**HARVARD UNIVERSITY**
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## DISSERTATION ACCEPTANCE CERTIFICATE

The undersigned, appointed by the

Division of Medical Sciences

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have examined a dissertation entitled

*The genetic basis of behavior in the blind Mexican cavefish, Astyanax mexicanus*

presented by Johanna Elizabeth Kowalko

candidate for the degree of Doctor of Philosophy and hereby certify that it is worthy of acceptance.

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# The genetic basis of behavior in the blind Mexican cavefish, *Astyanax mexicanus*

A dissertation presented

by

**Johanna Elizabeth Kowalko**

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

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Dissertation Advisor: Dr. Clifford Tabin Johanna Elizabeth Kowalko

# The genetic basis of behavior in the blind Mexican cavefish, *Astyanax mexicanus*

## Abstract

In recent years, considerable progress has been made towards understanding the genetic basis of the evolution of morphological traits. In contrast, relatively little is known about how behavioral traits evolve. *Astyanax mexicanus*, a species of fish that exists in both surface and cave forms, is an ideal system to study behavioral evolution. Surface and cave morphs of *Astyanax mexicanus* differ in a variety of morphological and behavioral traits. They are interfertile, allowing for genetic analysis of the evolution of these traits. Finally, *Astyanax mexicanus* exists in multiple, independently evolved cave populations, providing an excellent system for studying convergent evolution.

In this dissertation I have analyzed two behaviors that evolved in cave *Astyanax*: the loss of aggregation behavior, and a change in feeding posture. While surface *Astyanax* both school and shoal, cave populations do not display either of these behaviors. Surface *Astyanax* feed at an angle nearly perpendicular relative to the ground, while cavefish feed at a lower angle. I quantified these behaviors in three cave populations and compared them to

surface fish behavior. I performed quantitative trait loci (QTL) analysis to elucidate the genetics of these behaviors. Finally, I explored the contribution of morphological traits that have evolved in cave populations of *Astyanax* to these behavioral traits.

I found that multiple, independently evolved cave populations of *Astyanax* have lost schooling behavior and altered feeding posture. Loss of schooling behavior evolved through multiple genetic changes, and this loss results from both vision-dependent and vision-independent changes. Feeding posture, on the other hand, has not evolved through any changes in morphology analyzed here. Interestingly, two independently evolved cave populations have evolved changes in feeding posture through at least some different genetic loci.

Behavior in cavefish can evolve through multiple genetic changes, rather than through one gene of large effect. Changes in morphological traits can play a key role in behavioral evolution. Lastly, while convergent behaviors have evolved in different cave populations, these behaviors can evolve through different genetic loci. Together, this work provides fundamental insight into the genetic basis of behavioral evolution.

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## Dedication

To

my parents

my brother John

and Jeff

for your love and support

# Chapter One

# Introduction

Organisms are extremely diverse in both morphology and behavior. Underlying this diversity are genetic changes, which over evolutionary time have come to differentiate populations of organisms from one another and specify a diverse set of characteristics. Understanding the genetic basis underlying the evolution of morphological traits has been and continues to be a field of intense interest, where researchers have made considerable progress. Equally important, but much less well understood, is the genetic basis of the evolution of behavioral traits.

Understanding the genetic basis of behavioral evolution will elucidate many important evolutionary questions, including: Do behaviors evolve through changes in one gene of large effect or multiple genes of small effect? What are the contributions of the evolution of morphological traits, such as sensory systems, to the evolution of behavioral traits? When a behavior evolves multiple times, does it evolve through changes in the same genes? Do behaviors evolve through coding or regulatory changes? What is the contribution of natural selection to behavioral evolution? What are the developmental and genetic constraints on behavioral evolution?

In this dissertation, I attempt to understand some of these questions using the blind Mexican cavefish, *Astyanax mexicanus*, as a system to study the evolution of two behaviors – loss of schooling behavior and modification of feeding posture. As an introduction, I briefly discuss the genetic basis of behavioral evolution. I discuss the utility of cave organisms for the study of evolution, and give an overview of the organism discussed in this dissertation,

*Astyanax mexicanus*. I discuss what is currently known about the specific behaviors discussed in this dissertation, both generally and specifically in *Astyanax*. I conclude with an overview of the research presented in this dissertation.

## Genetics of the evolution of behavior

A behavior is defined as any of an organism's actions. Behaviors are important for many aspects of an organism's life, including mating and raising young, finding and obtaining food, selecting a habitat, and avoiding predators, to name a few. Why an organism behaves in a particular way is based on a large number of factors, including that organism's genetic makeup, neuronal circuitry, environment, perception of that environment, and past experiences. Given the large number of factors influencing behavior, it is unsurprising that the types of genes important for generating behaviors and their functions are diverse, including genes involved in sensory systems, emotion, motivation, nervous system development, and neural plasticity (Bendesky and Bargmann, 2011).

Behavioral evolution has been an area of interest for quite some time (Lorenz, 1958). Behaviors can evolve. For example, in response to a new environment, populations can evolve both morphological and behavioral traits as they adapt to new selective pressures (e.g. Spence et al., 2013). Behaviors can also play a role in the evolution of other traits. Behavioral shifts can allow organisms to exploit a new environments, thereby exposing

them to new sets of selective pressures, which in turn can affect evolution of other traits (Duckworth, 2009). Investigations into the genetic underpinnings of behaviors help further our understanding of behavioral evolution (Boake et al., 2002).

A significant amount of evidence, from classic studies of both laboratory animals and natural populations, has helped broaden our understanding of the genetic architecture underlying behavioral traits. Many previous studies have focused on determining the function of single genes through the analysis of mutants or candidate genes, as well as identifying many genes and genetic loci responsible for behavior through genetic mapping or gene expression studies (reviewed in Boake et al., 2002; Bendesky and Bargmann, 2011).

Quantitative trait loci (QTL) analysis is an important tool that can be used to elucidate the genetic basis of behavioral traits. QTL analysis involves phenotyping a group of related individuals, genotyping these individuals for markers distributed across the genome, building a linkage map to determine the relationship of these markers to one another, and mapping where the loci responsible for these phenotypes are within the genetic linkage map. QTL analysis has been used successfully to map the genetic basis of behavioral traits differing between inbred strains of laboratory animals such as flies (e.g. Edwards and Mackay, 2009) and mice (e.g. Sauce et al., 2012), as well as in natural populations such as sticklebacks (Greenwood et al., under review) and deer mice (Weber et al., 2013). QTL analysis can answer important

questions about behavior traits, including whether they evolve by changes of large or small effect, where these changes are located within the genome, and whether independently evolved populations evolve similar traits through the same or different loci. Furthermore, QTL analysis provides an initial step towards identifying the genes and genetic changes underlying behavioral evolution.

A few principles from this work have emerged that are relevant to the evolution of behavior. First, although single genes of large effect can underlie natural variation in behavior, many behaviors are controlled by multiple genes of smaller effect (Bendesky and Bargmann, 2011). Second, while genes responsible for variation in behavior in natural populations could be in many different groups of genes, many of the genes identified so far affect sensory systems, or neurotransmitters and neurotransmitter receptors (Bendesky and Bargmann, 2011).

To understand the origins and evolution of a given behavior, it is essential to understand the behavior from a broad perspective. Tinbergen established categories important for the study of any behavior. He held that to understand behavior, we must understand proximate mechanisms, the development of behavior and mechanistic, causal explanations for how behavior works, as well as ultimate mechanisms, including how behaviors affect survival and the evolutionary history of organisms and their behavior (Tinbergen, 1963). *Astyanax mexicanus* is particularly suitable to this type of analysis, due to both its habitat and the rich wealth of previous research. In

this dissertation, I contribute to aspects of the genetic underpinnings of behavioral evolution in *Astyanax* and attempt to integrate this with what is known about the ecology of the system, with the objective of moving closer to Tinbergen's goal of an integrated understanding of behavior.

## The cave as a system to study evolutionary biology

Caves are an excellent place to study evolution, in particular adaptation to a novel environment. The cave habitat provides several striking selective pressures, the most obvious of which is darkness. Animals within a cave must navigate, find food, avoid predators, and mate, all without visual cues (Culver and Pipan, 2009). The lack of light means there are not any primary producers, and most cave ecosystems, therefore, rely on food brought in from outside of the cave. This often, although not always, results in caves having reduced amounts of food (Culver and Pipan, 2009). For a number of environmental factors, such as temperature, caves represent a relatively constant environment with little variability compared to surface habitats. Other environmental factors, however, can be quite variable within caves, such as flood-dependent food availability (Culver and Pipan, 2009).

Similarities between cave habitats have led to a large amount of convergent evolution. Many cave organisms have evolved a similar set of troglomorphic, or subterranean-specific, traits: reduced eyes and pigmentation, elongated appendages, and enhanced non-visual sensory systems (Culver and Pipan, 2009). It is estimated that over 50,000

subterranean species exist, counting only those groups which include at least 50 identified subterranean species. These represent 21 invertebrate orders and 2 vertebrate groups, salamanders and fishes (Culver and Pipan, 2009). Furthermore, cave species do not appear to be a random sampling of surface populations that were swept into the caves by chance. This has led many cave biologists to speculate that certain populations are pre-adapted to cave life (Culver and Pipan, 2009).

There has been a considerable amount of debate about the role of natural selection in the evolution of cave-specific traits (Culver and Pipan, 2009). Some researchers have postulated that the loss of traits such as eyes and pigmentation results from lack of use (e.g. Darwin, 1859) and subsequent accumulation of neutral mutations (e.g. Wilkens, 1988). Others have argued that some regressive traits, such as eye loss, could have arisen under natural selection (e.g. Protas et al., 2007), either because they are, themselves, adaptive or through pleiotropic selection in favor of constructive traits (Yamamoto et al., 2009; Yoshizawa et al., 2012). Constructive traits, on the other hand, are thought to be adaptive in cave environments (reviewed in Culver and Pipan, 2009).

Cave organisms are interesting for the study of evolutionary biology for several reasons (Protas and Jeffery, 2012). Cave animals evolved from surface animals. Therefore, the direction of evolution is known. The surface counterparts of some of these cave species are extant, providing an opportunity for comparative studies, and in some cases to perform genetic

crosses. The environment in which cave animals evolved is relatively well defined, and, therefore, the ecological influences on cave traits are easier to study. Finally, the evolution of similar traits across a large number of species allows for the study of convergence.

## An introduction to *Astyanax mexicanus*

*Astyanax mexicanus* is a species of teleost fish that exists in both cave and surface forms. Cave forms of *Astyanax* were first described in 1936 (Hubbs and Innes, 1936). Both surface and cave forms of *Astyanax mexicanus* are extant, therefore comparisons can be made between derived (cave) and ancestral-like (surface) fish. Cave forms of *Astyanax* have evolved a variety of morphological and behavioral traits (reviewed in Jeffery, 2008). Furthermore, cave and surface forms are interfertile (Sadoglu, 1979), and can be bred together to explore the genetic basis of the evolution of these cave traits (Wilkens, 1988). Importantly, both cave and surface fish live and breed in the lab and have a relatively short generation time, 4-6 months (Jeffery, 2008), both of which facilitate genetic and developmental research.

A number of tools have been developed for *Astyanax mexicanus* because of its utility as a model system for evolutionary research. The genetic underpinnings of many cave traits have been mapped using the linkage maps established for quantitative trait loci (QTL) mapping (Protas et al., 2006, 2007, 2008; Gross et al., 2009; Yoshizawa et al., 2012; O'Quin et al., 2013). Genetic overexpression and knockdown techniques have been

used successfully in *Astyanax* to perturb gene expression during development (Yamamoto et al., 2004, 2009). Genomic tools exist, including a BAC library (Di Palma et al., 2007) and extensive synteny maps with zebrafish (Gross et al., 2008; O'Quin et al., 2013), that are useful for identifying candidate genes under QTL. Transcriptomic analyses have been performed comparing both adult (Gross et al., 2013) and developing (Hinaux et al., 2013) surface fish and cavefish. Most recently, the *Astyanax mexicanus* genome has been sequenced and is currently in the process of being assembled and annotated, which will allow for a more comprehensive analysis of specific genes and mutations underlying the evolution of cave traits.

