. Relict karsts serve as input karst systems, which means more meteoric water is sent beneath the ground and expelled out of springs at a lower elevation. This karstic evolution formed many of the caves that would become refuges for clutches of the *Astyanax mexicanus*, since they would capture ancestral surface populations and usher them underground.

The ever-changing karstic landscape of the Sierra de El Abra has created a plethora of caves with a variety of unique selective pressures, and these formations can be classified on a number of different features and traits. The best way to really capture the differences between these caves is to classify them based on their level of isolation. Isolation can be defined in two ways, either as isolation from the surface environment or isolation from other connecting caverns and karstic formations. Isolated caves, like those which were formed from the conversion of spring to caves with the dropping of the water table, are important for the study of cave animal evolution since in general higher degrees of isolation over longer periods of time result in more pronounced cave-like traits (Culver, 1982).

Cave Profiles Introduction

Karstic evolution has created cave formations with varying degrees of isolation. To display some of this variance, the profiles of two of the more important caves that have lineages of *Astyanax mexicanus* within them are below. La Cueva Chica, which is considered to lack isolation and La Cueva de El Pachón, which is one of the most isolated caves will serve as two examples. These profiles are modified from Mitchell, Russell and Elliott 1977, and a complete collection of cave summaries of the Sierra de El Abra can be found there.

La Cueva Chica Profile

La Cueva Chica, hereby referred to as the Chica cave, was the original cave where researchers found the blind Mexican cavefish. In 1936, fish collectors traveled into the caves to find new specimens to sell back in the states. After their discovery, the New York Aquarium visited in 1940 to also perform research. It was not until 1970 when Mitchell and other researchers visited the caves that an accurate survey was made. The previous two were considered to be erroneous due to discrepancies between individuals' recordings.

The Chica cave has been surveyed to a length of approximately 320 meters from its entrance, which is about 50 meters above sea level. It contains four distinct pools that seem to run into one another. After traveling about 90 meters, the dry floor of the cave runs into the first two pools. Pool I seems to drain into the nearby Pool II through an underground passage, and Pool II drains over a 50 meter incline and over a 2 meter drop into Pool III. Pool III then drains into the largest Pool IV, which ends in a siphon to another body of water (Mitchell, Russell and Elliott, 1977). The number of fish in this cave varies since it is not a closed system, but most of the fish reside in Pool IV (Figure I.4).

This cave is considered by many scientists to be atypical of most caves in the Sierra de El Abra region. There are a few reasons for this belief; the first being the large population of bats. The Chica cave has a significant bat population within it, so significant that early surveyors noted the cave as “a nightmare of slime and the stench of bats” (Mitchell, Russell and Elliott, 1977). While the importance of this feature will be highlighted in future sections, the presence of a bat roost can be considered a direct nutrient source to animals found within the Chica cave. This has drastically changed the way evolution has occurred in Chica compared to other caves in the region. The other major feature of Chica that is not found in other caves is the migration of fresh surface stock into the cave. It has been theorized that the siphon found in the back of Pool IV is a direct connection to a nearby river, the Rio Tampáon (Mitchell, Russell and Elliott, 1977). Although not experimentally confirmed, the siphon positioning in the pool, the pool’s elevation, and a noted sand bar, which would be created by a diverted water flow, found in a plausible location in the Rio Tampáon all strongly suggest a connection is present through the pool siphon. Additionally, the presence of surface and cave *Astyanax* in Chica signifies that surface fish are entering the cave in some fashion. Thus the cave is not considered an isolated entity.

La Cueva de El Pachón Profile

Unlike the Chica cave, which has an opening formed by runoff and is continually supplied by an external source of water, the La Cueva de El Pachón, hereby referred to as the Pachón cave, is completely removed from any external rivers or streams. Additionally, it seems that the single pool within the cave has no outflow passage, so the fish in the Pachón cave will not have the opportunity to ever leave. This isolated cave has resulted in some of the most drastically modified fish in the Sierra de El Abra, and therefore has produced the prime test subject for many researchers using *Astyanax*.

The Pachón cave entrance, which is a largely abandoned resurgence, is located high on the western slope of the Sierra de El Abra. The entrance is found about 210.5 meters above sea level, and appears to still act as a wet weather resurgence. The cave is one passageway that runs about 210 meters long (Mitchell, Russell and Elliott, 1977). After 160 meters of a dry, downward sloping ground, an 8-meter-deep lake is found that runs about 50 meters long. This single lake holds upwards of 8,500 blind *Astyanax* (Mitchell, Russell and Elliott, 1977) (Figure I.5).

Compared to the Chica cave, which possesses a massive bat roost, there are much less nutrient options for the Pachón fish. There is a small bat roost found about the cave, but it is much less populated than the Chica roost. The cave seems to gather discarded plant debris from an unknown location during rains. This debris helps to feed the fish within this underground pond (Mitchell, Russell and Elliott, 1977). Fish from the Pachón cave are considered to express the most drastic versions of troglobitic traits, and it is likely that the population diverged from the surface population a very long time ago.

The Pachón cavefish were likely trapped in the cave after the karstic landscape shifted from a single river-based drainage system. The position of the cave places it near the ancient river that cut across the Sierra de El Abra. Its elevation and cave formation suggest that it served as a spring to create a tributary for this drainage river (Personal Contact). As the water table dropped due to the erosion of the valley, the cave shifted from an output to an input site. If any ancestral surface fish were in the Pachón cave before this conversion, it is likely that they became trapped in this cave.

Requirements for Future Research

While there has been much interest in the Sierra de El Abra region in Mexico and a recent resurgence in the importance of *Astyanax* with the development of molecular biology techniques to study genetic differences between surface and cave populations, there is a notable lack of recent geological data from the region. To further understand the intricacies of *Astyanax mexicanus* and the dynamics which have resulted in the variances noted between different cave populations, a better understanding of the caves themselves is required. The most recent geological data that is readily available to the scientific community is a short study done in 1998 by a researcher named Camargo that was more interested in developing the region for oil extraction. The Sierra de El Abra requires a better knowledge of the geological features that are not easily seen by the naked eye.

One of the primary questions surrounding the Sierra de El Abra region is the extent of subterranean connections between different caves. The number of times a population interacts with other populations can affect the rate at which evolution occurs. If there are a large number of connective elements between caves, populations which may actually be extremely old can appear to be young.

There are two noted ancestral stocks of surface fish in the history of *Astyanax mexicanus*, an old and a new stock (Gross, 2012). The old stock populated the Sierra de El Abra either approximately at 8 Ma with the closure of a land bridge that allowed the fish to migrate north or the loss of a land bridge that connected Panama and Columbia 3.3 Ma. The new stock was thought to have originated approximately at 2.1 Ma (Gross, 2012; Espinasa and Borowsky, 2001). To determine these two ancestral stock ages, the level of connectivity and repeated mating between populations is a calculation factor. The rate of evolution depends on that fact, but many times the information must be inferred due to limits on understanding of the geological history of the region. It is impossible to pinpoint exactly when the old and new stocks were present in certain parts of the valley, but by gaining a better understanding of the current subterranean connective systems, we can better hypothesize the dynamics of inter-cave relations. These limestone caves are continuously changing due to repeat dissolution and precipitation of calcite. Without a clear picture of what is currently there we cannot hope to understand how these passageways may have changed over the years.

Besides gaining an understanding of how caves may be connected by subterranean passageways, another important feature that needs to be recorded is the age of these caves relative to their emergence. This could be done by dating various dripstone formations in the caves. While stalactite and stalagmite formations do not represent when a cave was first being created, they do serve to date when the cave first started to precipitate calcite. These dripstone formations serve a similar purpose as the rings within a tree trunk. They can give information such as age of the cave and can be interpreted to show environmental factor variation such as periods of warm and cold climates (Harmon, 1975). For example, Harmon (1975) collected two speleothem specimens from the Sierra de El Abra region. From these two samples, cold and warm climate changes could be determined to a degree of thousands of years. This level of detail may help scientists can gain a better understanding of what conditions populations may have been subjected to during different time periods. This may help to better classify and define differences between caves in the Sierra de El Abra.

While it is easy to see elements of geological research in the Sierra de El Abra that are currently lacking, it is important to understand why this region has limited research done on it. There is a general tendency of scientists to study topics that are relatively close to them. This is why the majority of the limestone caves in the United States and Europe are well mapped and understood, because they were easy to research. Scientists of the past were unable to amass large amount of data on the Sierra de El Abra due to the fiscal requirements of traveling to the region. However, with current scientists, the problem has shifted. Now, the issue is related to safety of the researcher in this part of the world. Mexico is no longer a simple destination for research as it was in the 1970's when Fish and Mitchell both made numerous expeditions down to study the karstic landscape. Current cartel violence and an inadequate state security force make any expedition to Mexico dangerous. Also, a lack of well-developed infrastructure and difficult terrain in the Sierra de El Abra make it tough to move equipment into the region (Mitchell, Russell and Elliott, 1977).

Conclusion

Although this thesis primarily is focused on the biological evolution that occurs within a cave environment, the evolution of the environment is a driving factor of this phenomenon. Abiotic selective pressures such as temperature, water flow rates, and even light availability all affect how these populations will evolve as they reside in these caverns. In the case of *Astyanax mexicanus,* some of the most unique troglobitic adaptations would never have been developed unless the geological landscape had been modified to form highly isolated caves.

The importance of this phenomenon highlights why scientists need more information on the geological history of the Sierra de El Abra limestone formation. Geologists can learn a lot from the karst formations in the region, and more context can be given to biologists to better understand the evolutionary dynamics that cause variation among populations of *Astyanax*.

Unless researchers can focus more attention to this region, truly understanding the origins of these animals will be similar to trying to get through a mythological labyrinth without a piece of golden thread guiding the way.

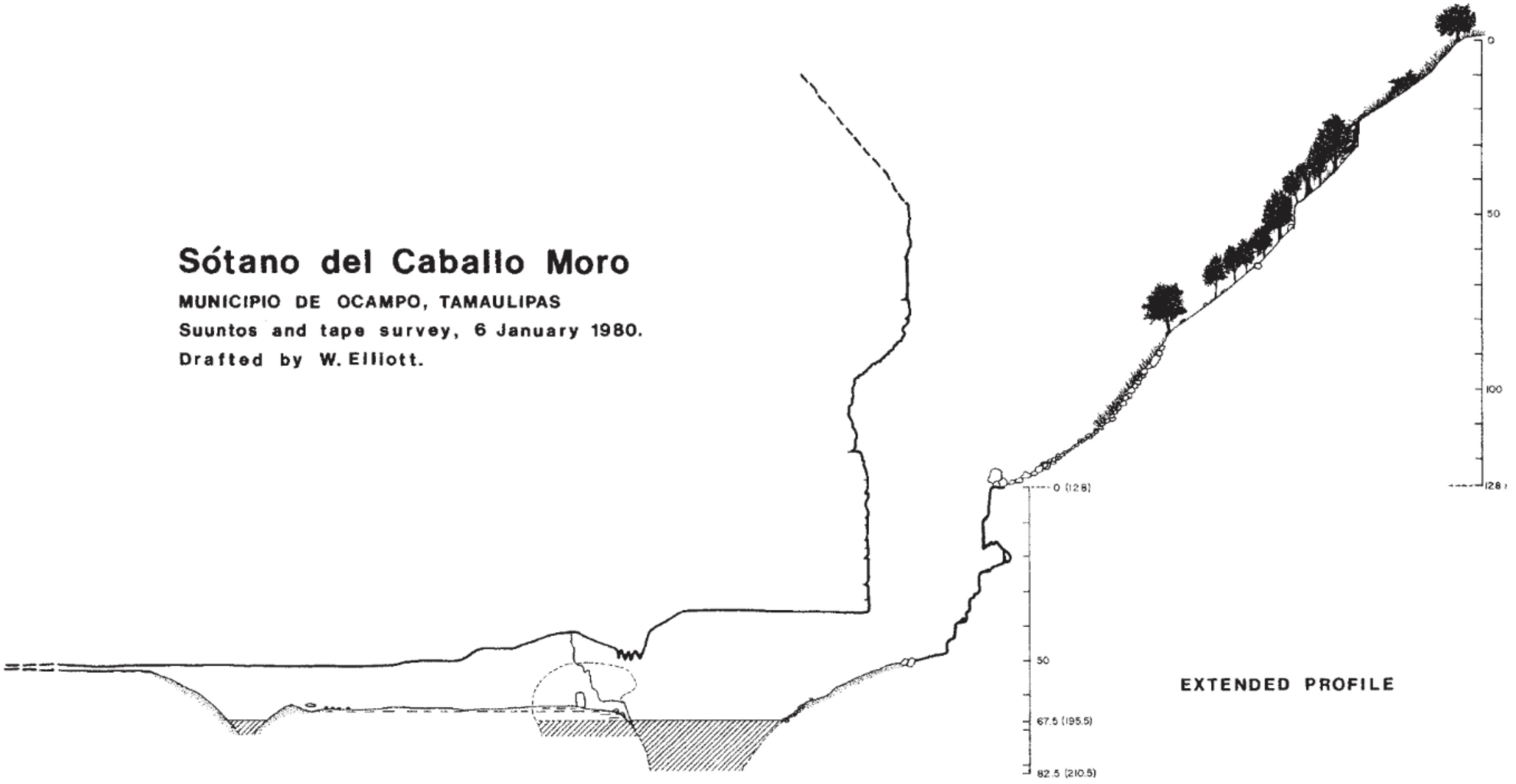

Figure I.1 – Cutaway View of Sótano del Caballo Moro - The Caballo Moro Cave, which is found at the base of a doline (sinkhole) is an example case of how the abiotic selective pressure of light can affect the level of adaptation in a cave environment. Both cavefish and surface fish reside in the pool found at the entrance of the cave, and both forms generally segregate based on the presence of light in the pool (Espinasa and Borowsky, 2000).