Like other cave animals, *Astyanax* is also a useful system for studying convergent evolution. At least 30 cave populations of *Astyanax mexicanus* exist (Mitchell et al., 1977; Espinasa et al., 2001). Each cave population is named after the cave it inhabits, and these populations share a number of morphological similarities (Figure 1.1). The origin and evolutionary history of these cave populations has been studied extensively (reviewed in Gross, 2012). Existing cave populations were colonized by two separate surface populations: one older, now extinct population, which colonized the El Abra cave populations including Pachón, Tinaja and Chica caves, and one younger population which colonized the Micos and Molino caves (Bradic et al., 2012). Furthermore, both invasions consisted of multiple, independent colonization

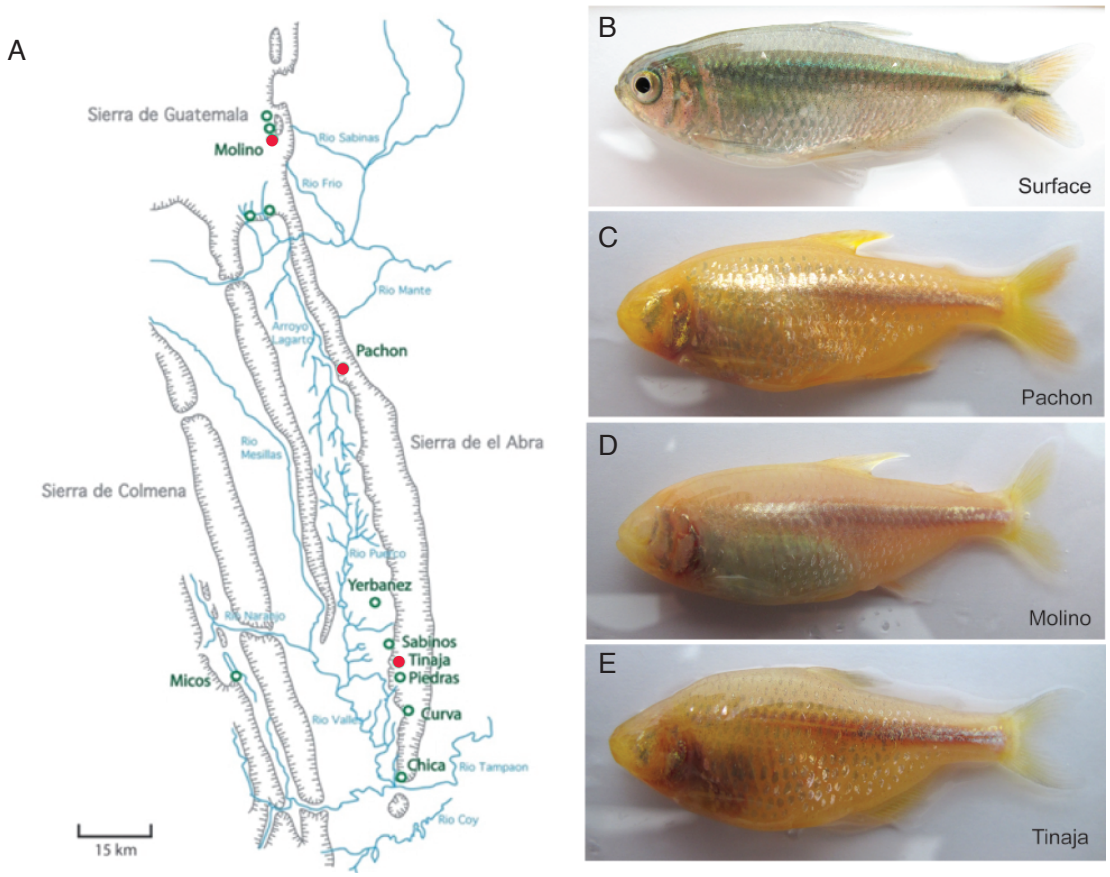


**Figure 1.1. Map of cave locations and examples of morphologies of surface and cave *Astyanax mexicanus*.**

A. Map of the Sierra de el Abra region of Mexico. Caves with cavefish are indicated by circles. Red circles indicate the cavefish populations used in this work. B. Surface fish. C. Pachón cavefish. D. Molino cavefish. E. Tinaja cavefish. Map adapted from Mitchell et al. (1977).

events. At least three invasions occurred from the old surface population - Pachón, Chica, and then the rest of the El Abra cave populations, and two invasions from the new population - Micos and Molino populations (Mitchell et al., 1977; Dowling et al., 2002; Strecker et al., 2003; Strecker et al., 2004; Bradic et al., 2012). Analyses of the genetics of different morphological traits have confirmed that many traits evolved independently in different caves (Wilkens and Strecker, 2003; Protas et al., 2006; Borowsky, 2008; Gross et al., 2009). Therefore, *Astyanax mexicanus* is useful for studying convergent evolution of traits.

Together, the evolutionary history, ecology and genetics of *Astyanax mexicanus* make it an ideal system for studying important questions about how traits evolve. So far, research on *Astyanax mexicanus* has elucidated some aspects of the evolution of certain cave traits, including the number and identity of genes underlying the evolution of traits, if these changes are due to coding or regulatory mutations, whether these traits have evolved by selection, or through neutral mutations, and the role of pleiotropy during evolution. The genetic and developmental underpinnings of some of the morphological traits have also been uncovered. This research has shed light on the genetic basis of independently evolved traits, including whether they evolve through the same or different genetic changes. I will now briefly review some of the morphological and behavioral traits evolved in cave *Astyanax mexicanus*, and what is known about their evolution.

Cavefish have evolved a suite of regressive morphological traits, including typical troglobite traits, such as a reduction in pigmentation and the eyes. Cavefish have a reduced number of melanophores. Additionally, their melanophores produce less pigment than melanophores in surface fish, and some cave populations are albino (Sadoglu, 1957; Sadoglu and McKee, 1969; Wilkens, 1988; Wilkens and Strecker, 2003). While the decrease in pigmentation cell number is a polygenic trait (Sadoglu, 1957; Wilkens, 1988; Protas et al., 2008), albinism is caused by one gene of large effect (Sadoglu, 1957; Protas et al., 2006). Interestingly, multiple populations of cavefish have evolved a reduction in the number of melanophore pigmentation cells and melanin content per cell through different mutations in melanocortin 1 receptor (Mc1r) (Gross et al., 2009), and albinism through different mutations in ocular and cutaneous albinism II (Oca2) (Protas et al., 2006). Furthermore, some of these mutations were coding, while others are likely regulatory (Protas et al., 2006; Gross et al., 2009). Loss of pigmentation has been hypothesized to occur through neutral mutations, rather than through selection against pigmentation (Wilkens, 1988). This may be the case for reduction in the number of pigment producing cells, as cave alleles at QTL responsible for pigmentation both increase and decrease the number of pigment producing cells, an indication that this trait may have evolved without selection (Protas et al., 2007). It has been speculated that albinism, on the other hand, could have evolved as an adaptation due to trade-offs. Excess L-tyrosine substrate no longer used to make melanin in albino animals could be

used to synthesize extra dopamine, which could play a role in adaptive behaviors such as foraging (Jeffery, 2006). Further investigations into the role of Oca2 in evolution are needed to resolve this interesting question.

Adult cavefish do not have external eyes (Figure 1.1). Larval cavefish develop eyes which degrade and eventually become covered by skin (reviewed in Jeffery, 2001). Reduction of the eyes in cavefish is the result of multiple developmental processes. The initial optic vesicle is smaller in cavefish relative to surface fish (Cahn, 1958), a process likely controlled by an expansion of the sonic hedgehog genes (Shha and Shhb) along the midline during cavefish development (Yamamoto et al., 2004). In addition, the ventral portion of the retina is never formed (Yamamoto et al., 2004), due to an early expansion of fibroblast growth factor 8 (Fgf8) and Shh (Yamamoto et al., 2004; Pottin et al., 2011). Many other aspects of eye degradation result from the apoptosis of the lens (reviewed in Protas and Jeffery, 2012). This has been demonstrated by early lens transplantations from surface fish to cavefish, which result in smaller but well-formed cavefish eyes, and lens transplantations from cavefish to surface fish, which result in largely degraded eyes (Yamamoto and Jeffery, 2000). Lens apoptosis, like the smaller optic vesicle, is a result of the expansion of Shh at the midline in cavefish (Yamamoto et al., 2004).

Eye reduction in cavefish is the result of many genetic changes (Protas et al., 2007; Protas et al., 2008). Furthermore, reduction of the eyes has occurred through at least some different genetic changes in multiple cave

populations (Wilkens, 1971; Wilkens and Strecker, 2003; Borowsky, 2008). Whether the reduction of the eyes in *Astyanax* has occurred through lack of use and therefore the accumulation of neutral mutations (Wilkens, 1971), or through selection for smaller eyes has been a matter of debate. All of the QTL responsible for eye reduction from a Pachón cave cross have cave alleles associated with a decrease in eye size. This is evidence that, at least in the Pachón cave population, eye reduction may be under selection (Protas et al., 2007).

Other regressive morphological traits in cavefish include a decrease in the size of the optic tectum (Soares et al., 2004), and in some cave populations a reduced number of ribs (Dowling et al., 2002), a reduction in the number of anal fin rays (Protas et al., 2008), reduced scales (Wilkens, 1988), and smaller suborbital bones (Yamamoto et al., 2003). Additionally, while surface fish display space-dependent growth, defined as the size of the environment having an effect on growth rate, multiple cave populations have decreased this effect (Gallo and Jeffery, 2012).

Cave *Astyanax* have also evolved constructive traits, many of which probably aid in foraging and navigation in the dark environment of the cave. The *Astyanax mexicanus* lateral line consists of both superficial and canal neuromasts, the functional units of the lateral line. Cavefish have an increased number of cranial superficial neuromasts, and increased size of both superficial and canal neuromasts relative to surface fish (Teyke, 1990;

Jeffery et al., 2000). These sensory organs most likely evolved to help cavefish locate food in the dark (Yoshizawa et al., 2010).

Cavefish have a number of other constructive adaptions thought to be associated with differences in foraging. They have an increased sensitivity to amino acids in the water (Protas et al., 2008), as well as skeletal traits potentially associated with feeding, such as wider jaws (Yamamoto et al., 2009) and an increased number of maxillary teeth (Yamamoto et al., 2003). Additionally, they have an increased number of taste buds. Taste buds in surface fish are confined to the lips and mouth, while in cavefish taste buds are also on the ventral side of the lower jaw, along the top of the head, and expanded laterally on the lip (Schemmel, 1974; Varatharasan et al., 2009). Interestingly, the expansion of taste buds and jaw width in cave *Astyanax* has been linked developmentally to the reduction in eye size. Shh expression at the midline, which reduces the size of the eyes, also increases the number of taste buds and the width of the jaw (Yamamoto et al., 2004, 2009). Furthermore, some QTL for taste buds and eye size overlap (Protas et al., 2007), supporting the idea that the same genes may play a role in the evolution of both of these traits. This provides a potential explanation for how selection could be acting to decrease eye size. There could be a trade-off between having eyes, and other, dark-adaptive traits, such as enhanced taste buds and jaw width, if these traits are controlled by the same genes as eye size (Jeffery, 2005).

Cavefish also have modified brain morphology relative to surface fish. They have a larger telencephalon, hypothalamus, and olfactory lobes relative to surface fish (Menuet et al., 2007), reviewed in (Retaux et al., 2008). Many of these changes might be linked to enhanced non-visual sensory systems, and they are at least in part controlled by the Shh and Fgf8 pathways (Menuet et al., 2007).

In addition to morphological differences, *Astyanax* cavefish have evolved a suite of behavioral differences. Some of these behaviors have may have evolved to help cavefish find food in the dark, which would suggest that they are adaptive. Cavefish probably lack macroscopic predators in the cave (Jeffery, 2008), and behaviors important for predator avoidance could, in principle, be lost in cavefish due to a release of selective pressures to maintain these traits.

Behaviors that may be linked to food finding include a vibration attraction behavior (VAB), present in some cavefish populations and nonexistent in the majority of surface fish (Yoshizawa et al., 2010). Cavefish are attracted to objects oscillating in the water, a behavior that is presumably a disadvantage in the surface fish habitat, which contains predators. In a dark, predator-free environment, however, this behavior could enhance their ability to find food (Yoshizawa et al., 2010). VAB relies on functional superficial neuromasts (Yoshizawa et al., 2010) and has evolved through multiple genetic changes. It has been linked genetically with an increase in the number of orbital neuromasts and a reduction in eye size, both of which

share overlapping QTL with VAB and each other (Yoshizawa et al., 2012). This finding is particularly interesting, as it offers another explanation for why the loss of eyes may have been under selection (Yoshizawa et al., 2012; Yoshizawa et al., 2013).

Reductions in sleep and aggression are also cavefish behavioral traits hypothesized to be important in foraging. Cavefish spend less time sleeping relative to surface fish. This behavior, reduced in multiple cavefish populations, and potentially regulated through β-adrenergic signaling, may have evolved to give cavefish more time for foraging (Duboue et al., 2011; Duboue et al., 2012). Many, although not all, cavefish populations also display reduced aggression (Parzefall, 1983; Burchards et al., 1985; Parzefall, 1985; Espinasa et al., 2005; Elipot et al., 2013). Reduction in aggression has been linked to an increase in the amount of brain serotonin in cavefish relative to surface fish, and may be controlled by Shh (Elipot et al., 2013). Reduction of aggression in cavefish may represent a behavioral shift, where cavefish forage rather than fight (Elipot et al., 2013).

Other behaviors in cavefish may have been lost as a result of a lack of predators in the cave, such as a depressed response to an alarm substance. Surface fish can sense an alarm substance released from broken skin of conspecific fish, and when alarm substance is placed into their water, surface fish avoid this area and display fear-associated behaviors. Some cavefish, however, display a reduced fear response in the presence of alarm substance (Fricke, 1987; Parzefall and Fricke, 1991).

*Astyanax mexicanus* has been used successfully to identify many interesting aspects of the evolution of both behavior and morphology. Morphological traits have been particularly well studied, and in a few cases, genetic loci and mutations that are likely responsible for the evolution of these traits have been identified. With the exception of vibration attraction behavior, the loci underlying the evolution of behavioral traits in *Astyanax mexicanus* are much less well understood.

## Shoaling and schooling behaviors

Social interactions occur in many species. These interactions range from mating and parental care to complex group aggregations such as flocks of birds and schooling fish (de Bono, 2003). The molecular basis of variation in social behavior in natural populations has been studied in a number of animals, including social organization in fire ants (Krieger and Ross, 2002), reviewed in (Robinson et al., 2008), pair bonding in voles (e.g. Winslow et al., 1993), reviewed in Donaldson and Young, 2008), and the onset of foraging behavior in bees (reviewed in Robinson et al., 2008). Although genes involved in social behaviors have been identified, little is known regarding the evolutionary genetics of these behaviors.

Schooling and shoaling are aggregation behaviors in fish. Schooling occurs when fish swim in parallel, exhibiting synchronized behaviors, e.g., changing direction together. Shoaling, on the other hand, occurs when fish aggregate, with or without exhibiting synchronous movement (Pitcher, 1983).

Many factors influence fish aggregation, including shoal size (e.g. Krause et al., 1997), species (e.g. Saverino and Gerlai, 2008), body size (e.g. Ward and Krause, 2001), kinship (e.g. Evans and Kelley, 2008) and familiarity (e.g. Barber and Ruxton, 2000). To aggregate, fish sense one another using multiple sensory systems. For example, without vision or a lateral line, another fish, saithe can school, although less efficiently than with all of their senses intact. Without both of these cues, however, these fish can no longer school (Pitcher et al., 1976; Partridge and Pitcher, 1980). Olfactory cues from conspecific fish also attract other fish (e.g. Hemmings, 1966).

Adaptive reasons for schooling and shoaling include predator avoidance, increased ability to sense the environment, and food acquisition. Predators appear to be distracted by a large number of similar-looking fish, and a predator's chance of successfully attacking prey is decreased when fish are in a school (for example Landeau and Terborgh, 1986). Modeling of movements of groups of prey fish indicates that predators are less likely to attack groups that display both attraction and orientation to conspecifics (Ioannou et al., 2012). Additionally, models of predator-prey interactions have shown that predator confusion, defined as when predators become less successful at attacking prey in a large, moving group, can lead to an increase in swarming behavior in prey over evolutionary time (Olson et al., 2013).

Shoaling increases group vigilance, which allows individual fish to spend more time feeding instead of being on the lookout for predators (Magurran et al., 1985). Groups also make faster, more accurate decisions

relative to those made by individuals during encounters with a predator (Ward et al., 2011). In addition to detecting predators, being in a school can improve a fish's ability to respond to cues in its environment (e.g. Berdahl et al., 2013). Aggregation can also increase food finding and feeding efficiency (e.g. Pitcher et al., 1982; Baird et al., 1991; Ranta and Kaitala, 1991).

Aggregation is not always beneficial to individual fish. Shoaling may result in increased competition with shoal mates (e.g. Ranta et al., 1993). During food deprivation, fish decrease their tendency to shoal (e.g. Krause, 1993; Plath and Schlupp, 2008) and fish spend less time handling their food when the size of the shoal they are in increases (Street et al., 1984). Additionally, some fish living in areas with low fish and bird predation do not school (Plath and Schlupp, 2008). Thus, both decreased food resources and decreased predation can lead to decreased tendency to shoal.

The genetic and neurological basis of shoaling behavior has begun to be examined. QTL mapping of reduced shoaling behavior in laboratory strains of zebrafish relative to wild fish has revealed that reduction of shoaling in domesticated zebrafish occurs through multiple genetic changes (Wright et al., 2006). Work on the neurological basis of shoaling behavior has implicated dopamine as important in this behavior (Scerbina et al., 2012; Mahabir et al., 2013; Saif et al., 2013).

*Astyanax mexicanus* is an excellent system to study the evolution of shoaling and schooling because there are distinct differences in the tendency


**Figure 1.2. Surface and cavefish in their natural habitats.**

A. Surface fish at Naciemiento del Rio Choy in San Luis Potosi state, Mexico. These fish live in high density and spend much of their time in shoals. B. Cavefish, shown here in the Piedras cave in San Luis Potosi state, Mexico, live in a lower density and are not seen in shoals or schools. Piedras photograph courtesy of Dr. William Jeffery.

to aggregate between cave and surface forms (Figure 1.2). Surface populations of *Astyanax* tend to shoal and school (Parzefall, 1983) while multiple cave populations do not school or shoal in the wild or the lab (Parzefall, 1983; Parzefall and Fricke, 1991; Gregson and De Perera, 2007), reviewed in Parzefall, 1985). Loss of shoaling behavior in cavefish does not appear to be explained solely by differences in vision, since when Piedras cave and surface fish are crossed, the portion of the F2 population that can see has variable shoaling tendency (Parzefall and Fricke, 1991). However, vision appears important for at least shoaling behavior. Surface fish do not shoal in the dark (Gregson and De Perera, 2007).

Reduction of shoaling has occurred in other species of cavefish (Timmermann et al., 2004; Plath and Schlupp, 2008) indicating a convergent loss of this behavior in cave environments across multiple species of fish. This derived reduction in shoaling behavior in cavefish may be due to either decreased food sources, a lack of predators, or both of these factors. Thus, loss of shoaling in cavefish could be due to selection against shoaling due to the adverse effects of food competition, or a loss of selection for shoaling in the absence of predators.

## Feeding behavior

Adaptation to feeding in specific environmental conditions, such as a cave, where food can be scarce and may be difficult to locate, is particularly important to cave organisms. In response to these challenges, cavefish have

evolved characteristics that increase their ability to find food in the dark. Seasonal floods often wash surface fish into the caves, where they become trapped during the dry season. Surface fish collected in the caves appear to be starving, and they die shortly after being trapped (Mitchell et al., 1977). These observations suggest that cavefish have evolved specific feeding adaptations that are greatly improved over the primitive surface fish traits in cave conditions.

Cavefish have an improved ability to find food in the dark. Pachón cavefish find food four times faster than surface fish in dark conditions and react faster when food enters the water (Hüppop, 1987). Some of this improvement may be due to differences in the non-visual sensory systems discussed previously, such as an enhanced lateral line (Teyke, 1990; Jeffery et al., 2000), an increased number of taste buds (Schemmel, 1974; Varatharasan et al., 2009), or the increased ability to sense amino acids in the water (Protas et al., 2008). Behavioral traits, such as vibration attraction behavior (Yoshizawa et al., 2010), also likely play a role in this enhanced ability to find food in the dark.

Cave and surface fish have an additional difference in feeding behavior that may play a role in food finding (Schemmel, 1980). In the dark, surface fish feed at an angle of ~80° relative to the bottom of the tank, whereas cavefish feed at an angle of ~56° (Figure 1.3). Crosses between Pachón cavefish and surface fish indicate a genetic basis for feeding angle that is

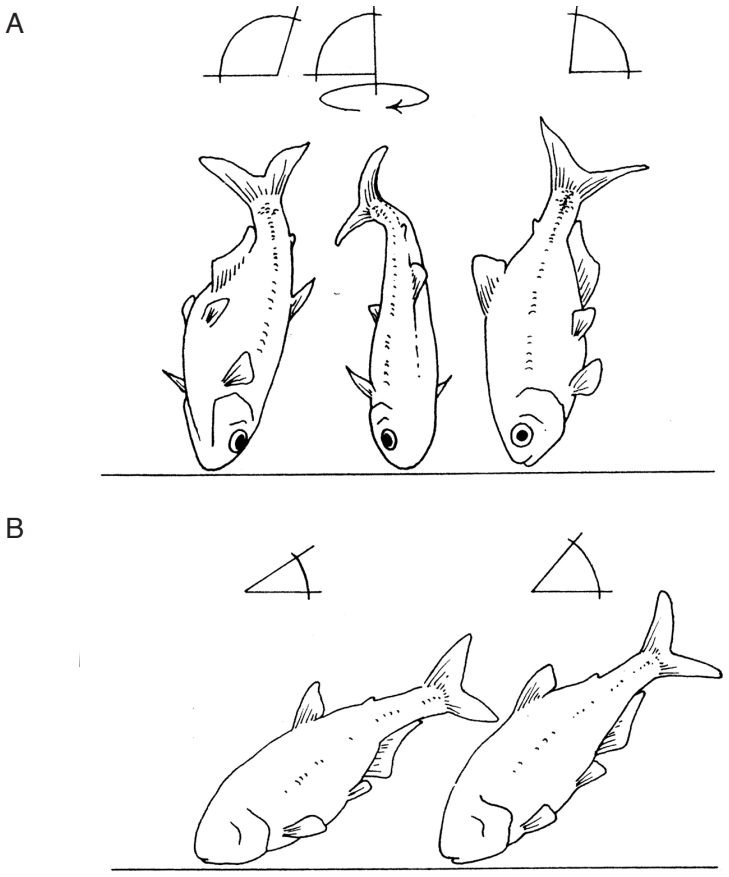


**Figure 1.3. Feeding posture in cave and surface fish.**

A. A schematic representation of feeding posture in surface fish. B. Feeding posture in Pachón cavefish. Figure from Schemmel (1980).

likely controlled by one gene contributing to a substantial portion of the phenotype with minor effects caused by multiple additional loci (Schemmel, 1980). Furthermore, feeding angle is consistent across multiple populations of cavefish, including those that independently evolved (Mitchell et al., 1977; Schemmel, 1980; Dowling et al., 2002; Strecker et al., 2004; Bradic et al., 2012). Crosses between Pachón and Sabinos cave populations resulted in genetic noncomplementation, indicating that feeding posture is controlled by the same genes or pathways in these populations. Additionally, feeding angle does not appear to be due to the overall increased number of taste buds in the Pachón cave population (Schemmel, 1980).

Although enhanced feeding efficiency may be caused by many factors, feeding posture likely plays a role in feeding efficiency in cavefish. Multiple, independently evolved cavefish populations have converged upon this trait, suggesting that it is adaptive, and therefore likely to effect foraging.

## Overview of the dissertation

*Astyanax mexicanus* is an excellent model system for understanding the genetic basis of the evolution of cave traits. The extreme environment of the cave, the existence of surface and multiple cave populations, the ease of husbandry in the laboratory, the existing genetic tools, and the extensive literature on *Astyanax* provide a strong foundation on which to base genetic behavioral studies.

In this dissertation, I study the genetic basis of the evolution of two behaviors – loss of schooling and feeding posture – in *Astyanax mexicanus*. First, I examine aggregation behavior in surface fish and multiple populations of cavefish using two behavioral assays, one to detect schooling behavior, and the other to detect shoaling behavior. I examine the contributions of two sensory systems that have evolved in cavefish, the visual system and the lateral line, to schooling and shoaling in surface fish, and to the evolutionary loss of schooling behavior in cavefish. By crossing surface fish and Tinaja cavefish and performing QTL analysis, I examine the genetic architecture underlying the evolutionary loss of schooling behavior in this cave population.

In addition, I examine the convergent evolution of feeding posture in two independently evolved cave populations, from the Tinaja and Pachón caves. Through a series of crosses and mapping, I examine whether feeding posture in these cave populations has evolved through the same or distinct genetic changes. Finally, I explore the contributions of the evolution of some craniofacial traits and sensory systems to the evolution of feeding posture.

In the appendices, I include additional work. This includes QTL for neuromast number and size and morphometric analysis of cave and surface forms of *Astyanax mexicanus.* Additionally, I present work on identification of mouse perichondrium-specific markers, and a study on identifying multiple cell types in micromass cultures.