This image is modified from the Association for Mexican Cave Studies database of cave maps. (http://www.amcs-pubs.org/maps/0971.pdf)

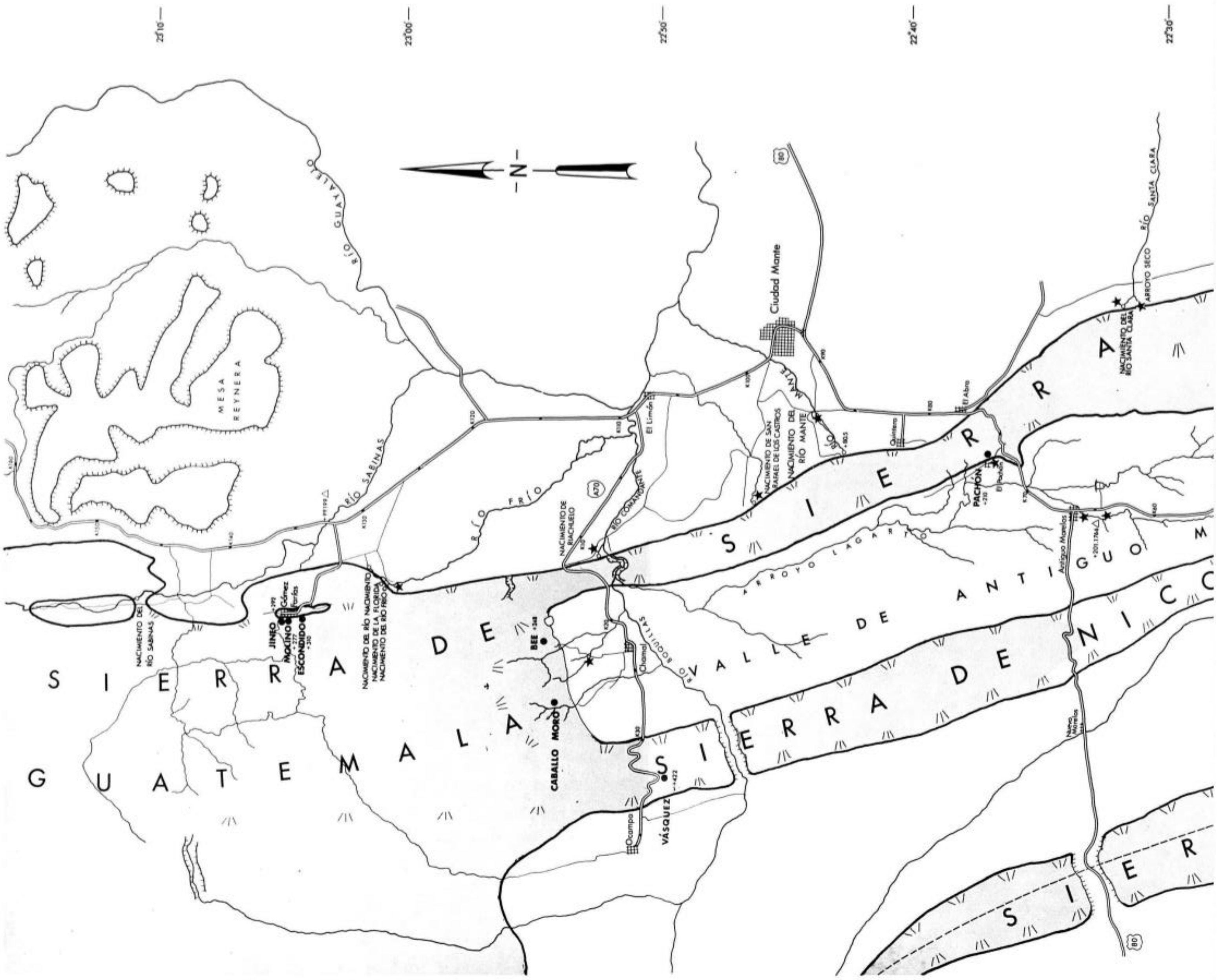

Figure I.2 – Map of the Region Surround the Sierra de El Abra – Karstic evolution of this region has resulted in a variety of surface and subsurface karst formations, some of which can be classified as isolated while others possess high levels of connectivity to the surface or other caverns.

This image is modified from Mitchell, Russell and Elliott, 1977

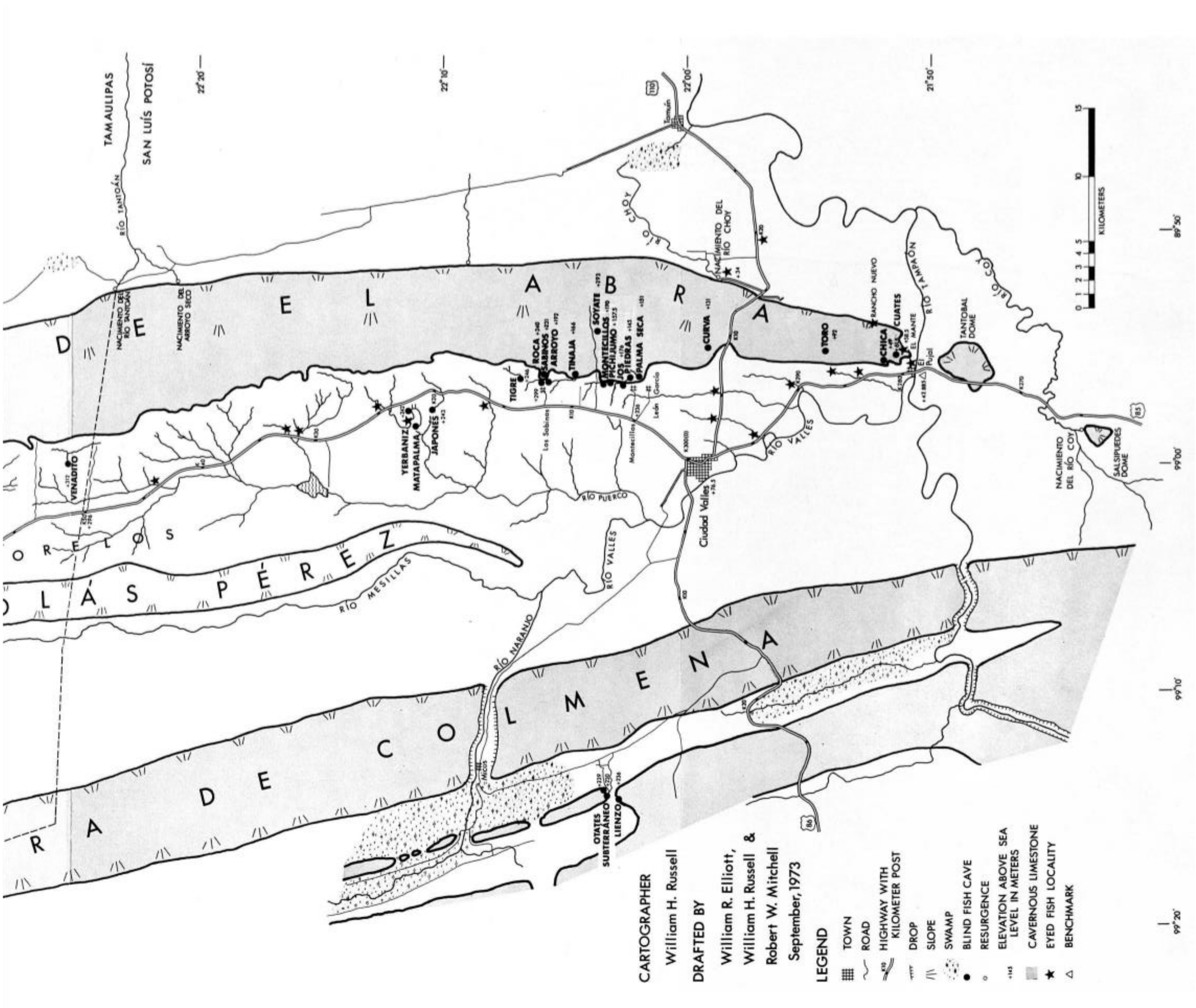

Figure I.2 Continued – Map of the Region Surround the Sierra de El Abra

This image is modified from Mitchell, Russell and Elliott, 1977

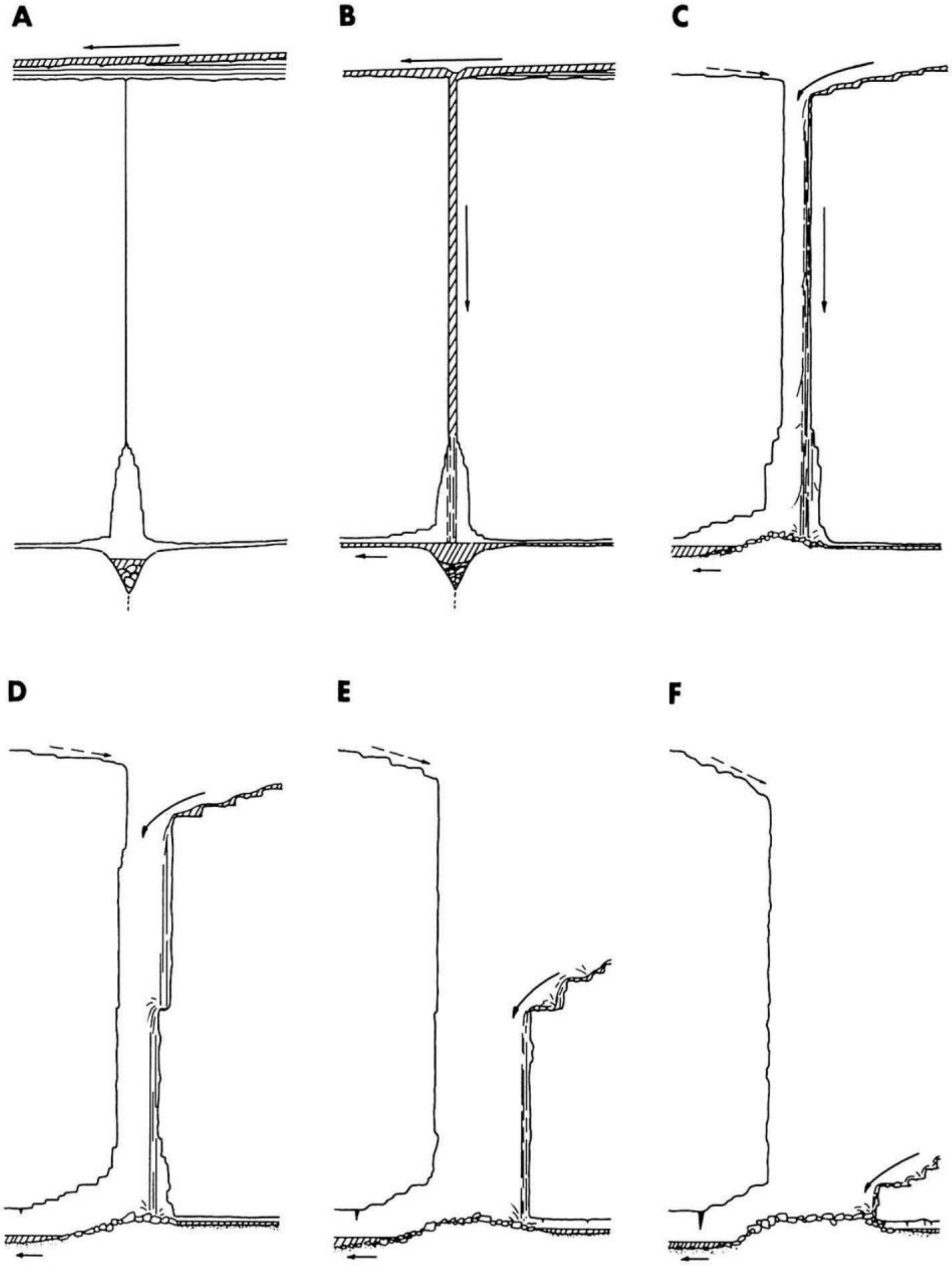

Figure I.3 – Diagram Representing the Development of a Stream Capture – A. A surface stream has developed on an impervious protective layer covering cavernous limestone. A fracture runs vertical to another rock bed, which will eventually form the sótano. **B.** The stream erodes through the protective layer, and water begins to travel down the fracture. **C.** The fracture has been weathered out to form a pit. The surface stream is now completely traveling underground. **D.** Continued dissolution has widened the pit even further and the horizontal cave opening has begun to widen as well. **E.** The stream continues to erode downwards, further lowering the stream wall. **F.** Erosion has downcut so far that the stream plane is at equal elevation with the cave entrance.

This image is modified from Mitchell, Russell and Elliott, 1977

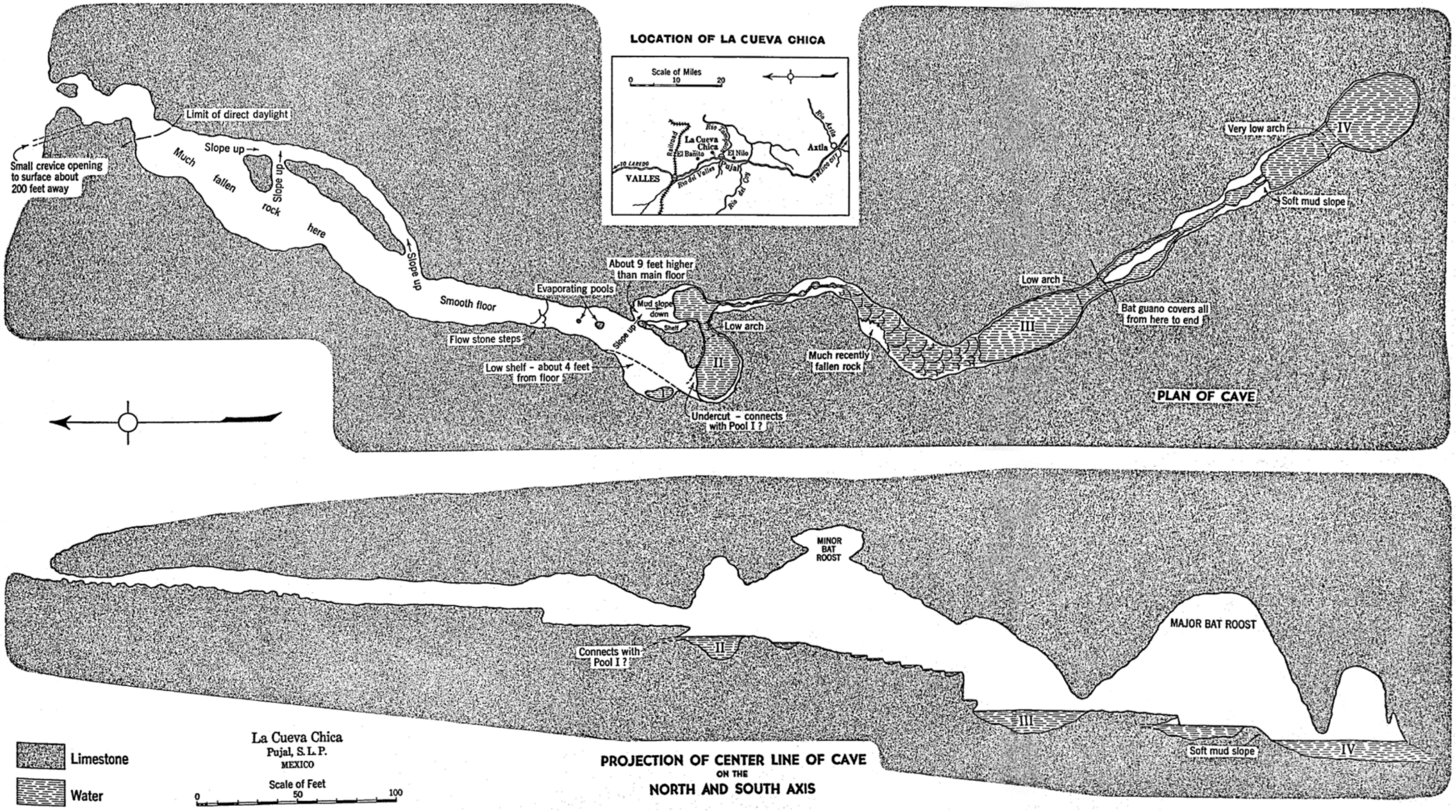

Figure I.4 – Surveyed Map of La Cueva Chica – This map diagrams the four pools of the Chica cave and also the siphon which is believed to connect to the Río Tampaón which is approximately 972 meters away (Mitchell, Russell and Elliott, 1977). The original survey of these caves (which is diagrammed here) was created by Breeder in 1940 as he led the expedition of the New York Aquarium Society. However, Mitchell (1977) argues that these diagrams are somewhat distorted, and the scales given are exaggerated.