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# Chapter 2

# Genetic analysis of the loss of schooling behavior in cavefish reveals both sight-dependent and independent mechanisms

# Genetic analysis of the loss of schooling behavior in cavefish reveals both sight-dependent and independent mechanisms

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This chapter contains the manuscript titled “Genetic analysis of the loss of schooling behavior in cavefish reveals both sight-dependent and independent mechanisms”, accepted at Current Biology. It has been modified to better fit the style of this dissertation. All of the experiments, with the exception of the dark preference experiments (pg 63), which were performed by Nicolas Rohner, were performed and analyzed by Johanna Kowalko. Text and figures were produced jointly by Johanna Kowalko and Clifford Tabin. Santiago Rompani developed the LabView programs used for data analysis. Tess Linden aided with data analysis. Brant Peterson, Emily Kay, Jesse Weber and Hopi Hoekstra provided reagents for and helped with analysis of the RAD-seq data and QTL mapping. Lens ablation experiments were performed in the laboratory of William Jeffery, with the assistance of Masato Yoshizawa. Richard Borowsky and William Jeffery provided *Astyanax mexicanus* for the experiments.

## Abstract

Background: Little is known about the genetic basis of the evolution of social behaviors, such as schooling in fish. *Astyanax mexicanus* is an ideal system in which to study this problem. This species is fragmented into several isolated yet interfertile populations. The surface populations of *Astyanax mexicanus*, living in rivers like their common ancestors, school, while derived cave populations of the same species have lost schooling behavior.

Results: We quantify schooling behavior in individual *Astyanax mexicanus* from one surface and three cave populations, as well as in crosses of surface fish and cavefish. We demonstrate a genetic basis of schooling behavior and report some of the first quantitative trait loci (QTL) for schooling behavior. Using a scototaxis vision assay, we find that the evolutionary modulation of schooling has both vision-dependent and independent components. We also quantify differences in the lateral line between cavefish and surface fish and conclude that these differences have a small effect on the evolutionary loss of schooling behavior in cave populations. We provide evidence that a monoamine may have played a role in the evolution of schooling behavior.

Conclusions: Vision is essential for schooling tendency in *Astyanax mexicanus*, while the lateral line has a small effect on this behavior. Schooling behavior has a genetic basis and is controlled by several QTL. As there are vision-dependent and independent bases of schooling behavior, and separate QTL underlying these processes, we conclude that schooling behavior in *Astyanax mexicanus* has evolved both through changes in

sensory systems and through changes in genetic loci that likely act downstream of sensory inputs.

## Introduction

In adapting to new environments, animals modify both morphological and behavioral traits. While studies have begun to identify the genetic basis of morphological adaptations (for example (Shapiro et al., 2004; Colosimo et al., 2005; Protas et al., 2006; Steiner et al., 2007; Protas et al., 2008)), far less is known about how behaviors evolve. Thus, fundamental questions remain unanswered about the genetic architecture of behavioral evolution. For example, does behavioral change depend on a small number of large effect loci or many loci of small effect? Little is known about the genetics underlying convergence of behaviors, and whether the same or different genes and pathways are utilized in the independent evolution of similar behavioral traits. Moreover, behavioral and morphological traits co-evolve in the face of new selective pressures and often depend on one another. Thus, one would like to understand how behaviors co-evolve with morphological traits, such as sensory systems.

Schooling behavior in fish is a trait that is dependent on environmental context, and most species of fish exhibit this behavior during some phase of their life cycle (Shaw, 1978). Schooling benefits fish in a variety of ways, including predator avoidance and foraging (Partridge, 1982; Pitcher et al., 1982; Magurran et al., 1985; Landeau and Terborgh, 1986; Baird et al., 1991;

Ioannou et al., 2012). However, in some situations schooling behavior appears to be less advantageous. For example, when food is scarce, fish tend to school less (Krause, 1993; Plath and Schlupp, 2008). When fish do school, they rely on the ability to sense one another. Both the visual system and the ability to sense water pressure and current through the lateral line have been implicated in schooling behavior (Hemmings, 1966; Pitcher et al., 1976; Partridge and Pitcher, 1980; Partridge, 1982).

While much is known about how and why fish school and shoal, little is known about the evolution of these behaviors. One exception to this is the reduction of schooling in domesticated zebrafish (Wright et al., 2006). The Mexican tetra, *Astyanax mexicanus*, provides an excellent opportunity to examine this question. *Astyanax mexicanus* exists in two forms, a sighted surface-dwelling form, and a blind cave-dwelling form. Not surprisingly, given the distinct environments they inhabit, cavefish differ from surface fish in a variety of morphological and behavioral traits. Morphological adaptations to life in the caves include an increased number and distribution of taste buds and cranial superficial neuromasts, regressed eyes and decreased or absent melanin pigmentation (Schemmel, 1974; Wilkens, 1988; Teyke, 1990). Cavefish also have a variety of modified behaviors, including reduced aggression and a decrease in time spent sleeping, a depressed response to alarm substance, an enhanced attraction to vibrations in their environment, modified feeding behaviors, and the absence of schooling (Schemmel, 1980; Burchards et al., 1985; Fricke, 1987; Parzefall and Fricke, 1991; Yoshizawa

et al., 2010; Duboue et al., 2011). While many of these behaviors have been studied to some extent, little is known about their genetic architecture.

Cave and surface forms of *Astyanax mexicanus* are interfertile, allowing for the genetic analysis of cave traits (Wilkens, 1988). In particular, quantitative trait locus (QTL) mapping has been used successfully to identify loci underlying the evolution of several morphological traits in these fish (Protas et al., 2006; Protas et al., 2007; Protas et al., 2008; Gross et al., 2009; Yoshizawa et al., 2012; O'Quin et al., 2013). Another advantage of studying evolution in *Astyanax mexicanus* is the existence of a number of independently evolved cave populations (Mitchell et al., 1977; Dowling et al., 2002; Strecker et al., 2004; Bradic et al., 2012; Gross, 2012). Fish from these distinct cave populations display similar morphological characteristics and behaviors, making *Astyanax mexicanus* an ideal system in which to study parallel and convergent evolution.

While the surface form of *Astyanax mexicanus* actively aggregate into schools and shoals, the cave forms have reduced this behavior (Parzefall, 1983; Parzefall, 1985; Parzefall and Fricke, 1991). The apparent absence of macroscopic predators in the caves relieves at least one selective pressure favoring schooling, suggesting that the loss of schooling behavior could be the result of relaxed selection. Alternatively, the scarcity of food resources in most caves potentially renders clustering of the fish disadvantageous, which would suggest that the loss of this behavior could be adaptive in the caves. The absence of schooling could also be a secondary consequence of the loss

of vision and/or changes in the lateral line system in cavefish, or a pleiotropic consequence of other adaptive neurological or morphological changes.

Here, utilizing two different behavioral assays, we establish that schooling behavior has been lost in three independently evolved cave populations. We place this behavior in the context of cavefish-specific alterations in sensory systems, including the lateral line, which does not appear to have a large effect on schooling evolution, and vision, which does affect this process. We assess vision by assaying scototaxis, or preference for the dark, a trait that is lost in cavefish. Using this dark preference assay, along with examination of eye morphology and experimental eye ablation, we show that vision is necessary for schooling behavior in *Astyanax mexicanus*, and that loss of the visual system in cavefish contributed to loss of schooling behavior. However, we provide genetic evidence for both vision-dependent and independent components in the evolutionary loss of schooling behavior. Finally, we provide evidence that dopamine may have modulated the loss of schooling behavior.

## Results

### Loss of schooling behavior in cavefish

Schooling and shoaling behaviors in fish occur when individual fish, perceiving and responding to their local environment, interact in the context of larger groups. By following a set of relatively simple rules on the local scale, their behaviors result in complex group patterns of collective motion (Couzin

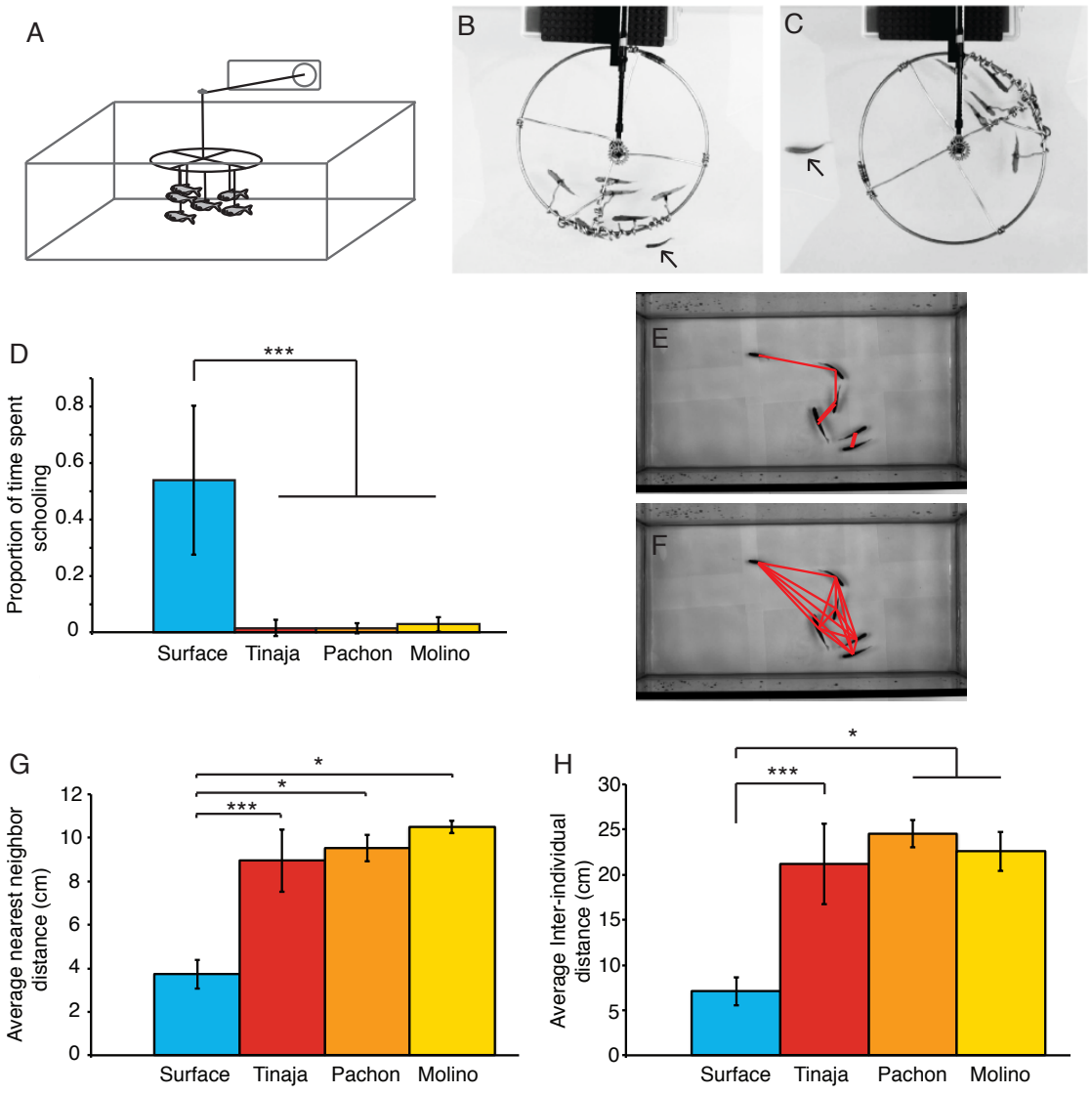
et al., 2002; Couzin and Krause, 2003; Sumpter, 2006). In order to quantify differences in this behavior, we use a relatively simple definition of schooling, the tendency of fish to synchronize their behavior, and swim in an oriented manner relative to one another (Pitcher, 1983). To quantify schooling behavior we first measured the tendency of fish to follow a model school of plastic fish (Wark et al., 2011) (Figure 2.1A). Surface fish follow the model school (Figure 2.1B and D). In contrast, three independently evolved cave populations (Mitchell et al., 1977; Dowling et al., 2002; Strecker et al., 2004; Bradic et al., 2012; Gross, 2012) from the Tinaja, Pachón, and Molino caves were significantly different from surface fish, and did not display schooling behavior (Kruskal Wallis: $H_4$=63.6, $p<0.001$; Mann-Whitney compared to surface: Tinaja: U=3, z=-6, $p<0.001$; Pachón: U=1, z=-4.6, $p<0.001$; Molino: U=4, z=-4.6, $p<0.001$; Surface: n=34, Tinaja: n=19, Pachón: n=9, Molino: n=10, F1s: n=12; Figure 2.1C and D).

We next utilized a shoaling assay to complement the model school assay. Shoaling behavior is defined as the tendency of fish to aggregate with other fish of the same species (Pitcher, 1983). This definition of shoaling includes schooling behavior. We used two measures of shoaling for each group of fish, the average nearest neighbor distance (NND) and the average distance between each fish and every other fish in the tank, the inter-individual distance (IID), (Figures 2.1E and F). Surface fish swam significantly closer together than fish from any of the cave populations by

**Figure 2.1. Cavefish have lost the tendency to school and shoal.**

A. Diagram of the model school behavioral assay. B. Image from a video of a surface fish. C. Image from a video of a Tinaja cavefish. Arrows indicate the position of the live fish. D. Average proportion of the time spent following the school in surface fish (n=34), and cavefish populations – Tinaja (n=19), Pachón (n=10) and Molino (n=10). Asterisks indicate p-values in a Mann-Whitney test. E. Method for quantifying average nearest neighbor distance. F. Method for quantifying average inter-individual distance. G. Shoaling measured as the average nearest neighbor distance (in centimeters) for each fish in a group. Groups of six fish each were measured for surface (9 groups), Tinaja (9 groups), Pachón (3 groups), and Molino (3 groups) fish. Asterisks indicate p-values in a Mann-Whitney test. H. Shoaling measured as the average inter-individual distance (in centimeters) for the groups in G. Asterisks indicate p-values in a Mann-Whitney test. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Figure 2.1, cont.



NND (Kruskal-Wallis: $H_3$=18.8, p<0.001; Mann-Whitney test compared to surface: Tinaja: U<0.001, z=-3.6, p<0.001; Pachón: U<0.001, z=-2.5, p<0.05; Molino: U<0.001, z=-2.5, p<0.05; Surface: n=9 groups, Tinaja: n=9 groups, Pachón: n=3 groups, Molino: n=3 groups; Figure 2.1G) and by IID (Kruskal Wallis: $H_3$=17.4, p<0.001; Mann-Whitney compared to surface: Tinaja: U<0.001, z=-3.6, p<0.001; Pachón: U<0.001, z=-2.5, p<0.05; Molino: U<0.001, z=-2.5, p<0.05; Figure 2.1H). Average standard length of the fish in each group was not significantly correlated with NND within surface fish (Pearson's correlation: R =0.43, p=0.24, n=9 groups, Figure 2.2A) or within Tinaja cavefish (Spearman's correlation: rho=-0.42, p=0.27, n=9 groups, Figure 2.2B).

Taken together, these results indicate that multiple, independently evolved natural populations of cavefish have decreased the tendency to aggregate compared to the surface population. Cavefish have lost the tendency to swim oriented to one another, or school, as well as decreased the tendency to congregate in a group, or shoal.

**Genetics of schooling behavior**

Surface fish raised in isolation follow the model school, responding similarly in the assay to group-raised fish ($t_{36}$=-0.5, p=0.61; group-raised n=34, isolation-raised n=4; Figure 2.3A). Thus, schooling behavior in *Astyanax*, as measured in this assay, is not learned and likely has a genetic basis. To study the inheritance of this behavior, we crossed surface fish and Tinaja cavefish to generate F1 hybrid fish. F1 hybrid fish follow the model

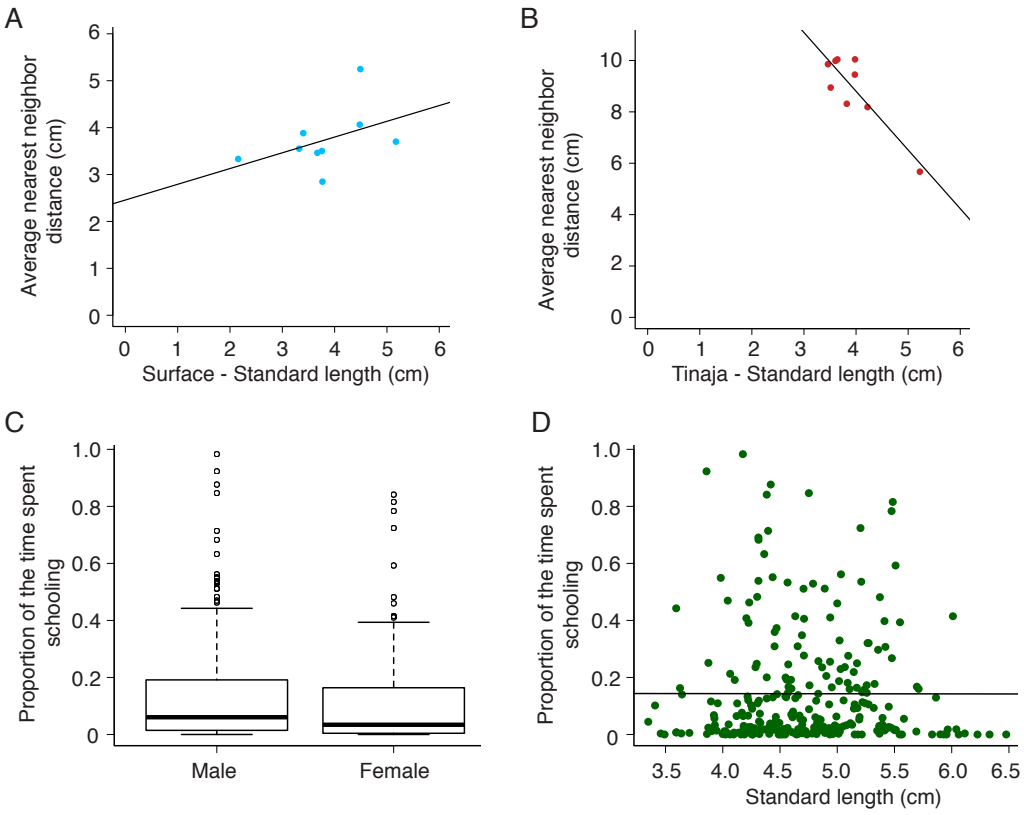



**Figure 2.2. Effects of standard length and sex on schooling and shoaling behaviors.**

A. Average standard length compared to average nearest neighbor distance for shoals of surface fish (n=9). B. Average standard length compared to average nearest neighbor distance for shoals of cavefish (n=9). C. Proportion of time spent schooling of male and female F2 fish (n=252). D. Plot of the distribution of the average proportion of time spent schooling as a function of body length, in centimeters, in 271 F2 fish.

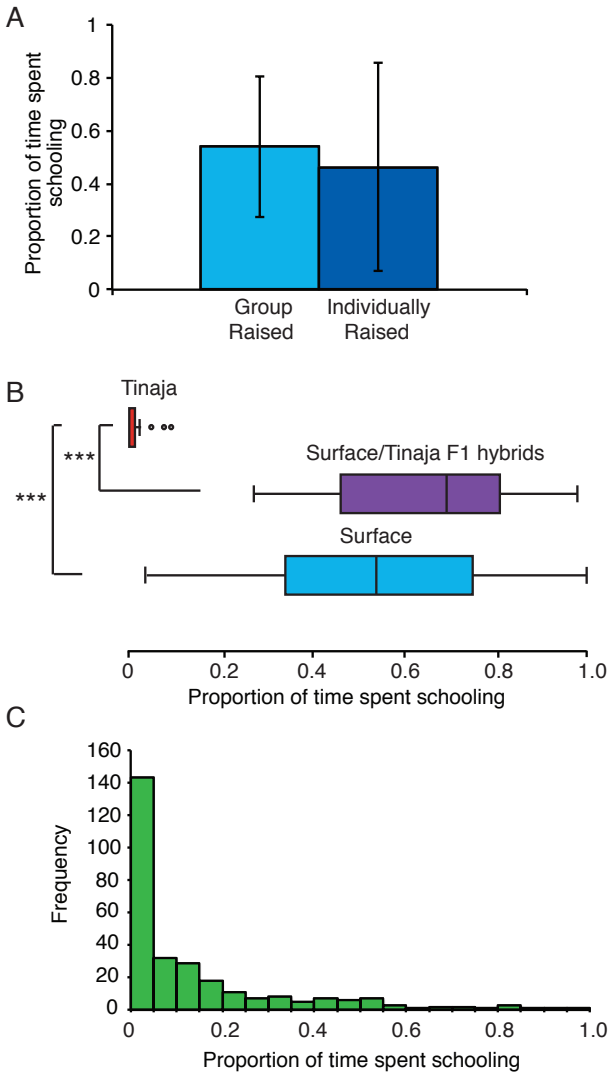



**Figure 2.3. Genetics of schooling behavior.**

A. Proportion of time spent schooling in individually raised (n=4) and group raised (n=34) surface fish. B. Distribution of the proportion of time spent schooling in surface fish (n=34), surface/Tinaja F1 hybrid fish (n=12), Tinaja cavefish (n=19). Asterisks indicate p-values in a Mann-Whitney test. C. The distribution of the average proportion of time spent schooling across five trials of 287 F2 fish from a surface/Tinaja F1 hybrid intercross. All error bars indicate standard deviation. *p<0.05, **p<0.01, ***p<0.001.

school, similar to surface fish, but significantly different from Tinaja fish (Mann-Whitney compared to F1: surface: $U=155.5$, $z=-1.2$, $p=1.0$; Tinaja: $U<0.001$, $z=-4.8$, $p<0.001$; Surface: $n=34$, Tinaja: $n=19$, F1: $n=12$), indicating that tendency to school segregates as a dominant trait (Figure 2.3B).

To probe the genetic architecture of this trait more deeply, F1 hybrid fish were intercrossed to generate F2 fish. F2 fish vary widely in their behavior, ranging from an apparent complete lack of schooling behavior similar to cavefish, to a strong tendency to follow the model school, similar to surface fish (Figure 2.3C). These results strongly indicate a polygenic basis for this behavior. Tendency to school in F2 fish differed based on sex (Mann-Whitney: $U=6669$, $z=-2.1$, $p<0.05$, $n=252$; Figure 2.2C) and was not correlated with size (Spearman's rho=0.05, $p=0.37$, $n=271$; Figure 2.2D).

**An enhanced lateral line in cavefish does not contribute significantly to loss of schooling behavior**

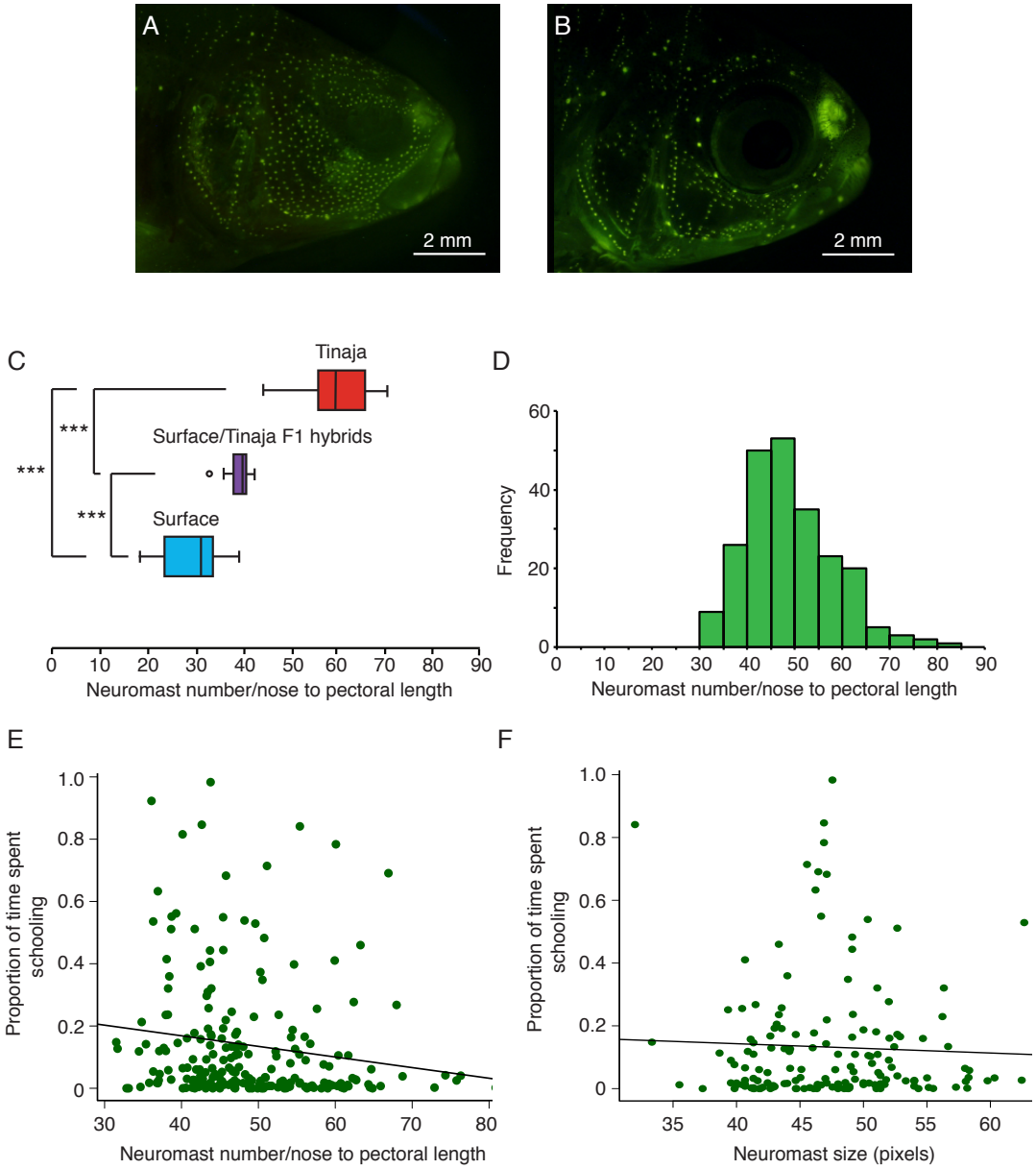
The lateral line and the visual system have been implicated in schooling behavior in other fish species (Hemmings, 1966; Pitcher et al., 1976; Partridge and Pitcher, 1980; Partridge, 1982). Cavefish have an enhanced lateral line and have lost their eyes (Wilkens, 1988; Teyke, 1990), either or both of which could influence the observed differences in schooling behavior between these populations.