This image is modified from the Association for Mexican Cave Studies database of cave maps. (http://www.amcs-pubs.org/maps/3154.pdf)

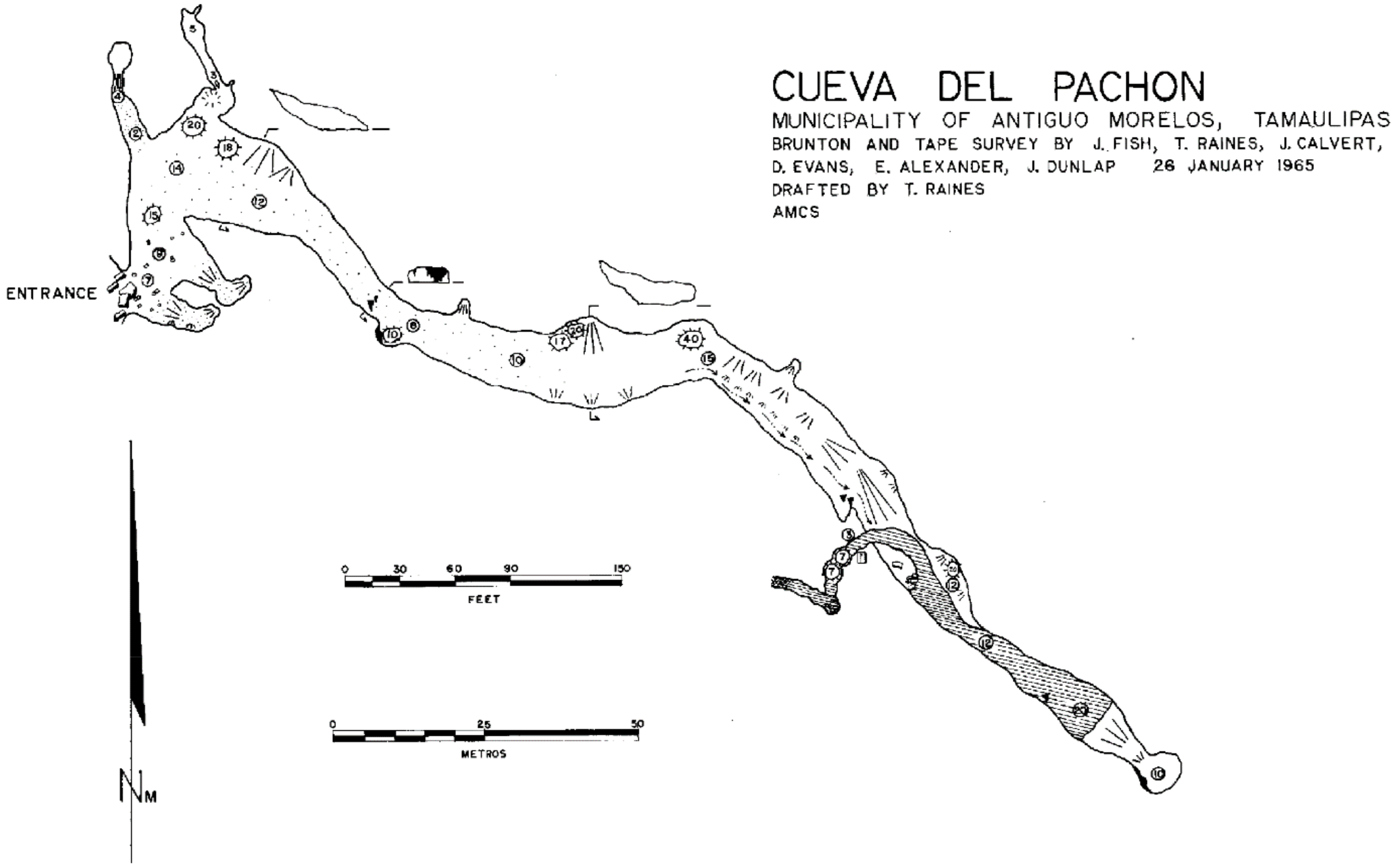

Figure I.5 – Surveyed Map of La Cueva de El Pachón – This map diagrams the isolated Pachón cave found on the western face of the Sierra de El Abra Formation. The cave is likely an abandoned resurgence point (spring) and is considered to be one of the most isolated cave systems in the Sierra de El Abra region (Mitchell, Russell and Elliott, 1977).

This image is modified from the Association for Mexican Cave Studies database of cave maps. (http://www.amcs-pubs.org/maps/0001.pdf)

II TROGLOBITIC CHARACTERISTICS

When studying organisms that live within cave environments, one obvious place to begin is with the modified phenotypes that the creatures have evolved. It takes no more than a quick glance at these species to realize that the selective pressures of their environment are drastically different than those found on the surface. These modified phenotypes are referred to as troglobitic, and the species that have evolved them are called troglobites.

Before examining the common trends found in troglobitic adaptations, it is important to first define and examine the specific selective attributes of a cave ecosystem. Abiotic cave system attributes that may affect selection have been previously discussed within Section I, so here we will examine the biotic characteristics of karstic cave systems.

Nutrient Sources within Cave Systems

As discussed in Section I, one of the defining characteristics of a cave ecosystem is the reduction or total absence of light. Without this energy input source, the food chain loses its base, photosynthesis-capable producers. Although there are a few chemolitho-autotrophic bacteria found within these systems, the rate of reproduction of these microorganisms is too slow to adequately sustain a cave ecosystem (Culver 1982). Thus, cave organisms are primarily scavengers or decomposers, surviving on nutrients that enter the cave system from the surface. The majority of carbon for cave organisms is derived from five primary sources: organic waste, microorganisms, cave insect feces, bat guano (and other cave-dwelling mammal’s feces) and cave insect eggs (Culver 1982).

The use of organic waste as a carbon source is a method that is primarily employed by cave microorganisms; microfungi, bacteria, protozoa, etc. Usually, plant detritus such as branches, leaves, and other parts are washed into the cave system and are used by these microorganisms as a food source. This food source can have effects on the abundance and activity of microorganism populations in these caves. In the Mammoth Cave system in Kentucky, Feldhake and Vestal (1983) found that the amount of organic material positively correlated with the activity levels of microbe populations. This higher amount of organic material therefore results in larger populations of these microorganisms since the selective pressure of food availability is lessened, and a larger population of organisms can be sustained.

Additionally, plant detritus has been correlated with activity in caves of non-troglobitic organisms. In caves with highly varying flow rates, large amounts of plant detritus are left behind to act as a resource for the isopod *Ligidium elrodii*. Although it is not limited to cave systems, this species can survive in a cave as long as the plant waste levels are high enough (Culver 1982).

The microorganism populations that develop when organic waste is placed into caves can also serve as an energy source for larger troglobitic organisms. Various insects and crustaceans can inhabit these caves, and microorganism colonies serve as nutrients for these larger invertebrates. Not all types of microorganisms contribute equally, however. For example, in a series of caves in Virginia, fungi populations have been shown to directly correlate with abundance of invertebrate populations, but bacterial and other microorganismal levels do not seem to have the same definitive results (Culver 1982).

Besides organic waste and microorganisms, the presence of non-troglobitic organisms in cave systems has been shown to impact cave adaptation. These animals serve as a nutrient delivery service and provide sustenance for a variety of troglobitic organisms. Trogloxenic organisms, which live near and around caves and cave entrances, will migrate into the caves and consume food and defecate there. This type of interaction greatly impacts the level of adaptation troglobitic organisms undergo in these caves, and many variations of species employing coprophagia, consumption of fecal matter, for energy acquisition are not found in caves devoid of migrating animals (Culver 1982).

One primary example of this phenomenon is the population of *Astyanax mexicanus* that is found within the La Cueva Chica (hereafter Chica) cave system. The Chica lineage of *Astyanax mexicanus* was the first of the eyeless cave fish populations to be found within the Sierra de El Abra region. The cave has a medium-sized bat population within it that has been noted to defecate within the water source housing the Chica lineage (Mitchell, Russell and Elliott 1977).

This specific lineage is important to biologists primarily due to its apparent evolutionary history. It is difficult to accurately determine the age or time of population events for *Astyanax mexicanus*. However, scientists have used a variety of different genetic analysis tools to determine that there were at least two separated ancestral surface stocks that seeded multiples caves (Ornelas-García, Domínguez-Domínguez and Doadrio 2008). These two events resulted in what have been commonly referred to as the “old” and “new” stocks.

The Chica fish are of interest because there is disagreement about whether this location was one of the first or last caves that was invaded by surface fishes. Bradic, et al. (2012) used comparative genetic techniques to examine the genetic diversity of the Chica population and compare it to other cave populations in the Sierra de El Abra region. What they found was that while the cave system had one of the most genetically diverse populations, it was similar in genetic distance from both the old and new stocks, meaning that it had to have been populated very early relative to other caves in the region. This confusing pattern may be in part explained, in part, by the large bat population in the La Cueva Chica. This phenomenon increases the nutrient concentration of the cave system allowing for repeated migration events from the surface fish populations. Thus, guano produced by the bat population acts as a stabilizer for non-adapted fish to allow them to reproduce and not suffer extinction, which would be inevitable without a solid nutrient base (Gross 2012; Bradic et al. 2012).

Predation in Caves

Although scavenging and decomposing are considered the primary methods of nutrient acquisition for many organisms found within a cave environment, there have been some records of predation within caves between troglobitic species. Most of these predator-prey examples are found between insect species. For example, cave beetles have been noted to consume troglobitic cricket eggs when they have been deposited within a sandy substrate (Culver 1982; Howarth 1983). This interaction is lost, however, when the sandy substrate is missing. In this situation, the cave cricket deposits its eggs in a tougher substrate that the beetle cannot excavate, resulting in a lack of a predator/prey relationship (Culver 1982; Howarth 1983).

Other invertebrates, such as cave spiders and millipedes, have also developed different predator/prey relationships. Some of these relationships occur closer to the entrance of the cave to exploit prey that accidentally wander into the environment, while at other times the interaction occurs deep within the cave network (Howarth 1983).

Enhanced vs. Repressed Phenotypes

Before examining some of the characteristics cave organisms have evolved in a cave ecosystem, it is important to first clarify exactly what I mean by the terms enhanced and repressed. For phenotypes that are considered enhanced, adaptations have occurred that directly respond to the lack of nutrient availability found in the cave environment. When a phenotype is classified as repressed, the expression of the phenotype occurs at a quantifiably lower level than the expression in the surface form of the animal. Adaptation is a non-directional feature that increases survival in a given environment, thus both enhanced and regressed phenotypes can be classified as adaptations. Also, when discussing how these traits have developed, a trait that is becoming repressed is undergoing regression, while enhanced phenotypes are formed through enhancement.

Regressive evolution of traits is a phenomenon typical of cave organisms, and has been a major driving force behind early studies on these animals. The basic characteristics of these repressed phenotypes will be explained in this section, but the various theories behind how this particular type of evolution occurs will be the primary topic of Section III.

Non-Visual Sensory Enhancements

From the perspective of a species that obtains the majority of its information through visual means, it is difficult for us to imagine how a species may interact with an environment where complete darkness is a defining feature. Cave organisms possess exaggerated versions of a variety of sensory systems that allow them to function in these lightless settings.

Unlike many other environments found on the surface of the planet, caves are considered to be relatively stable in their physical characteristics. As noted in Section I, characteristics such as temperature and current flow rates typically vary little on a yearly basis (Culver 1982). This usually results in troglobitic sensory system adaptations that are more sensitive to fluctuations in these characteristics than surface counterparts.

One example of this phenomenon can be noted in structure and sensitivity of the lateral line system in *Astyanax mexicanus*. The lateral line system functions through the use of neuromasts. These specialized hair cells connect to an external cupula which moves relative to water movement (Bleckmann and Zelick 2009; Yoshizawa et al. 2013). They function in a similar manner as a computer joystick, and the force applied on the stem of the hair cell is recorded at the base of the neuromast. There are two types of these neuromasts within the lateral line system. The canal neuromasts (CN) are located within fluid-filled pores, which are arranged in various branching patterns across the fish body (Bleckmann and Zelick 2009). CN are arranged around the supraorbital and infraorbital areas around the ocular socket, and also may run along the trunk of the body (Bleckmann and Zelick 2009). The other type of neuromasts are superficial neuromasts (SN). These specialized hair cells are free-standing organs found throughout a fish body, but they will typically be localized near morphologic pits and crests found on the organism.

In the troglobitic forms of *Astyanax*, both the CN and SN have been noted to have increased in cell size, but only the SN seem to have increased the cell number relative to the surface form (Yoshizawa et al. 2010). This increase is most prevalent in the region where an eye would be located on the surface fish, specifically dorsal to the supraorbital canal (Yoshizawa et al. 2013).