Cavefish have enhanced the size and number of superficial cranial neuromasts, the sensory organ of the lateral line system, relative to surface fish (Teyke, 1990; Jeffery et al., 2000) (Figure 2.4A and B). It was, thus, possible that the larger numbers of cranial neuromasts in cavefish provide a

**Figure 2.4. Relationship between schooling behavior and the lateral line system.**

A. Cranial neuromasts in a Tinaja cavefish. B. Cranial neuromasts in a surface fish. Neuromasts are visualized using DASPEI. C. Distribution of cranial neuromast number corrected for size in surface fish (n=21), surface/Tinaja F1 hybrid fish (n=7), and Tinaja fish (n=21). D. Distribution of cranial neuromast number corrected for size in the F2 population (n=227). E. Proportion of the time spent schooling as a function of number of cranial neuromasts corrected for size in the F2 population (n=214). F. Proportion of the time spent schooling as a function of neuromast diameter (in pixels) in the F2 population (n=154). *p<0.05, **p<0.01, ***p<0.001.

Figure 2.4, cont.



sensory input that helps repel them from one another, leading to avoidance of conspecifics and hence a decrease in the tendency to school.

Surface fish indeed have significantly fewer cranial neuromasts than cavefish (one-way ANOVA: $F_{2,46}$=99.2, p<0.001; Surface: n=21, Tinaja: n=21, F1: n=12; Games-Howell Surface compared to Tinaja: p<0.001, Figure 2.4A-C). F1 hybrid fish have an intermediate number of cranial neuromasts, significantly different from both surface (Games-Howell p<0.001) and cavefish (Games-Howell p<0.001). The F2 population (n=227) ranges in number of cranial neuromasts, with a peak between the number observed in F1 and cavefish (Figure 2.4D).

To determine if the number of cranial neuromasts has an effect on schooling behavior, we compared the body size-corrected number of neuromasts to the proportion of time spent schooling for each fish in the F2 population (Figure 2.4E). The number of neuromasts in F2 fish accounted for a statistically significant amount of variation in the proportion of time schooling, but the effect size was quite small (Spearman's rho=-0.22, p<0.001, n=214). In addition to superficial neuromast number, we also measured superficial neuromast diameter in F2 fish, and found no correlation between this measure and the proportion of time schooling (Spearman's rho=0.04, p=0.64, n=154, Figure 2.4F). Thus, the increased number and size of neuromasts that evolved in response to the cave environment did not have a large effect on the evolution of schooling tendency.

**Neuromast ablation does not have a significant effect on either schooling or shoaling behavior**

To determine the extent to which the lateral line system is required for schooling and shoaling activity in *Astyanax*, fish were treated with 0.002% gentamicin to ablate neuromast function (Song et al., 1995; Van Trump et al., 2010; Yoshizawa et al., 2010). Surface fish did not show a significant difference in behavior in the absence of neuromasts, as assessed either by schooling (Mann-Whitney: U=191, z=-0.5, p=0.63; treated: n=21, untreated: n=21; Figure 2.5A) or by shoaling as measured by NND ($t_{10}$=-1.03, p=0.33; treated: n=6 groups, untreated: n=6 groups; Figure 2.5 B), or by IID ($t_{10}$=-1.01, p=0.34; Figure 2.5C). Tinaja cavefish also did not swim significantly farther apart from one another by NND ($t_{10}$=-1.69, p=0.12; treated: n=6, untreated: n=6, Figure 2.5B) and IID ($t_{10}$=-1.15, p=0.28, Figure 2.5C). While not significant, NND and IID in both surface and Tinaja fish were greater in treated fish compared to controls. Therefore, it is unlikely that an enhanced lateral line drives the evolution of loss of schooling or shoaling behavior in cavefish.

**Vision is essential for schooling behavior**

Visual function is important for schooling and shoaling behavior in a variety of fish species, either independent of or in conjunction with lateral line function (for example (Pitcher et al., 1976; Partridge and Pitcher, 1980; Gregson and De Perera, 2007)). It has been previously reported that in *Astyanax mexicanus,* surface fish placed in the dark show a reduction in

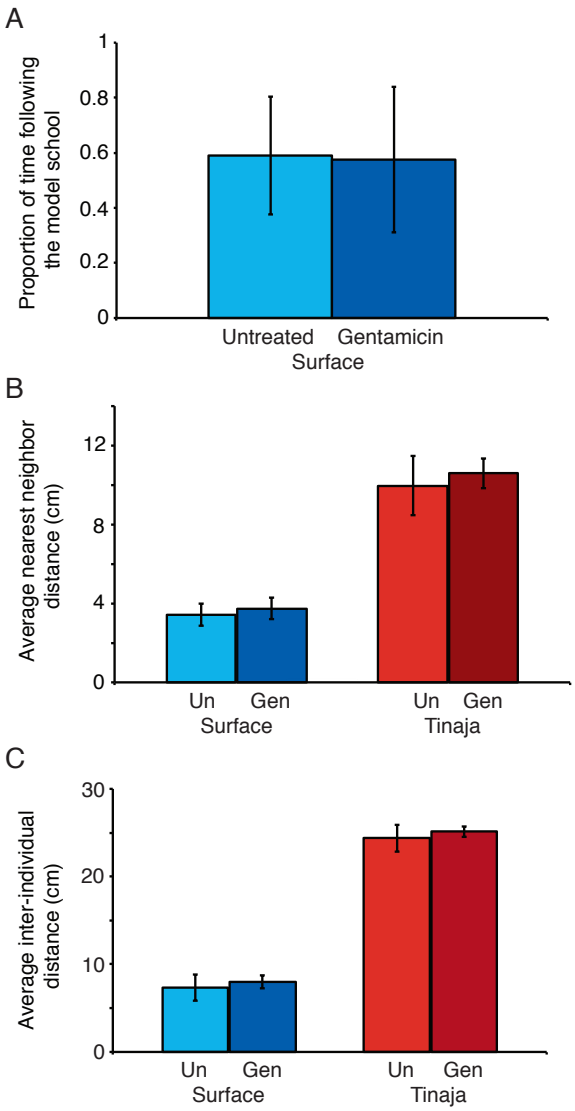


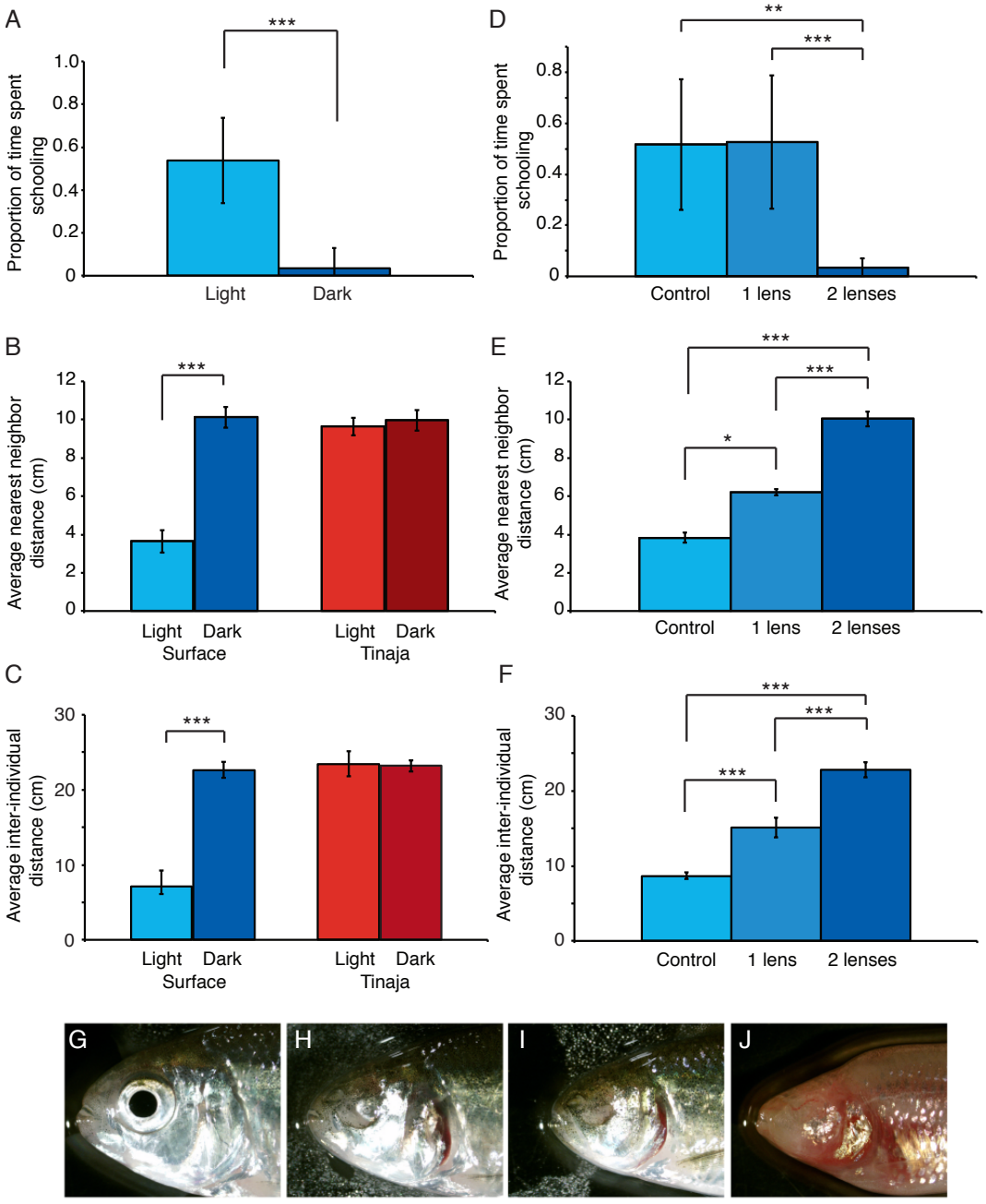

**Figure 2.5. Effects of neuromast ablation on schooling and shoaling behavior.**

A. Proportion of the time spent schooling in surface fish treated with 0.002% gentamicin (n=21) and untreated (n=21). B. Average nearest neighbor distances for surface fish groups treated with 0.002% gentamicin (n=6) and untreated (n=6) and Tinaja fish groups treated (n=6) and untreated (n=6). C. Average inter-individual distances for the groups in B.

**Figure 2.6. Vision is required for schooling and shoaling behavior.**

A. Proportion of time spent schooling of surface fish in the light (n=12) versus the dark (n=10). Asterisks indicate p-value in a Mann-Whitney test. B. Shoaling nearest neighbor distance measured in groups of six of surface fish (5 groups) and cavefish (5 groups) in the light and the dark. Asterisks indicate p-value in a paired t-test. C. Shoaling inter-individual distances measured in groups from B. Asterisks indicate p-value in a paired t-test. D. Surface fish with zero (n=7), one (n=12) or two (n=8) lenses removed assayed with the model school. Asterisks indicate p-value in a Games-Howell test. E. One group each of one- (5 trials), two-lenses (5 trials) removed, or control fish (2 trials) assayed for shoaling by nearest neighbor distances. F. Inter-individual distances for groups from E. G. Eye size in control surface fish. H. Partial eye degradation in surface fish with lenses removed. I. Complete eye degradation in surface fish with lenses removed. J. Eye degradation in cavefish. Error bars indicate standard deviation. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Figure 2.6, cont.



shoaling (Gregson and De Perera, 2007). We verified this result in our shoaling assay. Groups of surface fish in the dark swam significantly farther apart compared to the same groups in the light as measured by both NND (paired t-test: $t_4$=-17.2, p<0.001, n=5 groups; Figure 2.6B) and IID (paired t-test: $t_4$=-15.2, p<0.001; Figure 2.6C). As expected, cavefish were unaffected by the change in lighting conditions both by NND (paired t-test: $t_4$=-1.2, p=0.31, n=5 groups; Figure 2.6B) and by IID (paired t-test: $t_4$=0.45, p=0.67; Figure 2.6C). To determine if schooling behavior in surface *Astyanax* also requires vision, surface fish were assayed with the model school. Schooling behavior in surface fish was completely lost in the dark compared to in the light (Mann-Whitney: U=1, z=-4, p<0.001; light: n=12, dark: n=10, Figure 2.6A).

Loss of surface fish schooling in the dark could be due to a learned reliance on vision for schooling behavior. If this were the case, fish that lost vision early in development might school in the absence of sight. Cavefish develop eyes, which undergo apoptosis and degenerate (Wilkens, 1988; Jeffery and Martasian, 1998; Jeffery, 2001). Cavefish eye degradation can be phenocopied in surface fish by removing lenses at 36 hours post fertilization (hpf) (Yamamoto and Jeffery, 2000). To test if surface fish that lost visual function during development would school, one, two, or no lenses were removed in surface fish. Fish eyes lacking lenses ranged in their adult morphology from a small, degraded eye to a complete absence of an eye, resembling cavefish (Figure 2.6G-J).

Lens removal had a significant effect on both schooling (one-way ANOVA: $F_{2,24}$=13.9, p<0.001; control: n=8, one lens removed: n=12, two lenses removed: n=7; Figure 2.6D) and shoaling measured by NND (Welch ANOVA: F=253.9, p<0.01; control n=2 trials, one lens removed: n=5 trials, two lenses removed: n=5 trials; Figure 2.6E) and IID (one-way ANOVA: $F_{2,9}$=127.1, p<0.001; Figure 2.6F). Surface fish with both lenses removed schooled significantly differently from control fish and fish with one lens removed, regardless of their amount of eye degeneration (planned-contrast test: $t_{14.1}$=-7.8, p<0.001; Games-Howell test compared to control fish: p<0.01 and one-lens removed fish: p<0.001, Figure 2.6D). Additionally, groups of two-lenses removed surface fish swam significantly farther away from one another compared to control fish (NND Games-Howell test: p<0.001, Figure 2.6 E; IID Games-Howell test: p<0.001, Figure 2.6F). Surface fish retaining one eye were indistinguishable from control fish in the model school assay (Games-Howell test: p=0.996, Figure 2.6D). However, fish with one eye had intermediate nearest neighbor distances in the shoaling assay, significantly different from both control (NND Games-Howell test: p<0.05; IID Games-Howell test: p<0.001) and two-lenses removed fish (NND Games-Howell test: p<0.001, Figure 2.6E; IID Games-Howell test: p<0.001, Figure 2.6F). These results demonstrate that visual function is necessary for schooling and shoaling in surface forms of *Astyanax mexicanus*.

**Vision-dependent and independent loss of schooling tendency in F2 fish**

Since visual function is required for schooling behavior in *Astyanax mexicanus*, the ancestral fish would have lost the ability to school immediately upon entering the pitch-dark cave environment. Thus, cavefish may have evolved their decreased tendency to school in our assays solely as a consequence of their loss of eyes. Alternatively, loss of schooling behavior may have become fixed in these fish through additional changes, independent of the loss of vision. To distinguish between these possibilities, visual function was assayed in the F2 population.

Visual function in the F2 population can be approximated using the external morphology of the eyes. Both eye diameter ($t_{8.7}$=-13.1, p<0.001; Surface: n=20, F1: n=5) and pupil diameter ($t_{4.9}$=-9.0, p<0.001) are significantly reduced in F1 hybrid fish compared to surface fish (Figure 2.7A). Nearly all F2 fish (n=283) have eyes and pupils, although most of them are smaller than surface fish eyes (Figures 2.7B and C). Eye diameter and pupil diameter are highly correlated in the F2 population (Pearson's R = 0.81, p<0.001, n=283, Figure 2.7D). Proportion of time schooling in the F2 population is weakly to moderately positively correlated with both eye size (Spearman's rho=0.27, p<0.001, n=270) and pupil size (Spearman's rho=0.35, p<0.001, n=270, Figure 2.7E and F). However, there are individual fish with relatively large eyes and pupils who still did not demonstrate schooling behavior. This suggests that while schooling requires visual function, there may be an independent genetic basis for loss of schooling.

**Figure 2.7. Relationship of eye and pupil size in F2 fish.**

A. Eye size and pupil size in surface fish (n=20) and F1 hybrid fish (n=5) from a surface/Tinaja cross. Eye and pupil size are corrected for body length. Error bars indicate standard deviation. B. Distribution of corrected eye diameter in the F2 population (n=285). C. Distribution of corrected pupil diameter in F2 fish population (n=285). Both eye and pupil diameters were corrected for the expected size of the eye or pupil of a surface fish of the individual's body length. D. Relationship between eye and pupil size in the F2 fish population (n=283). E. Average proportion of time schooling in the F2 population (n=270) as a function of eye size. F. Average proportion of the time schooling in the F2 population (n=270) as a function of pupil size.

Figure 2.7, cont.

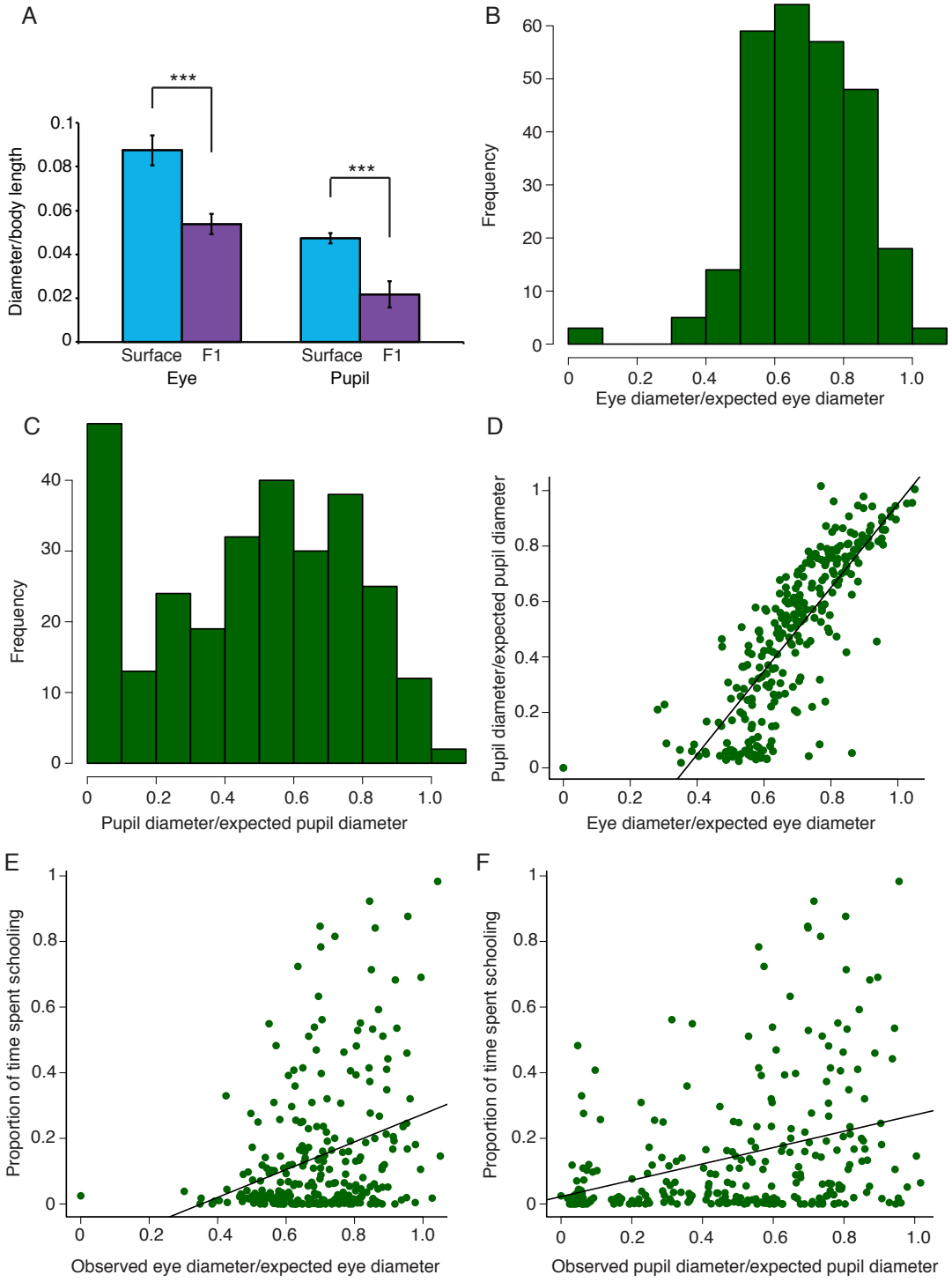
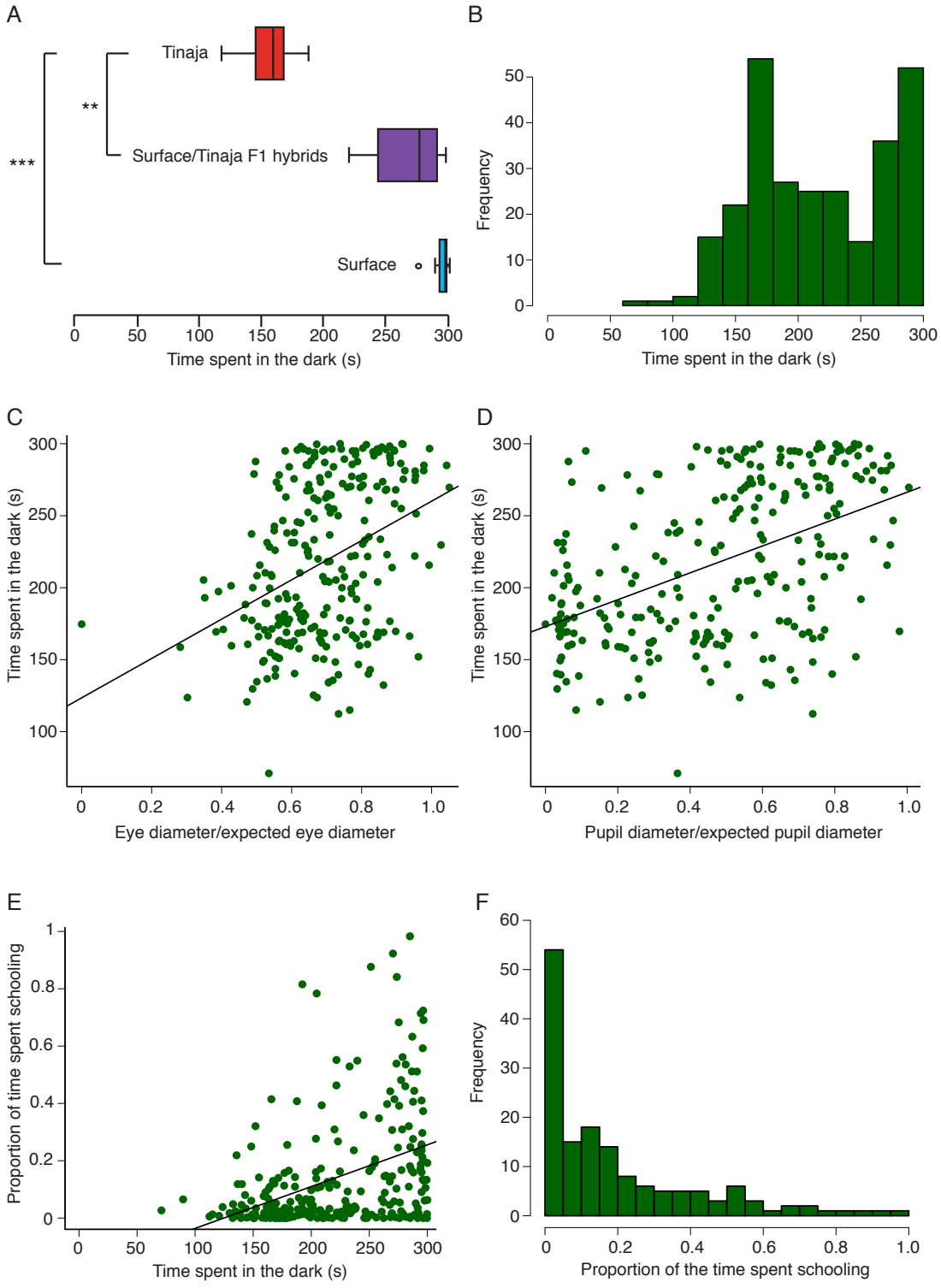


**Figure 2.8. Dark preference and schooling behavior.**

A. Surface (n=9), Tinaja (n=14), and Surface/Tinaja hybrid F1 (n=4) individuals in an assay for dark preference. Dark preference was quantified as the number of seconds spent in the dark out of a total of 300 seconds. Asterisks indicate p-values in a Mann-Whitney test. B. Distribution of average time spent in the dark across 3 trials for F2 population of fish (n=275). C. Relationship between eye diameter and time spent in the dark (n=265). D. Relationship between pupil diameter and time spent in the dark (n=265). E. Average proportion of the time spent schooling as a function of dark preference in the F2 population (n=266). F. The distribution of the tendency to school in seeing F2 fish, defined as spending an average of 200 seconds in the dark (n=151). *p<0.05, **p<0.01, ***p<0.001.