The increase in size of the SN cells has evolved a difference in vibration attraction behavior (VAB) in the troglobitic form of *Astyanax* (Yoshizawa et al. 2013). VAB is a prey-seeking method used by these fish to locate organisms that have fallen into cave pools; however, the behavior is absent in the surface form. VAB is not present in these surface fish due to a risk of predation (Protas and Jeffery 2012). By increasing the frequency of movement towards vibrating sources, the fish increase their risk of predation in streams and ponds. In the cave ecosystem, where predators of *Astyanax* are nonexistent, the selective pressure against VAB development is absent, thus the larger SN are an adaptive benefit to prey discovery in the cave environment.

Along with an expansion in the lateral line system in *Astyanax,* increased size of other nonvisual sensory organs is a common trend. The troglobitic form possesses a statistically larger average naris (nostril) size, which correlates with an increased chemosensory ability to discover food (Bibliowicz et al. 2013; Wilkens 1988).

Taste bud numbers and morphology have also increased and expanded, respectively, in the troglobitic Astyanax *mexicanus*. The surface form possess taste buds around the lip region on the face, while the cave fish taste buds are distributed through an area that extends a considerable distance away from the mouth and up the front of the face (Wilkens 1988) (Figure II.1). This noted expansion in taste bud positioning and concentration begins in the cave form during the developmental stage when eye development normally occurs in the surface fish. Therefore, it may be the case that the resources normally budgeted for eye development have been repurposed for taste bud formation instead (Varatharasan, Croll and Franz-Odendaal 2009).

An increase in size or expansion of physical characteristics is not a trend that is limited to cave organisms. Modification of a physical trait is an evolutionary adaptation that has been recorded in a variety of different species. For example, Emlen and Nijhout (2000) compiled a large amount of data on beetle adaptations and how various stimuli can cause morphological adaptations. What was found was that under artificial selective pressures, enhanced morphologies were found in subsequent generations. Specifically in beetles, this exaggerated morphology shift was recorded in horn length of the animal. While the actual relationship pattern (linear, exponential, logarithmic, etc.) varied based on the trait, enhanced morphologies were a consistent trend in response to selection (Emlen and Nijhout, 2000).

Elongation and expansion of sensory organs originally was considered a fairly common trend in cave animals, and is not confined to vertebrates. For example, the crustacean *Gammarus minus* can be found in surface springs and cave pools and has been used extensively for troglobitic cave trait research. Troglobitic *Gammarus* have much larger antennae than ones obtained from springs (Culver, Kane and Fong 1995; Culver 1982). Antennae are primarily used by *Gammarus* and other crustaceans for olfactory capabilities, and therefore their elongation is believed to aid in locating food in the dark environment.

Central Nervous System Reorganization

As the sensory organs or peripheral nervous system features of many troglobitic cave species are enhanced to better interact with the cave environment, enhancements to the central nervous system, the brain, also evolve to reflect these sensory changes. I refer to these organizational changes as an overall enhancement to troglobitic animals primarily due to their conjunctive effect in adaptation. By limiting the scope of observation to specific portions of the central nervous system, one could make an argument that reduction occurs instead of enhancement. For example the optic lobe in *Astyanax mexicanus* and other cave fishes is reduced relative to their surface ancestors. However, by defining the scope as the entirety of the central nervous system, the restructuring of the brain’s organization is an enhanced phenotype in cave forms of *Astyanax*.

As noted above, the optic lobe in cave animals is decreased in size. Cavefish of a variety of species as well as cave crustaceans routinely have this adaptation in their central nervous system (Culver 1982; Culver, Kane and Fong 1995). Since brain tissue requires a substantial amount of energy for maintenance, reduction in the size of the optic lobe likely evolves for energy conservation (Wilkens 1988). However, the driving force behind this shift is related to the actual regression of the eye, which will be discussed later in this section.

Along with this reduction there is an increase in the size of the olfactory lobe. In *Astyanax mexicanus*, this increase is believed to result from an increase in the naris size. An increase in the size of olfactory lobe accommodates an increased number of receptor cells found in the olfactory epithelium (Protas and Jeffery 2012). The increase in receptor cell number requires more input processing in the brain, so the increase in size probably evolves in response to a need for this processing power.

Astyanax and other species of cavefish also seem to have an increase in portions of the brain associated with balance, optical avoidance, and spatial memory. Portions of the brain such as the telencephalon (memory development or activity level regulation), cerebellum (motor skills and muscle coordination), semicircular canals (balance regulation and rotary movement detection), eminentia granularis (special portion of the fish brain for lateral line input), and other tactile sections of the brain are similarly increased in size (Culver 1982; Protas and Jeffery 2012; Gross 2011).

Although studying the differences in size between brains of cave and surface animals has been a primary discussion point for adaptations in troglobitic animals, research to understand the effects of these recordable size differences is lacking. However, with more precise tools and more advanced technology, scientists examining cave organisms have started to determine what effect these size differences have on animals' function.

One example of this was a recent study to determine the functionality of the telencephalon in *Astyanax mexicanus*. Riedel (1998) tested the hypothesis that the telencephalon was analogous to the human hippocampus, and therefore served as a memory center in the brain. If this were true, it would have given an anatomical origin for the noted increased spatial memory of the species (Culver 1982). When a lesion was applied to the telencephalon, no difference was recorded in the time it took for the fish to become acclimated to an unfamiliar environment, but general activity levels were lowered when these lesions were put into place. The study also examined the role of the olfactory lobe in relation to the telencephalon, and when a lesion was placed to separate these two sections of the brain, the cavefish showed hyperactivity (Riedel, 1998). Both results highlighted a network of complex interactions that regulated activity levels, but did not show any change in the memory capabilities of the fish. Thus it can be assumed the telencephalon does not function analogous to the human hippocampus and instead has some regulatory effect on basal activity levels (Riedel, 1998). As with the telencephalon, the effects of reorganizing the central nervous system through cave evolution are generally unknown, meaning they are promising research areas for future study.

Modifications in Metabolic Efficiency

Since troglobitic animals have developed and adapted numerous ways to find food within the nutrient-poor cave environment, one might also suppose that these animals also would have evolved ways to better store those nutrients. By lowering the organism's standard metabolic rate (SMR), average energy spent while active, and resting metabolic rate (RMR), average energy spent while resting/asleep, troglobites could theoretically survive longer on a smaller amount of food and also have more energy available for reproduction (Culver 1982).

One example of this pattern is the troglobitic crayfish *Cambarus setosus*. The cave crayfish showed lower metabolic rates and survived 3.5 times longer than its surface version, *Cambarus rusticus,* on the same amount of oxygen (Burbanck, Edwards and Burbanck 1948; Culver 1982; Gross 2011). This pattern was also repeated in the cave crayfish genus *Procambarus* (Dickson and Franz 1980), along with the fact that adenosine triphosphate (ATP) turnover rate was significantly longer. These turnover rates are correlated directly with metabolic activity because ATP serves as an important coenzyme for energy transfer in most intracellular functions (Knowles 1980). One important fact that the *Procambarus* study highlighted, however, was a pattern that affects all troglobitic adaptations. The degree of adaptation that the organism displayed was negatively correlated with food availability (Culver 1982; Dickson and Franz 1980). When a population of animals is undergoing selection on a trait, the strength of selection that is placed on that population will generally[5] correlate with the extent of phenotypic change.

An example of this phenomenon is the degree of starvation resistance that is found in two cave beetle species from Mammoth Cave National Park in Kentucky. Both *Neaphaenops tellkamfi* and *Pseudoanopthalmus menetriesii* are foraging cave beetles, which consume cricket eggs as their primary source of food (Kane and Poulson 1976). When food sources become limited in each cave system, *N. tellkampfi* had approximately fifty percent less weight loss on average than *P. menetriesii*. The major difference between both of these species is that throughout the year, *P. menetriesii*'s environment has a larger cricket to beetle ratio than *N. tellkampfi*. This difference in selection pressure has directly affected the level of adaptation for starvation resistance (Culver 1982; Gross 2011; Kane and Poulson 1976).

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[5] A few factors such as genetic variation and pleiotropic effects may cause this correlation to become masked in the population.

In some cave species, metabolic efficiency is not even noted. In *Astyanax mexicanus,* for example, only the extremely divergent Pachón population, originating in La Cueva del Pachón in the northern section of the Sierra de El Abra, showed a decrease in oxygen consumption that is considered synonymous with increased metabolic efficiency (Wilkens 1988). All other surface and cave strains showed normal or even increased metabolic rates. Some research has even suggested that there is an increase in metabolic activity of certain strains of *Astyanax* (Culver 1982; Gross 2011). These high metabolic levels are found within caves where large bat roosts and large amounts of food availability are present, again highlighting the importance of degree of selective pressure on severity of adaptation.

While *Astyanax* does not show an adapted metabolic system in all cave strains, some troglobitic fish are able to survive for longer periods of time, with restricted oxygen and food availability relative to their surface ancestors. Specifically, Pachón cavefish lose significantly less body weight when placed under starvation conditions (Rose and Mitchell 1982; Wilkens 1988). Pachón cavefish have been recorded to have about 36% of their body weight in fat stores, while only 9% of the surface fish weight is due to fat (Wilkens 1988). This difference not only helps to explain why the Pachón are better at surviving starvation for a longer period of time, but it may also help to explain why it has a lower oxygen consumption rate compared to other *Astyanax*. The energy requirements for body fat maintenance are much lower than other tissues and organs in the body, and therefore require less oxygen (Wilkens 1988). Since metabolic rates are calculated by comparing total body weight to oxygen consumption, it is very likely the metabolic rates calculated are skewed due to a difference in body fat percentage (Wilkens 1988). This information also indicates that it is very likely that metabolic efficiency and fat storage mechanisms and regulation are very distinct genetic pathways.

Other Enhancements in Troglobites

Although there are a variety of different troglobitic adaptations that are found in both cave vertebrates and invertebrates, there are a few specific adaptations that have been found in only certain species. These adaptations, while not widespread, show some of the more complex nuances of cave life and also another selective pressure that is especially relevant for non-aquatic animals.

Noted in Section I, an important feature of caves is their increased humidity levels. When moisture content increases in an organism's environment, the homeostasis that is maintained within their body fluid network will shift as well. Important salts and minerals can be pulled from the animal's cellular network due to the principles of osmotic regulation. Losing these salts and minerals is not good for the organism, and without ways to regulate this loss, they will die.

To prevent salt loss, cave arthropods have enhanced systems for humidity detection and mechanisms to excrete excess water (Howarth 1983; Culver 1982). A combination of these adaptations has been found in the cave beetle family *Catopidae*, which has developed longer, more complex internal antennal vesicles and a more permeable cuticle (Lucarelli and Sbordoni 1978). The permeable cuticle allows for water to passively leave the body, and the vesicles are used to amplify the antenna's ability to detect humidity level changes (Culver, 1982). Since the permeable cuticle is not a controllable feature, it is possible that the increased antennae are used by the beetle to determine when humidity levels are lower than normal in the cave. A cuticle with increased permeability would result in the beetle becoming easily dehydrated in this situation, so the beetle can prevent dehydration by moving closer to open cave waters or deeper within the cave where humidity levels are higher.

Cave fish do not need to adapt to high moisture levels as arthropods and other insects do, but a very specific feeding behavior has been observed in *Astyanax* which confers a direct survival advantage. Besides having adapted their central and peripheral nervous systems to better detect food, cave fish feed in a different manner than their surface ancestors.

The surface *Astyanax* collects food by orienting its body perpendicular to the bottom of the body of water. It uses its sight to target the food and consume it. The cave form, however, angles its body at an angle closer to 55º (Wilkens 1988) (Figure II.2). When both are fed within a completely darkened environment the cave fish have a much better success rate at actually finding and consuming the food (Hüppop 1987). This behavior has been found to have a genetic basis (Protas and Jeffery 2012), and is one of the first quantified, genetic-based behaviors that has been deemed to significantly increase cave survivability in the species (Schemmel, 1980).

Repressed Troglobitic Traits

While specific adaptions are easily noted and explained in cave organisms, it is usually difficult for biologists to determine how traits regress in a population, specifically when no apparent selective pressure is found. The hypothesis behind how cave organisms have obtained these repressed traits will be the focus of Section III, but before discussing how these organisms evolve these traits, we should first give some examples of the effects of regressive evolution within a cave ecosystem.

Loss of Pigmentation

Pigmentation patterns of surface organisms are a prime example of a phenotype that undergoes a large amount of selection. Surface animals have large and varied uses for these visual signals including camouflage, warning off predators, or sexual displays (Yokoyama and Yokoyama 1996). However, in the cave ecosystem, where light is not able to interact with the pigmentation of these animals, many organisms do not suffer the selective disadvantage that is associated with a loss of pigmentation or albinism.

In most situations, albinism or lack of pigments in the skin is developed through a variety of different mutations throughout the genome. Pigmentation in most animals occurs through the use of a special type of cell called a chromophore. Chromophores vary on their pigment coloration. Xanthophores (yellow), erythrophores (red), leucophores (white), and iridophores (silvery/iridescent) are found in various organisms in all environments on the planet (Sugden et al. 2004), but for many fish the primary chromophore that determines skin coloration is the brown/black pigment-producing melanophore (Wilkens 1988).

The melanophore cell contains vesicles called melanosomes that are filled with a dark brown or black pigment called eumelanin. Under certain conditions, these melanosomes will collect together near the center of the melanophore cell body. Concentration of these vesicles helps to create various color patterns and pigment changes (Sugden et al. 2004; Wilkens 1988), which are controlled by both environmental and physiological cues (Wilkens 1988).

In *Astyanax mexicanus*, there are a variety of different mutations that have been attributed to develop an albino phenotype, however, the gene *Oca2*, ocular and cutaneous albinism type II, seems to be the primary genetic target for the albinism in a variety of species. Mutations of the gene prevent the eumelanin pigment from correctly forming (Rinchik et al. 1993), but they do not have any effect on the physiological functioning of the melanophore (Wilkens 1988). This means that the melanosome vesicles will still aggregate near the center of the melanosome with certain stimuli, but the lack of pigment means no morphological change will occur (Wilkens 1988).

The *Oca2* gene is the primary explanation for albinism in a few lineages of *Astyanax,* but it does not explain all the pigmentation variations that are seen throughout the species in the Sierra de El Abra. Other mutations are noted to affect the number of overall melanophores instead of the eumelanin (Gross, Borowsky and Tabin 2009). Also, although melanophores are the primary chromatophore for pigment of *Astyanax* and other fishes, mutations have been noted in the secondary chromatophores as well (Protas and Jeffery 2012). These mutations do not always manifest themselves as the albino phenotype, but they still result in a variation in pigment that would be difficult to possess outside of a darkened, cave environment.

Multiple mutations that cause pigment shifts are not just unique to *Astyanax mexicanus*. *Asellus aquaticus,* an isopod crustacean, also has shown multiple genetic targets that result in albinism or reduced pigment formation (Protas and Jeffery 2012). This trend in pigmentation regression to include a variety of different genetic targets is an important example of the Neutral Mutation theory, but we will delve more into this phenomenon in Section III.

Lack of Ocular Development

Just as reduced pigmentation or albinism is a defining feature of many troglobitic organisms, a lack of eye development also is indicative of cave animals. The defining feature of a cave, the complete absence of light, results in repressed eye development in a variety of species. Many cavernous beetles have no visible external manifestations of the eye, though some retain small patches of photosensitive pigment (Culver, 1982). The cave salamander, *Proteus anguinus,* initially begins development of an eye, but then it undergoes a lytic process and sinks into the ocular orbits (Rétaux and Casane, 2013). Even non-cave animals that live in complete darkness have repressed eye development, such as the naked mole rat, *Heterocephalus glaber*, or star-nosed mole, *Condylura cristata.* Both animals have partially or completely repressed eye development (Emerling and Springer, 2014; Rétaux and Casane, 2013). Lack of eye development is one of the best examples of regressive evolution because it happens in so many different species.

Genetic and morphological pathways that different organisms use to repress eye development are quite diverse. Even within the same species, there can be various genetic targets that result in a lack of eye development when modified. This complexity is a reflection of the complexity of the eye structure itself.

One of the best-known examples of eye development loss is *Astyanax mexicanus*. Compared to the relatively large eye found on the surface fish, the complete lack of any external ocular structures is quite striking. The loss of the eye in the cave form of *Astyanax* can be classified through two distinct defects: controlled destruction (apoptosis) of the lens, or an underdeveloped retina (Rétaux and Casane, 2013). These two pathways of repression seem very distinct because during early development, the lens and retina develop from different embryological layers, the ectoderm and neural plate respectively (Rétaux and Casane, 2013). Normally, this layering results in tissues and organs that are completely separate from one another, so the fact the eye is made up from multiple embryonic layers highlights the eye's morphological complexity.

The lens is a flexible structure that is used to focus light to create a clear image of the environment. The initial embryonic structures develop in a similar manner as the surface fish, but it is not until approximately two days after fertilization that the lens undergoes apoptosis. One possible explanation of this destruction is that an anti-apoptotic factor is under-expressed or has undergone a loss-of-function mutation (Protas and Jeffery, 2012). Conversely, overexpression of a pro-apoptotic factor during that stage of development could activate apoptosis (Protas and Jeffery, 2012). Either situation would result in the destruction of the developing lens cells.

Along with lens apoptosis, reduction of the retina size and function is also a method for eye-development repression in *Astyanax* (Rétaux and Casane, 2013). Compared to the apoptosis of the lens, these regressions are much less destructive modifications (Rétaux and Casane, 2013). This idea is supported by the fact that surface retinal cells can be implanted into the cavefish retina and develop a morphological eye. Additionally, when lens apoptosis is occurring it is likely that retinal cell-signaling molecules are not supplied to the cavefish retina (Rétaux and Casane, 2013). All neurological connections and layer formation of the eye are created normally, so the survival of the retinal cells seems to be the only defect with the retina.

These two types of eye defects, which usually are noted to both be present in *Astyanax* lineages, have recently been thought to possess a common genetic factor. The Hedgehog signaling pathway is one of the initial developmental pathways that is used by an embryo for early development in a large number of animals (Bitgood and McMahon, 1995). In the cave form of *Astyanax* the Hedgehog pathway has been recorded to have increased expression relative to the surface form of *Astyanax* (Rétaux and Casane, 2013). Hedgehog signaling has downstream effects on gene expression, and the overexpression of the pathway could likely affect the apoptotic signals to the ocular lens and the retinal cell survival signals (Rétaux and Casane, 2013). Besides effects on eye development, Hedgehog signaling also may affect many of the traits discussed earlier in this section: brain formation (Rétaux and Casane, 2013), vibration attraction behavior (VAB) (Yoshizawa et al., 2010), and naris size / olfaction receptor cell size

(Bibliowicz et al., 2013). These complex interactions suggest that eye regression is not developed separate from other traits, and other enhancements have influenced the loss of the eye. This interaction also will be categorized in Section III.

Behavioral Traits of Cave Organisms

Besides morphological and structural changes, cave organisms have also been noted to exhibit behaviors that differ from their surface counterparts. Specifically in *Astyanax mexicanus,* vibration attraction behavior and feeding angle behavior, which relate to feeding and energy acquisition, have already been noted earlier in this section; however, cave organisms also exhibit variations on behaviors that do not directly involve food acquisition. Troglobitic *Astyanax* have been recorded having a lower level of aggressive behavior (Wilkens, 1988), a lack of schooling behavior (Wilkens, 1988), and no apparent sleep cycles (Protas and Jeffery, 2012), the latter which correlates with a possible loss of circadian rhythm cycles (Moran, Softley and Warrant, 2014).

For behavior analysis to occur within *Astyanax* and other troglobitic species, one important factor needs to be taken into account when using animals that have been raised in a laboratory. The presence of extraneous light may cause variation in activity that can influence behaviors. The complete lack of darkness in laboratory test settings may have some implications for all research on cave animals. However, the presence of light is much less likely to cause discrepancies on physical trait changes since those changes have been acquired over thousands and sometimes millions of years of evolution. This timeline contrasts the shifts seen in activity levels and other behavioral changes since these traits change on a minute-to-minute basis. For example, under twelve hour dark-light cycles, *Astyanax mexicanus* was noted to have an increase in activity level when the fish was placed into a tank that had a shifting light-dark cycle, it had an increase in overall activity level compared to when it was placed into a tank on a dark-only cycle. The light modified the activity variable significantly (Figure II.3), whereas morphological differences were not apparent when comparing these differences in cycles.

The noted activity level difference is likely caused by the pineal organ. The pineal organ is a light-sensitive gland in the brain that has some control over hormone secretion, which regulates activity levels by modifying melatonin levels in the presence of light (Ekstrzm and Meissel, 1997). When light is present, the pineal organ prevents the production of melatonin, and activity levels are increased due to its suppression. While this light-sensitive regulation is normally due to a retina/pineal organ interaction (Ekstrzm and Meissel, 1997), the transparency of the cave fish skin and the lack of adequate functional retinal tissue likely means the presence of light directly affects pineal organ function.

This interaction requires further study and experimentation, but as future research with *Astyanax mexicanus* and other troglobitic animals begins to expand into behavioral studies in addition to morphological studies, the presence of light in the experimental setting is something that will have to be noted to ensure more accurate results. Researchers cannot simply discount the presence of light in a study because the testing organism does not possess the traditional light-sensitivity that their surface counterparts have.

Conclusion

Cave organisms are a very interesting set of animals. Found within a dark, nutrient-poor environment, they have developed traits to better thrive in this setting. Sometimes, these traits, such as sensory capabilities, brain organization, and some metabolic functions, are considered enhanced and have resulted in physical characteristics that are exaggerated compared to their surface counterparts, for the adaptation results in a survival benefit to the species. Conversely, other traits, such as ocular structures and pigmentation, are completely lost in the troglobitic forms of the animals. The regression of these traits is somewhat confusing to scientists, who have yet to give this evolutionary dynamic a succinct cause. The evolutionary theories behind this regression and the confounding aspects surrounding it will be the topic of the next section.

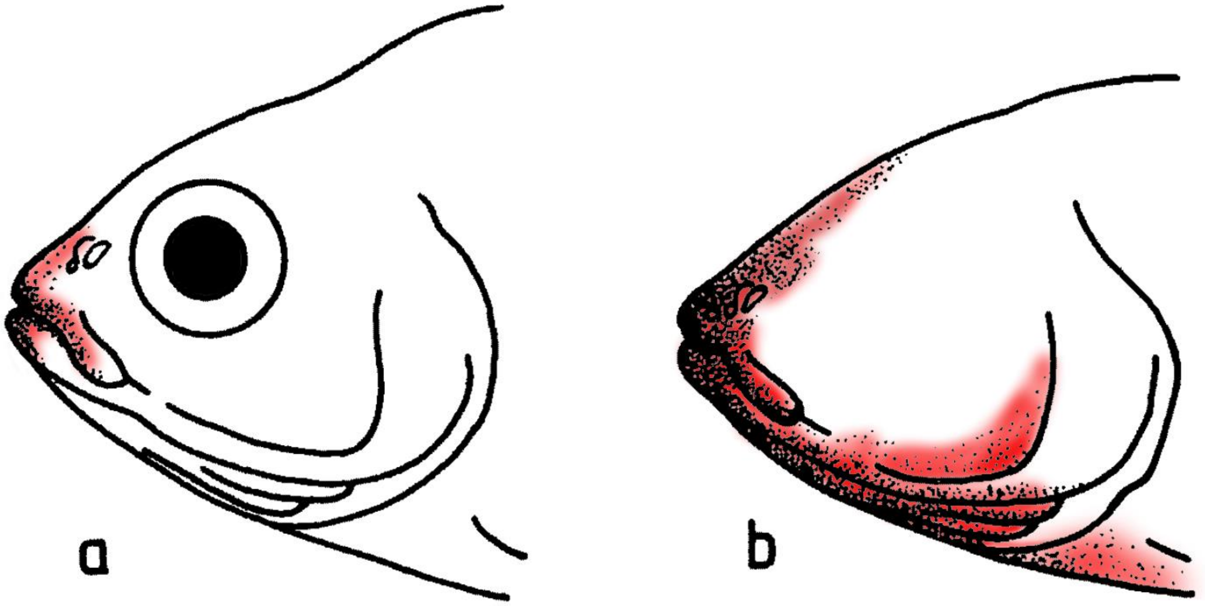

Figure II.1 – Distribution of Taste Buds in *Astyanax mexicanus*

Astyanax mexicanus has different levels of taste bud distribution as denoted by the red coloring. (a) Surface fish possess a relatively smaller amount of taste buds that are more localized around the mouth than (b) the cavefish. This enhancement is thought to occur in the cave form due to the excess energy saved from not producing an eye (Wilkens, 1988).

This image has been modified from Schemmel, 1967.

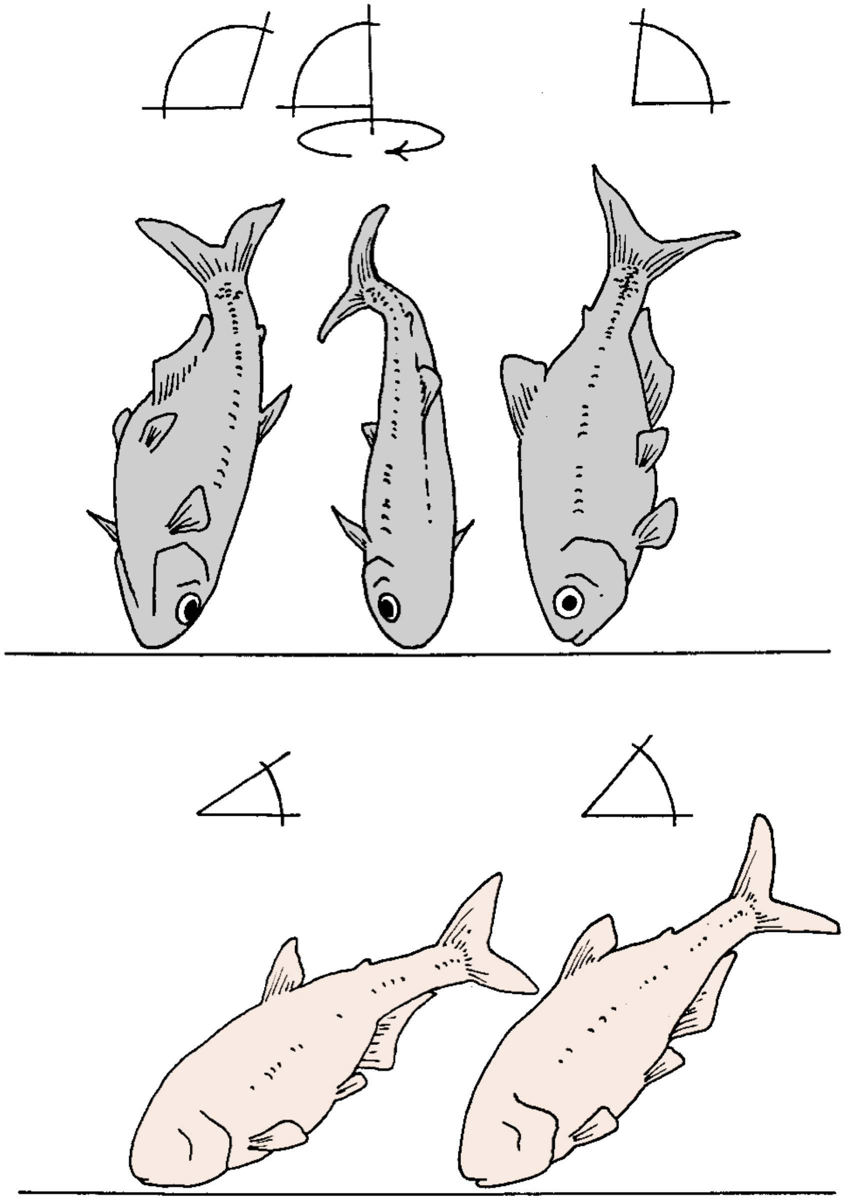

Figure II.2 – Feeding Methods in *Astyanax mexicanus*

Astyanax mexicanus has been noted to feed at a 55° angle relative to the ground. This feeding angle allows the cavefish to better collect food in the darkened environment (Hüppop, 1987), and is one of the first behavioral differences in the species that is found to have a genetic base (Protas and Jeffery, 2012)

This image has been modified from Schemmel, 1967.

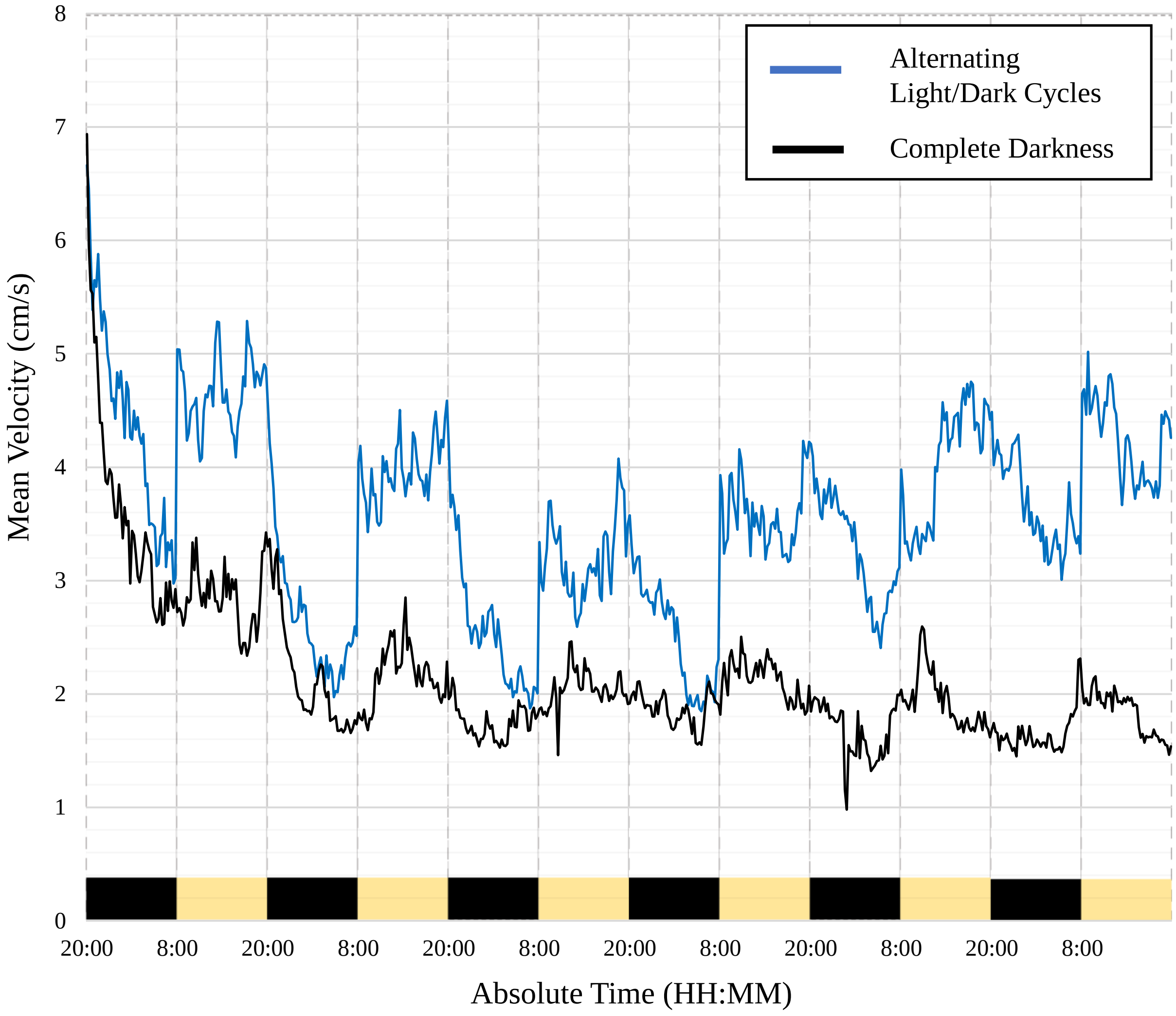

Figure II.3 – Activity Level Comparison of Cave Form *Astyanax*

Using activity tracking software, activity levels were recorded over a span of six days with a group of six cavefish (n=6). The first trial had the fish placed in a tank under cycling, eight-hour, light/dark cycles. In the second trial, the fish were placed in complete darkness for six days. Mean velocities were calculated for every fifteen minute interval, and a statistically higher velocity was noted when the cavefish was placed under light/dark conditions (Tukey's Test; $p \leq 0.047$). This variation is possibly attributed to the photosensitivity of the pineal organ, which is more exposed due to the lack of pigmentation of the cavefish (Wilkens, 1988).

This data was recorded and analyzed with Brian Carlson on 7/25/14

III THEORIES OF REGRESSIVE EVOLUTION

The first-ever documentation of a cave species was recorded by a Chinese author in 1541 (Gunn, 2004). He had found a population of cave fish while spelunking one day and wrote about the peculiar, eyeless animals swimming in an underground pool. About a hundred years later in 1689, a Slovenian man in a small town recorded that a nearby spring had been bringing odd animals from beneath the earth. The locals proclaimed they were the offspring of dragons that lived underground, and they caused flooding when they grew larger. These dragon offspring were actually cave salamanders that had been washed out of their cave environments (Culver, Kane and Fong, 1995). Many years after these initial cave creature discoveries, scientists today believe there are somewhere between 50,000 - 100,000 unique cave-dwelling animal species (Culver and Holsinger, 1992), and most of these organisms are blind and lack pigmentation (Culver, Kane and Fong, 1995). The pattern of regressive phenotypes seen in these animals has intrigued and baffled scientists for generations. Even with advances in molecular biology and genetic techniques, the simple question of "why did this organism lose its eye?" is still as hotly debated as it was a hundred years ago.

There are three primary hypotheses that try and explain why troglobitic organisms have acquired regressive traits. Some researchers believe that neutral mutation - random mutations that accumulate over time in an animal - has developed these traits. When these mutations are tolerable, meaning they do not impact reproductive success, this method of genetic variance due to chance is called genetic drift (Pierce, 2010). Others believe there are direct selective benefits of these regressive traits, and organisms have acquired these troglobitic characteristics from the unique selective pressures in a cave (Rétaux and Casane, 2013). Recently, some scientists hypothesize that certain traits are connected through a common genetic target (Rétaux and

Casane, 2013). This gene would have multiple effects on the organism, and that selection for one function of the gene would cause regression in another function. This multifunction aspect of a single gene is called pleiotropy, and is a common feature found in most genes. Before we take the time to examine these various hypotheses and the pros and cons of each model, we first should take a look into the history behind the study of regressive evolution and the thoughts that came before these three hypotheses to gain a better understanding of how points of view have shifted about the origins of these unique creatures.

History of Evolutionary Theory of Regressive Traits

While many scientists enjoy citing Darwin and his “wrecks of ancient life” to begin paper introductions, the first theorist who examined cave organisms through the lens of evolutionary biology was the Frenchman Jean-Baptiste Lamarck (Culver, Kane and Fong, 1995; Gross, 2011). Lamarck proposed the idea of adaptive evolution, or the belief that an individual organism will become modified throughout its lifespan to respond to certain environmental pressures. These adaptations are then passed onto the individual’s offspring, which will continue to be modified, or remain in stasis if the modification adequately satisfies the environmental pressure. This method of adaptation would result in very rapid evolution over a short period of time (Gross, 2011). Surprisingly, Lamarck barely discussed cave species in his work, even though they seem to be a perfect example of environmental adaptation (Culver, Kane and Fong, 1995).

It was not until Lamarck's later work and the resurgence of Neo-Lamarckism[6] in the 1860's that cave organisms became an example species for Lamarckian evolution.

The problem with the explanation given by Lamarck and Neo-Lamarckians is the idea of individual adaptation itself. Evolution occurs over generations within a population of organisms not over the lifespan of a single one. Our current Darwinian view of evolution, which helped to show the problems with Lamarckian evolution approximately 150 years ago, focuses on the function of natural selection on a population.

While Lamarck did not discuss cave organisms until his successors delved deeper into his writings after his passing, Darwin was fascinated by cave animals. He even highlighted their presence in *On the Origin of Species* (Culver, Kane and Fong, 1995; Darwin, 1859). Using natural selection as an explanation for adaptation was easy for Darwin when examining enhanced traits and some regressive traits as well. For example, Darwin explained the loss of the wings on some beetles found in island habitats by noting there may be a predator-prey relationship on the island that selects against the ability of flight for the beetles (Culver, Kane and Fong, 1995).

Although Darwin discussed natural selection for regression and cave organisms on countless occasions, he never seemed to relate the two. This may be due to his inability to formulate a theory on the regression of the eye. While Darwin continually noted the importance

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[6] Lamarckian evolution in this time period was much different than the standard idea of adaptive evolution that Lamarck had created. Neo-Lamarckians believed in orthogenesis, or the idea that all external changes were mediated internally. While this theory still holds some credence in the realm of genetics, Neo-Lamarckians attributed the control of this internal force to not be dictated by external forces at all. Instead, they believed that this internal force was controlled by a higher, theological power. To contrast natural selection and "survival of the fittest", ontogeny was the "controller of fitness" (Culver, Kane and Fong, 1995).

of natural selection in relation to fitness, he could not seem to postulate a fitness benefit in the loss of the ocular structure. Instead, he proposed a “Lamarckian” explanation for the loss of the eye, which was that disuse caused the eye formation to be lost within the population (Culver, Kane and Fong, 1995; Gross, 2011).

Although both Darwin and Neo-Lamarckians attributed the regression of the eye to disuse of the organ, the major disagreement between the two groups was not “how” but “how long”. Darwin argued that loss of the eye would require a substantial amount of time to be acquired by an entire population, because the primary mode of modification occurred after every generation. On the other hand, Lamarckians believed that the loss of the eye was a relatively quick process, and troglobitic traits could be established within a few generations (Culver, Kane and Fong, 1995).

As Lamarck’s hypothesis was pushed aside after Mendel’s work on genetics was rediscovered, the method of using the degree of adaptation as a “clock” for the timing of population divergence is still applied in research today. By assuming that evolutionary rate follows some mathematical pattern, whether linear, exponential, logarithmic, etc., researchers have used this method to date cave animals time since divergence from a surface population. This method gives us not only a better understanding of the organism’s evolutionary history, but serves as a geological “stop watch” to determine the age of various cave systems as well.

During the decline of Lamarckian evolution, two important researchers postulated ideas that are still congruent with beliefs on regressive evolution. The first, Alpheus Packard, was a Lamarckian evolutionist who noted a relationship between the regression and enhancement of different traits (Culver, Kane and Fong, 1995). Packard paid attention to the fact that the lack of an eye structure, no matter what the species, seemed to always correlate with an increase in other non-visual sensory organs. This correlation between regression of one trait and expansion of another is an important feature of troglobites that many other researchers missed, and the fact that this pattern is still studied today highlights that fact. The other researcher, A.M. Banta, believed that cave species had entered into caves due to phenomenon he called preadaptation (Culver, Kane and Fong, 1995). The idea of preadaptation is that certain individuals within a population may be slightly better at functioning in the opening of a cave mouth or darkened environment, so they tend to reside in that area. Those individuals reproduce with nearby individuals (who also are preadapted) and produce offspring that can thrive deeper within the cave. This process continues until the population becomes split between cave and surface individuals. Offspring born deep within the cave that possess surface qualities are unable to survive in the darkened environment, so they do not reproduce and pass along their surface characteristics. Banta originally ascribed this preadaptation to the idea of orthogenesis – all external changes to an organism are regulated by internal processes[7] – but as Lamarckian evolution dwindled, he modified his theory to include the possibility of natural selection (Banta, 1907; Culver, Kane and Fong, 1995).

Preadaptation is no longer considered a viable theory for regressive evolution for a variety of different reasons. There is still noted interbreeding between cave and surface fish in the wild populations of the Sierra de El Abra (specifically the Chica cave; Romero, 2011), so how could the divergence begin in the first place if interbreeding is still occurring? There has also been difficulty in ascribing what specific features may be considered preadaptive. Also, if there was preadaptation, why has the cave version of the fish undergone such drastic external,

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[7] Many Lamarckians believed this internal regulation was controlled by a theological being or higher power.

internal, and behavioral adaptations (Romero, 2011)? However, it was not until the past fifteen years, with the application of molecular biology techniques, that these criticisms have come forward.

The importance of Banta's theory, even if it is not relevant today, was that preadaptation allowed Darwinists an access point to begin thinking more about regressive evolution. By attributing natural selection to regressive traits that seemed to have no fitness advantage, he led others to think about how these morphological changes happen if not through preadaptation.

Neutral Mutation Theory / Genetic Drift

After Lamarckian evolution took a backseat to Darwinian evolution as an explanation for the regressive traits found in cave animals, one of the first hypotheses was the neutral mutation theory. To understand the neutral mutation theory, it is first important to understand the idea of genetic drift. Natural selection is the idea that certain mutations are selected for within a population because they increase fitness for the species. There is a degree of pressure exerted on a population, and the individuals that can withstand that pressure are able to survive and pass on their genetic information to their offspring. Genetic drift is the opposite situation, which occurs when a trait has no selective pressures acting upon it. There is no beneficial or deleterious effects of modifying a gene, so random modifications occur naturally and are sometimes fixed within a population. At a point of fixation, no other version of a trait will exist in a given population, so all individuals will be homozygous for that trait (Pierce, 2010).

There are a few requirements that need to be satisfied for this dynamic to occur. The first is that a significant amount of time is needed to fix a regressive trait through genetic drift (Culver, 1982, Gross, 2011). Since genetic drift only occurs at generational points and requires completely random mutation to occur, a significant period of time is needed to fix the mutation in the population. Besides a longer period of time, genes that can be affected by genetic drift cannot have a large number of pleiotropic roles in the animal (Gross, 2011). Pleiotropy is when a gene has multiple roles in the functions of an animal, so genes that have many of these pleiotropic effects are much more likely to resist genetic drift, since a mutation in them would more likely be maladaptive and selected against.

Although neutral mutation through genetic drift is considered to be completely random, these stipulations have resulted in a few genetic areas that are found to be recurring targets for genetic modification (Gross, 2011). For example, the *Oca2* locus in *Astyanax mexicanus* has been noted to show high sequence variability when comparing surface and cave forms of the fish. The *Oca2* gene functions as a transmembrane channel that allows certain materials to pass into pigment cells called melanophores. These materials are used to create the pigment molecules that color an organism's exterior. Without this specific channel, the materials cannot enter the melanophores and the pigment is not produced, inducing albinism. The specificity of the *Oca2* gene's function is a good indicator that it is experiencing genetic drift. Also, researchers have found that modifications to the *Oca2* gene also follow an important pattern of genetic drift, in that not all mutations in the gene result in a loss of pigment (Gross and Wilkens, 2013, Protas et al., 2007). If genetic drift is truly considered to result from a lack of selection, there should invariably be some mutations that result in enhanced or modified traits instead of just loss.

Albeit true randomness should result in an equal distribution of regressed and constructive traits, but it has been shown that it is easier to destroy the functionality of a gene through modification that it is to enhance it (Culver, 1982). This pattern helps to satisfy the issue of time required for these modifications to occur. It would take a much longer period of time to develop these regressive traits if enhanced traits were also developed at the same pace.

The neutral mutation theory does seem to serve as a good explanation of regressive evolution, but there are some issues found in cave organisms that it cannot resolve. Although the tendency of many mutations is to focus on regressive or loss-of-function modifications, the reduction in time still does not sufficiently satisfy the time requirements needed to develop certain regressive phenotypes (Culver, 1982). Additionally, while pigmentation may show variability in enhanced and repressed traits, other traits, such as eye development, do not follow that pattern. To my knowledge, there is no recorded example of enhanced visual systems in troglobitic organisms. Other research shows that unlike pigmentation where genes do not always follow a consistent cave allele/surface allele breakdown, many eye-function genes do follow a strict cave/surface division (Protas et al., 2007). This means that a cave fish may possess an allele of a pigment gene found in surface fish, but is extremely unlikely that it will possess the surface allele for an eye gene[8]. Therefore, the neutral mutation theory serves as an explanation for some of the regressive traits found in cave species, but other explanations are needed for other regressive traits.

Direct Selection and Energy Cost Benefits

If the neutral mutation theory and genetic drift require no selection to be present to occur, the second theory that tries to explain regressive evolution is that direct selection occurs resulting in loss of the trait. The main argument behind this theory is based in the idea of energy efficiency. For example, if an organism does not appropriate energy for the formation of the eye,

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[8] This correlation varies based on the line and specific strain of cave fish under examination.

then there should be excess energy available that it can administer to bolster other functions that confer a fitness benefit. Since these organisms are already found in a nutrient-poor environment, any energy conservation would likely benefit its survival (Culver, 1982).

There is some research that supports the idea of direct selection. For example, there may be energy conservation involved in preventing the cellular reproduction required for full formation of the lens in the eye (Strickler, Yamamoto and Jeffery, 2007) or maintenance of the retinal tissue (Wong-Riley, 2010). In addition, the redistribution of energy to different traits to confer a fitness benefit is not unique to cave animals. Ground beetles have been shown to develop smaller eyes and cranial features to develop more prominent horns for mating and defensive purposes (Emlen and Nijhout, 2000).

Just as with genetic drift, though, energy efficiency does not adequately explain all facets of regressive evolution. Some traits, which would theoretically be the first to become adapted in a low-nutrient environment, barely demonstrate any adaptation. As discussed in Section II, metabolic efficiency is not a common trend in *Astyanax mexicanus* (Gross, 2011). Also, if direct selection were the primary method of regressive evolution, then the caves with the lowest amount of nutrient availability should see the highest amount of troglobitic adaptation. This trend is not consistent within *Astyanax mexicanus* when comparing it to the level of troglobitic characteristics of another cave fish *Caecobarbus geertsii* (Culver, 1982). *Caecobarbus* is considered to live in a much more nutrient poor cave system than *Astyanax* does, yet the level of adaptation between the two species seems to suggest they reside in ecosystems with similar energy availability (Culver, 1982). Direct selection does allow for the explanation of a few trends found in regressive evolution, but just like the neutral mutation theory it does not explain everything.

Indirect Selection through Pleiotropy

Direct selection in many ways is very simple to understand. A gene produces a product that causes a trait to grow or shrink, and if that trait is beneficial the gene is selected for or against accordingly. However, with more research and understanding of how genetics works, scientists have realized that there is not one gene for every trait, and that in many cases a single gene can have effects throughout the organism. This idea of a gene having effects on various traits is called pleiotropy. It is an important concept for cave species because it may serve as an explanation to the relationship that is seen when organisms regress some traits while enhancing others.

In cave fish, a very strong example of this phenomenon is the pleiotropic relationship between taste buds and eye size (Yamamoto et al., 2009). It is thought that the pleotropic gene that causes this tradeoff may be one of the hedgehog signaling pathway genes. As discussed in Section II, the hedgehog signaling pathway is one of the initial developmental pathways that is used by an embryo for early development in a large number of animals (Bitgood and McMahon, 1995). Specifically, the *sonic hedgehog* (*shh)* gene has regulatory effects on both eye development and taste bud formation (Yamamoto et al., 2009). The effects of *shh* are positively correlated with taste bud formation, so more *shh* expression results in a larger quantity of taste buds. This pattern is opposite in eye formation, which is repressed the more *shh* is expressed in the cave fish (Rétaux and Casane, 2013). Additionally, the *shh* pathway has upstream effects on other various enhanced traits in *Astyanax mexicanus* such as chemosensory nerve cell counts (Bibliowicz et al., 2013) and vibration attraction behavior (VAB) enhancement (Yoshizawa et al., 2010). This may mean that the *shh* pathway has multiple pleotropic effects that have indirectly selected for the regression of the eye, while selecting for enhanced phenotypes in *Astyanax*.

Before highlighting some of the issues with pleiotropy and indirect selection, I want to discuss a variation on this hypothesis that is coming to light with more advanced molecular biology methods. Instead of indirect selection occurring through a single gene with multiple pleotropic effects, there is a possibility that indirect selection may occur collaterally due to positioning of certain genes. If a gene is placed proximal to another gene that is considered evolutionarily disadvantageous, the silencing of that gene may not be limited to only it. There may be repressed expression of surrounding genes as well. Gene regulation is not a simple on/off switch. Spillover of silencing could be an important factor to more thoroughly explain indirect selection. Although this interaction has not been confirmed within a vertebrate model, studies using the bacteria *E.coli* have shown that indirect silencing can occur (Kahramanoglou et al., 2010).

The difficulty in the indirect selection hypothesis is the complexity of the hypothesis itself (Culver, 1982). It is very easy to theorize that genes may have multiple pleiotropic effects, but it has not been until the past five years that methods to adequately test these genetic networks was feasible and cost-effective. This hypothesis requires much more experimentation and direct evidence to document its role in regressive evolution. However, it serves as the most promising of the three hypotheses for future research because, unlike direct selection and neutral mutation theory, it potentially explains many aspects of cave evolution. Specifically, indirect selection can explain how traits can evolve at a rate similar to selection without conferring the direct energy conservation benefit required of both genetic drift and direct selection.

Lack of Mutual Exclusion and Epigenetics

Although I have presented the three primary theories behind regressive evolution as individual theories, in all likelihood there probably is a large amount of overlap between each on a variety of different regressive traits. It is highly plausible that neutral mutation may occur up until a point when a trait becomes modified in a way that confers a direct fitness benefit. Then, direct selection may amplify that specific mutation, which in turn could have pleiotropic effects and regress other traits of the species. The regressive evolution dynamic is a complex phenomenon, and because of this complexity it is likely that it does not occur through just one method (Gross, 2011; Jeffery, 2003).

In addition to these theories, I would like to also take some time to discuss the possibility of a fourth theory to explain regressive evolution. While noted earlier in this section that Lamarck's adaptive evolutionary theory was dismissed around the turn of the 20th century, recent data shows that there is a possibility of non-DNA based inheritance mechanisms that can pass information from parent to offspring. This method is through the regulatory mechanisms of gene expression, commonly referred to as epigenetics.

Epigenetic research has just recently found a footing in labs, and theories surrounding these gene regulating methods have begun to surface. Originally, it was believed that when a sperm fertilized an egg cell, the combined genetic information underwent a period of gene regulation cleanup where all past silencing or enhancing imprints are removed from the cell, placing it into a completely unregulated, totipotent[9] state (Pierce, 2010). There has been research

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[9] Totipotent refers to a cell that has not become specialized or has a determined function. Totipotent cells are the opposite of determined cells, which are a specific cell type that has been modified for a specialized function. Stem cells are considered totipotent, while skin, liver, heart, etc. are considered differentiated cells (Pierce, 2010).

that suggests that epigenetic states are not completely removed during this period (Bonduriansky and Day, 2009), and data has begun to arise that shows epigenetic patterning from parents may be transferred vertically to their offspring after all.

One important point of this phenomenon is that the epigenome – the unique patterning of epigenetic regulation – is a variable system that is modified based on the actions of the individual. For example, it has been shown in humans that smoking can show variations of their epigenome that reflect their poor habit (Launay et al., 2009). So if the epigenome can be modified during an individual's lifespan and there is reason to believe the epigenome is not completely removed during fertilization of an offspring, then there is the possibility that an individual's actions have an effect on their children's livelihood (Bonduriansky and Day, 2009).

Specifically in cave fish, it is likely that the lack of light in the cave environment may have caused inherited epigenetic effects, which may cause various regressive traits to be developed over time. One primary target of this theory could be related to the melatonin signaling pathway found in fish. Melatonin is a hormone that is found in a large number of both animals and plants, which is secreted by the brain when it is dark. This hormone has a large number of functions, but in fish it has been found to specifically modify reproductive behavior, growth and development, and even general activity levels (Falcón et al., 2010).

An increase in production of melatonin may be able to shed some light on the modifications that are seen in *Astyanax mexicanus* and other cave fish. The melatonin receptor functions in a negative feedback loop; receptor activity is restricted when melatonin is at higher concentrations and upregulated when melatonin is found in lower concentrations (Witt-Enderby et al., 2003). If this down regulation is continuously occurring then it could be that the receptors develop a tolerance to high concentrations of melatonin. This pattern is not unheard of, and is the primary reason why individuals who regularly abuse heroin are unable to manage pain with morphine injections (He et al., 2002). If that melatonin receptor tolerance confers an epigenetic shift to restrict the production of melatonin receptors, it could possibly be passed down to the offspring of the cave-trapped *Astyanax*, causing future offspring to develop a higher tolerance to melatonin. If this is the case, then the various effects of melatonin may result in modifications to the fish and its offspring repeatedly.

To test this theory, a variety of different experiments would need to be performed. First, tests would need to be performed to confirm whether or not there are significant differences between the *Astyanax* cave and surface forms' epigenomes. Looking at methylation patterns would be an ideal initial experiment, since many methods that have been developed over the past decade to characterize the human epigenome target methylation patterns (Rakyan et al., 2004).

If differences are found in methylation patterns, the next step would be to determine if living in a darkened environment has any effect on the epigenome of *Astyanax*. To do this, surface forms would need to be introduced into a dark environment and reside there from the onset of adulthood to death. This would give significant amount of time for the epigenome to theoretically be modified in that individual. To account for the possibility that epigenome modifications occur only during embryonic development (Bonduriansky and Day, 2009), surface fish in an identical darkened environment should be allowed to breed and produce offspring. Methylation patterns would need to be categorized prior to insertion into the dark environment, post-insertion, and also after the dark-only reared offspring had developed into adulthood. Then comparative analysis would have to be performed to see what effects residing and rearing in darkness has on the epigenome.

The theory of epigenetics is surprisingly Lamarckian, and therefore many scientists are quick to dismiss the idea of non-genetic modes of inheritance. However, the importance of the lack of light in these evolutionary labs is a variable that should not be ignored by scientists in the future when discussing these animals' origins. The power of darkness is quite strong, and likely has more of an effect on these organisms' development than we currently appreciate.

Conclusion

Cave organisms have baffled scientists for hundreds of years. From the early theories that cave salamanders were actually dragon offspring to the more complex thoughts created by Lamarck and Darwin, these organisms have not ceased to amaze scientists today. Whether these animals have developed their characteristic regressive traits through neutral mutation and genetic drift, direct selection through energy conservation, or indirect selection through pleiotropy, there is likely a lot more occurring to develop these specific traits than we currently understand. Future research will likely let us peer deeper into the depths of these caves and truly understand how and why they produce such pronounced regressive phenotypes.

IV ANALYSIS AND CRITIQUE OF TROGLOBITIC RESEARCH

As noted in Section III, troglobitic animals have interested scientists for hundreds of years. With a fairly strong resurgence of the topic in the 1970's and an ongoing expansion currently occurring with the development of molecular biology techniques, troglobitic animals are establishing a foothold in research labs across the globe. Initial research has focused on evolution and developmental topics, but more and more researchers have begun to apply more questions to these animal models, questions that have been tough to answer with current systems and methods. The benefits of the cave animal research model are numerous, but along with these benefits some restrictions are found as well. The goal of this section is to not only highlight the requirements a cave species must satisfy for effective scientific research, but also the pros and cons of using these specific models in the first place.

Criteria for Evolution and Developmental Studies

Over the past ten years, over six phyla of animals contain species that have functioned as troglobitic research specimens (Juan et al., 2010). From salamanders to spiders, a variety of cave organisms have become topics of scientists' interest. As noted in previous sections, there are a large variety of cave organisms. However, not all of these animals are good research subjects. There are limits to what type of research can be done on these animals if they do not fit an appropriate criteria.

The field of evolution and developmental science, referred to at times as Evo-Devo, is an area of biological research that has repeatedly used cave animals as research subjects. The confusion that regressive evolution creates with these animals is a problem that is difficult to pass up for many researchers. Protas and Jeffery (2012), reviewed the use of cave animals as Evo-Devo model systems and outlined the characteristics a troglobitic species needs to possess in order to be considered a plausible model for this type of experimentation. In most settings, these requirements for evolution and development research would likely apply to other areas of biology as well. Of the species we have discussed in the previous sections, the blind Mexican cavefish, *Astyanax mexicanus*, the cave amphipod, *Gammarus minus*, and the isopod crustacean, *Asellus aquaticus*, all fit the required characteristics (Protas and Jeffery, 2012).

The first and primary requirement that Protas and Jeffery (2012) note for any troglobitic animal to be considered for evolution and development research is the presence of both a cave and surface form of the animal. There are many species of cave animals that do not possess a surface counterpart. The cave salamander, *Proteus anguinus*, would not serve as a very benficial research model for this reason, for it is the only species in the genus *Proteus* on the planet. It may be that the surface form was lost to extinction or has not been discovered yet, but without the ability to compare the species to a surface form that shares a recent common ancestor, research is not possible.

It also serves no benefit to have a surface counterpart that is incapable of breeding and creating viable offspring with the cave form. To obtain information about the genetic variation causing distinct cave-based traits, labs breed cave and surface forms together to create an array offspring with a variety of combinations of cave and surface traits. This is done in one of two ways. Both methods begin with a cave and surface parent breeding to create a first generation of mixed-origin fish. These offspring, which all resemble the surface fish with smaller eyes, then can be bred in one of two ways. In a testcross, a fish that is determined to only contain cave-based genetic alleles, is bred with members of the first generation (Pierce, 2010). A backcross will take original parent that spawned the first generation and incestuously breed it with its offspring (Pierce, 2010). In both a backcross and testcross, the second generation of offspring reared from the first will display a variety of different physical traits. Some animals may be completely albino with full eye development, while other may possess the exact opposite features. These fish can be categorically separated based on these physical traits, and then genetically sequenced to find genetic variation that correlates with the physical trait variation. (Figure IV.1) Without the ability to interbreed, scientists can still do base comparisons between the cave and surface forms, but they cannot argue for functionality of that gene in the same manner than an approach that allows them to cross the cave and surface forms of the species.

The second criterion to determine whether a troglobitic species is suitable for Evo-Devo research is the ability to collect the species from its natural habitat (Protas and Jeffery, 2012). As noted in Section I, there are caves all over the surface of the planet. While some caves are easily accessible, some are not. For example, in caves with large bat roosts, the presence of airborne fungal disease from the large amounts of bat guano may make the caves unsafe (Protas and Jeffery, 2012). Some caves also possess dangerous levels of ammonia or hydrogen sulfide, so spelunking into these caverns becomes difficult without extensive protective equipment (Sarbu, 2000).

Besides physical limitations on specimen acquisition, there also may be legal limitations as well. Many troglobitic species are so unique that they fall under the endangered classification. Laws limit researcher from obtaining adequate sample sizes of some of these animals and may also hinder the ability to resupply laboratory stocks if an accident occurs. Without an easily obtainable and legal source of specimens, certain troglobitic species cannot be used as research specimens.

If a species has both a cave and surface form and is easily accessible and obtainable, another requirement to use it for research is how the animal functions outside of its natural habitat and within a laboratory (Protas and Jeffery, 2012). Feeding behavior, water or air temperature requirements, and habitat attributes all require time and effort to understand and implement. In the case of some troglobites, the effort required for this is not worth the perceived benefits by some researchers. Besides proper care of troglobitic species, researchers also must understand how to maintain laboratory populations through breeding. Characteristics such as number of times an individual can reproduce, what environmental cues are required for breeding, and offspring hardiness can all make maintaining a research population difficult. If the species lives for an extremely long period of time or has a gestation period that makes it difficult to breed them relatively quickly, the likelihood that the species can serve as a research specimen is low.

Species that can fulfill all of these requirements make fantastic research models. Their resiliency and unique troglobitic properties allow experiments to be performed that are not possible using other species that have not been genetically modified. Besides the three model species listed above (*A. mexicanus, A. aquaticus, and G. minus*) a few other species fulfill these requirements and have promising futures for troglobitic research. Planarians, a type of non-parasitic flatworms, which have been hailed as a target species for upcoming genetic research (Newmark and Alvarado, 2002), have a troglobitic form that is lacking eye spots and pigment (Buchanan, 1936). An entire genus of Hawaiian planthopper[10], *Oliarus*, has formed troglobitic features such as albinism, reduced wing size, and increased antennae size (Bilandžija, Ćetković

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[10] The planthopper is a type of leaf-resembling insect that travels in a similar manner to a grasshopper (Bilandžija, Ćetković and Jeffery, 2012).

and Jeffery, 2012). Both of these species have surface counterparts which have been the focus of laboratory work in the past, meaning they are not difficult to integrate into a researcher's laboratory. In addition, they both allow for large specimen populations due to their small size. While not all troglobitic animals are useful as research specimens at this time, the prospects of using new, suitable species shows great promise.

Benefits of the Troglobitic Model System

The unique traits and specific dynamics that were required to create troglobitic species are some of the most particular selective pressures of any ecosystem. The unique qualities of these species incite a sense of curiosity that is almost a requirement for any researcher to want to investigate a particular phenomenon. However unusual, though, there are more benefits to studying troglobitic species besides the fact that they are different and interesting.

One primary benefit of cave species research is how it is used to determine gene function. Typically, genetic research follows a "bottom up" method of determining gene functionality. Geneticists can use chemicals called mutagens that cause a variety of types of DNA damage and modification in a species. A researcher will take a large number of developing, for example, and expose them to the mutagenic compound. Then they let the mice develop and see what types of physical alterations appear. After a target trait is affected, they then will compare these changes to the genetic modifications the mutagen made and try to determine what functions those genes have in the organism. This method does produce results, but has a variety of issues associated with it. The nonspecific aspect of mutagen-based modification associated with this method may make it likely that certain genetic targets are missed. Also, certain genetic mutations sometimes result in drastic physical changes, while others are completely silent. Many times, run a battery of tests with hundreds of test subjects, which mitigates these concerns. However, that in itself highlights another issue, that this “bottom up” method requires an extremely long time scale to create results.

Troglobitic genetic research is more of a “top down” approach. The natural laboratory represented by the cave environment has performed the genetic knockout for the researchers already. All that is required is to determine what mutations have been made to show the change in physical traits. Besides saving time, this method also has the opportunity to highlight genes that may be missed by chance through the traditional method of genetic research.

In addition to the method of genetic research, another simple yet important benefit of the troglobitic model has to do with the directionality of the changes in the species. There is a known polarity to the trait changes of cave animals that isn’t as obvious in other species. While there is disagreement on the method of eye loss in cavefish, for example, no researcher would ever make the argument that the eye remnants in the cavefish are actually the emerging novelties of an eye in the species (Jeffery, 2001). This directional modification helps many scientists working in Evo-Devo to focus their discussions on the mechanisms behind regressive evolution, instead of arguing whether it or not it is occurring. The definitive loss of functionally in these species is a huge benefit to our understanding of evolutionary theory, which is more difficult to explain and analyze in other situations.

The final benefit that is not as apparent when examining troglobitic species research has to do with its impacts outside of the laboratory. Troglobitic animals help to serve as a very strong example of evolutionary theory and biological research for the public. The physical traits associated with the cave and surface lineages and the peculiar array of traits found after the species has been crossbred together is a very visible phenomenon (Figure IV.2). You do not need to be able to understand a complex series of data sets or have a degree in biology to understand the differences between the two forms of these animals. Additionally, the stark images also serve to help instill a sense of wonder and excitement about the sciences in the general public. To better recruit students to study the sciences, displaying examples of current scientific research that intrigues them is an effective tool. From personal experience, the peculiarity of the eyeless *Astyanax* has served as an interesting discussion topic for many people outside of the sciences. The opportunities to use this research to not only answer questions about the world around us but also get other people intrigued about those questions is a major benefit of troglobite research.

Issues and Restrictions of Cave Animal Research

Although the benefits of cave animal research can give scientists a platform for both important research discoveries and public relation boons, there are some negatives associated with troglobitic research as well. The first highlights the “double-edged sword” effect of increased public visibility. Troglobitic research can help to easily intrigue members of the general population, but increased exposure also allows a greater opportunity for misinterpretation. Many individuals have used these cave animals as “evidence” of anti-Darwinian or creationist theories. These arguments stem from a lack of understanding of the basic details of modern evolutionary theory. For example, some anti-evolutionists try to use the process of genetic drift to show that Darwin was incorrect in his arguments on natural selection. They argue that because cavefish development has not been directed through natural selection, thus Darwinian Theory is disproven by these species[11]. Others incorrectly assume that evolution is a "means to an end" in the sense that it only can result in new or beneficial traits to a species. These arguments demonstrate dramatic misunderstandings of modern evolutionary theory. Directionality is not a feature of evolution, and theoretically a population of cave animals could (if given enough time and the correct selective pressures) develop into a fish population resembling their surface ancestors. Therefore, researchers using these model systems need to ensure that the messages they present to the general public are clear and difficult to misinterpret to prevent these arguments from gaining traction.

The other issue that arises with the use of troglobitic species as research models has to do with the disruption that is caused when humans interact with these animals. Original specimens of *Astyanax mexicanus* were brought to the United States from Mexico in the 1940's. Researchers share and spread different species of *Astyanax*, so the majority of the laboratory specimens across the United States and in other countries outside of Mexico are cavefish that have never resided in an actual cave (personal contact). The importance of this fact depends on the research being performed. For morphological research, it is unlikely that millions of years of genetic modification will be reversed within a span of approximately eighty years in laboratories that have lights. As discussed in Section II, for behavioral research the effects that light has on activity levels is possibly more influential and may cause experimental error.

The issue is that scientists do not know what type of effect laboratory rearing has on *Astyanax*. It may be that the effects are minimal and the cavefish are very similar to those still found in the Sierra de El Abra caves, or it may be that laboratory rearing has caused changes that

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[11] I would like to point out that Darwin's Theory of Evolution, put forth in *On the Origin of Species,* has been added to and modified numerous times since its publishing. Current evolutionary theory is very different than what was written almost 180 years ago.

directly affect the results found in these laboratories. Scientists refer to the caves these fish originate from as natural laboratories due to their isolation and controlled elements, and by removing the fish from these locations they have modified this natural experiment. To determine if this change has affected the integrity of these species as a research tool, comparisons need to be made to cavefish that have been reared naturally in a cave system. Genetic variation tests need to be performed to determine if a laboratory setting has a positive or negative effect on the rate of genetic modification compared to the cave system. This test could be performed through a standard full-genome genetic comparison of a set number of individuals. The comparison would look for genetic differences that may have been created by laboratory rearing. In addition to this test, gene expression profiles and epigenome comparisons also should be performed to see if there are any expression variations that are not accompanied by genetic sequence variation. The final tests should examine behavioral modification to see if laboratory and natural cave organisms respond differently to an array of sensory inputs, such as light, food, and current flow rates. These tests would help to determine what effect, if any, controlled laboratory rearing of these animals has caused.

Admittedly, it is unlikely that tests such as these are of paramount importance at this point in time. The majority of the recent research on cavefish and other troglobitic species has been focused more on morphological differences between the cave and surface forms of the animals. Albinism, eye loss, and other traits likely took an extended period of time to evolve and become fixed, and it is unlikely that laboratory rearing could have changed the base genetic variations that have caused these trait differences. However, as research on troglobitic species expands into other areas it is likely that these tests would need to be performed in order to ensure that variation induced by the laboratory environment does not invalidate the troglobitic aspect of these specimens.

Conclusion

Studies using troglobitic animals as a research model have the potential of leading to a better understanding of various aspects of evolution and development. Although not all of the animals found within caves make efficient and effective research models, the ones that do have great promise for future experimentation. Additionally, the visible differences between the cave and surface forms of these animals can serve as an effective tool to help increase public interest in the sciences, as long as the information is portrayed in a manner that is difficult to misinterpret. The biggest question that arises from the use of these species is how much of an effect rearing cave animals in a lab has on their troglobitic integrity. No matter the answer to this question, cave animals have developed and will continue to develop our overall understanding of the differences between the cave and surface environments, which can help us to take full advantage of the results of these natural experiments in cave "laboratories."

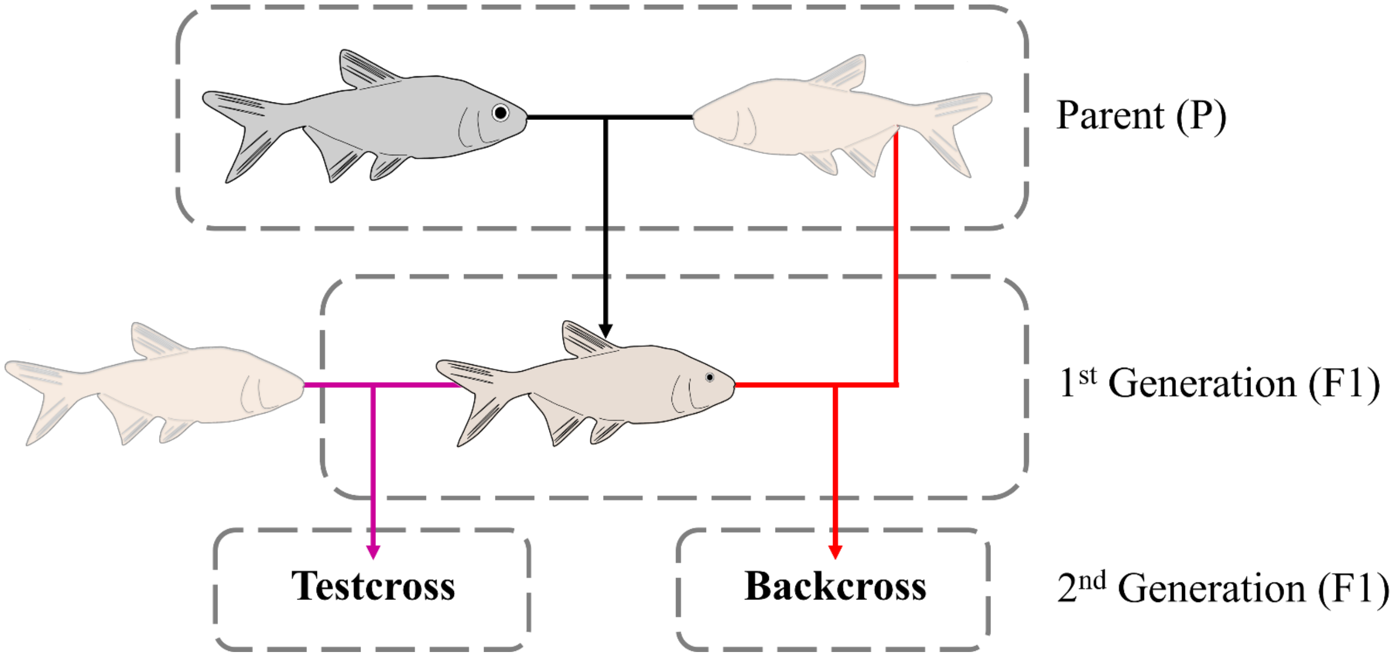

Figure IV.1 – Diagram Representing Testcross vs. Backcross – To do comparison genetic testing, scientists employ either the backcross or testcross to create a variety of physical traits. Both methods begin by breeding a surface and cave form of the animal together to form a mixed first generation (F1). After this breeding, researchers can either breed individuals from the F1 group back with a cave parent or with another confirmed cave animal (Pierce, 2010). Both methods result in the same 2nd generation of offspring, which can be viewed in Figure IV.2.

Figure IV.2 – F2 Offspring Raised from Testcross or Backcross – These images are results of a successful backcross of testcross method used to create a population of fish that possesses individuals with mixed cave and surface traits. These fish can be genetically sequenced to find possible genes that may have an effect on the physical variations found among these individuals.

This image has been modified from Gross, Borowsky and Tabin, 2009.

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