Figure 2.8, cont.



Of course, F2 fish with large eyes and pupils may still lack visual function, and fish with smaller eyes may be able to see. To more directly test visual function, fish were tested for their ability to sense light. Surface fish display strong negative phototaxis, spending nearly all of their time in the dark. Tinaja cavefish behave significantly differently (Kruskal Wallis: $H_2$=175.6, p<0.001; Surface: n=9, Tinaja: n=14, F1: n=4; Mann-Whitney Surface compared to Tinaja: U<0.001, z=-4, p<0.001; Figure 2.8A), showing no preference for either the dark or the light. Surface/Tinaja F1 hybrids display strong negative phototaxis, not significantly different from surface fish (Mann-Whitney: U=5, z=-2, p=0.15) and significantly different from Tinaja fish (U<0.001, z=-3, p<0.01; Figure 2.8A).

Dark preference in the F2 fish population had a bimodal distribution (n=275, Figure 2.8B). Dark preference in the F2 population was moderately positively correlated with both eye diameter (Speaman's rho=0.36, p<0.001, n=265) and pupil diameter (Spearman's rho=0.47, p<0.001, n=265, Figures 2.8C and D). This is consistent with the hypothesis that smaller eyes and pupils are associated with less visual function.

Dark preference was also moderately positively correlated with the tendency to school in the F2 population (Spearman's rho=0.42, p<0.001, n=266, Figure 2.8E). A large proportion of non-schooling fish had no dark preference, or spent around 150 seconds in the dark. This indicates that many of the F2 fish that displayed no tendency to school have little visual function. However, some F2 individuals that showed a strong dark

preference did not show any tendency to school, suggesting that there has been a loss of the tendency to school in cavefish independent of vision.

In order to test for factors that affect schooling behavior independently of vision, a population of F2 fish with visual perception needed to be defined. Light-perceiving F2 fish were defined as those fish with an average time spent in the dark of 200 seconds (2/3 of their time) or more out of a 300 second trial. This cutoff would include F1 and surface fish, but exclude Tinaja cavefish. Because dark preference could reflect multiple aspects of visual response, this cutoff likely excludes some fish that can see. However, this conservative method allowed us to definitively identify a subset of light-perceiving fish, and exclude all fish that can no longer perceive light.

Light-perceiving fish (n=151), defined in this manner, were then analyzed for their propensity to school. Interestingly, within this group of light-perceiving F2 fish, many do not display schooling behavior (Figure 2.8F). However, the proportion of F2 fish with the lowest schooling tendency (less than five percent of the time) is significantly less than in the general F2 population, from 50% of the total F2 population to 35% of the light-perceiving portion of the F2 population. This is likely because many of the F2 fish that lacked the ability to see failed to school for that reason, and were excluded from this subgroup.

To confirm that there is a non-visual component to the loss of grouping behavior in the cavefish, groups of light-perceiving and non-light-perceiving F2 fish were tested in the shoaling assay. Light-perceiving, schooling groups

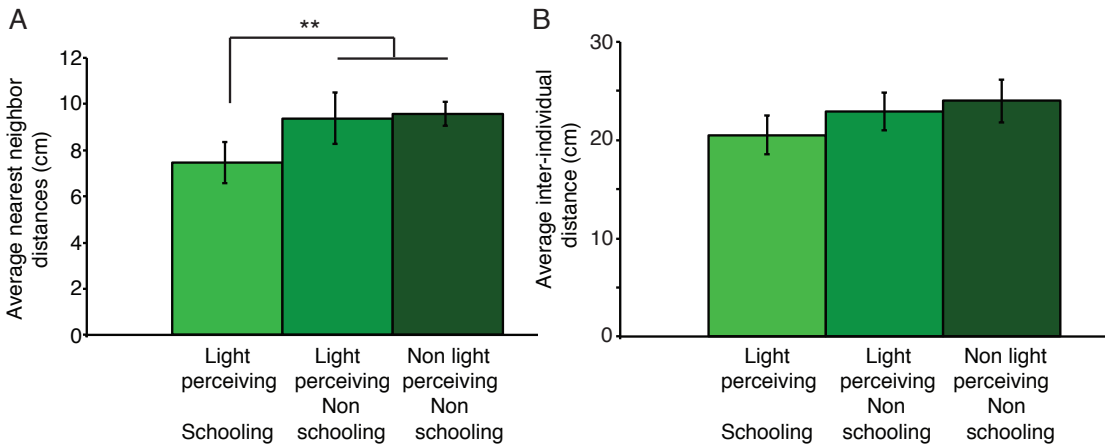



**Figure 2.9. Shoaling in F2 fish.**

A. F2 fish were separated into groups based on visual function and tendency to school. Groups of 6 fish of light perceiving, schooling fish (6 groups), light perceiving, non-schooling fish (4 groups) and non-light perceiving, non-schooling fish (3 groups) were tested for shoaling behavior by nearest neighbor distance. Asterisks indicate p-values for a planned-contrast test. B. F2 fish (from A) assayed for shoaling by inter-individual distance. All error bars indicate standard deviation. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

of fish swam significantly closer to one another compared to light-perceiving, non-schooling fish groups and non-light-perceiving, non-schooling groups by NND (one-way ANOVA: $F_{2,10}$=7.65, p<0.05; planned-contrast test: $t_{10}$=-3.91, p<0.01; Schooling: n=6 groups, Light-perceiving, non-schooling: n=4 groups, Non-light-perceiving, non-schooling: n=3 groups; Figure 2.9A). These groups were not significantly different by IID, although they trended in the same direction as NND (one-way ANOVA: $F_{2,10}$=3.5, p=0.07, Figure 2.9B). Furthermore, a mixed group of F2s, containing 2 fish from each of these categories, produced a NND of 10.17 and an IID of 22.27. This NND is outside the range of the light-perceiving, schooling F2 fish. This confirms that a subset of F2 fish maintain visual function, but do not have a tendency to aggregate.

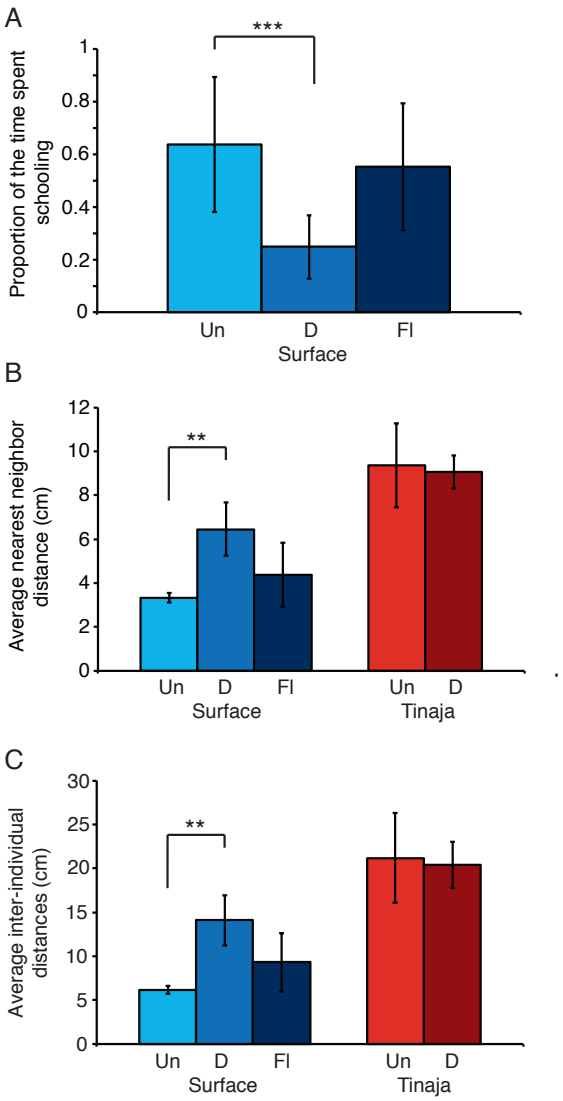
**The roles of monoamine neurotransmitters in schooling behavior**

Recent research has shown that there are differences in levels of monoamine neurotransmitters between cave and surface *Astyanax mexicanus* (Strickler and Soares, 2011; Elipot et al., 2013). In order to determine if these differences could have an effect on schooling behavior, we treated cave and surface fish with two inhibitors, (R)-(-)-Deprenyl hydrochloride and fluoxetine hydrochloride. Both of these drugs result in an increase in serotonin levels in the brain (Elipot et al., 2013). However, (R)-(-)-Deprenyl hydrochloride targets monoamine oxidase (MAO), inhibiting the breakdown of multiple monoamines.

**Figure 2.10. Role of monoamine neurotransmitters in schooling and shoaling behavior.**

A. Proportion of the time spent schooling in untreated (Un, n=21), 10uM Deprenyl treated (D, n=12), and 14 uM Fluoxetine treated (Fl, n=21) surface fish. Asterisks indicate p-values in a Mann-Whitney test. B. Average nearest neighbor distance in groups of untreated (Un, n=6 groups), 10uM Deprenyl treated (D, n=6 groups), and 14 uM Fluoxetine treated (Fl, n=5 groups) surface fish and untreated (Un, n=6 groups) and 10 uM Deprenyl treated (D, n=6 groups) Tinaja cavefish. Asterisks indicate p-values in a Games-Howell test. C. Average inter-individual distances for groups from B. Asterisks indicate p-values in a Games-Howell test. All error bars indicate the standard deviation. *p<0.05, **p<0.01, ***p<0.001.

Figure 2.10, cont.



The treatments result in significant differences in schooling behavior (Kruskal Wallis: $H_2$=18.4, p<0.001; Untreated: n=36, Deprenyl: n=12, Fluoxetine: n=22). (R)-(-)-Deprenyl treatment results in a significant decrease in schooling behavior (Mann-Whitney: U=46, z=-4, p<0.001) while fluoxetine does not significantly affect schooling relative to control fish (Mann-Whitney: U=313, z=-1.3, p=0.38, Figure 2.10A). In addition, (R)-(-)-Deprenyl (Welch ANOVA: $H_2$=18.4, p<0.01; Untreated: n=6 groups, R-Deprenyl: n=6 groups, Fluoxetine: n=5 groups; Games-Howell: p<0.01) but not fluoxetine (Games-Howell: p=0.35) results in significantly greater separation between fish in the shoaling assay using NND (Figure 2.10B). The results for IID are similar (Welch ANOVA: $H_2$=21.6, p<0.01; R-Deprenyl Games-Howell: p<0.01, Fluoxetine Games-Howell: p=0.21; Figure 2.10C). In contrast, the Tinaja cavefish in the shoaling assay were not affected by treatment with (R)-(-)-Deprenyl hydrochloride when measured by NND (Mann-Whitney: U=0.9, z=-1.4, p=0.18; Untreated: n=6 groups, Treated: n=6 groups, Figure 2.10B) or by IID (Mann-Whitney: U=12, z=-0.96, p=0.39, Figure 2.10C). While we have not tested for differences in brain monoamine levels here, this data suggests that an increase in brain monoamine levels, but not specifically brain serotonin levels, decreases the tendency to school.

**QTL mapping of schooling behavior**

Finally, we performed QTL analysis to map the regions of the genome underlying the loss of schooling behavior in the Tinaja cavefish. We generated a novel linkage map of 330 SNPs falling onto 28 linkage groups

**Table 2.1. Summary of QTL statistics.**

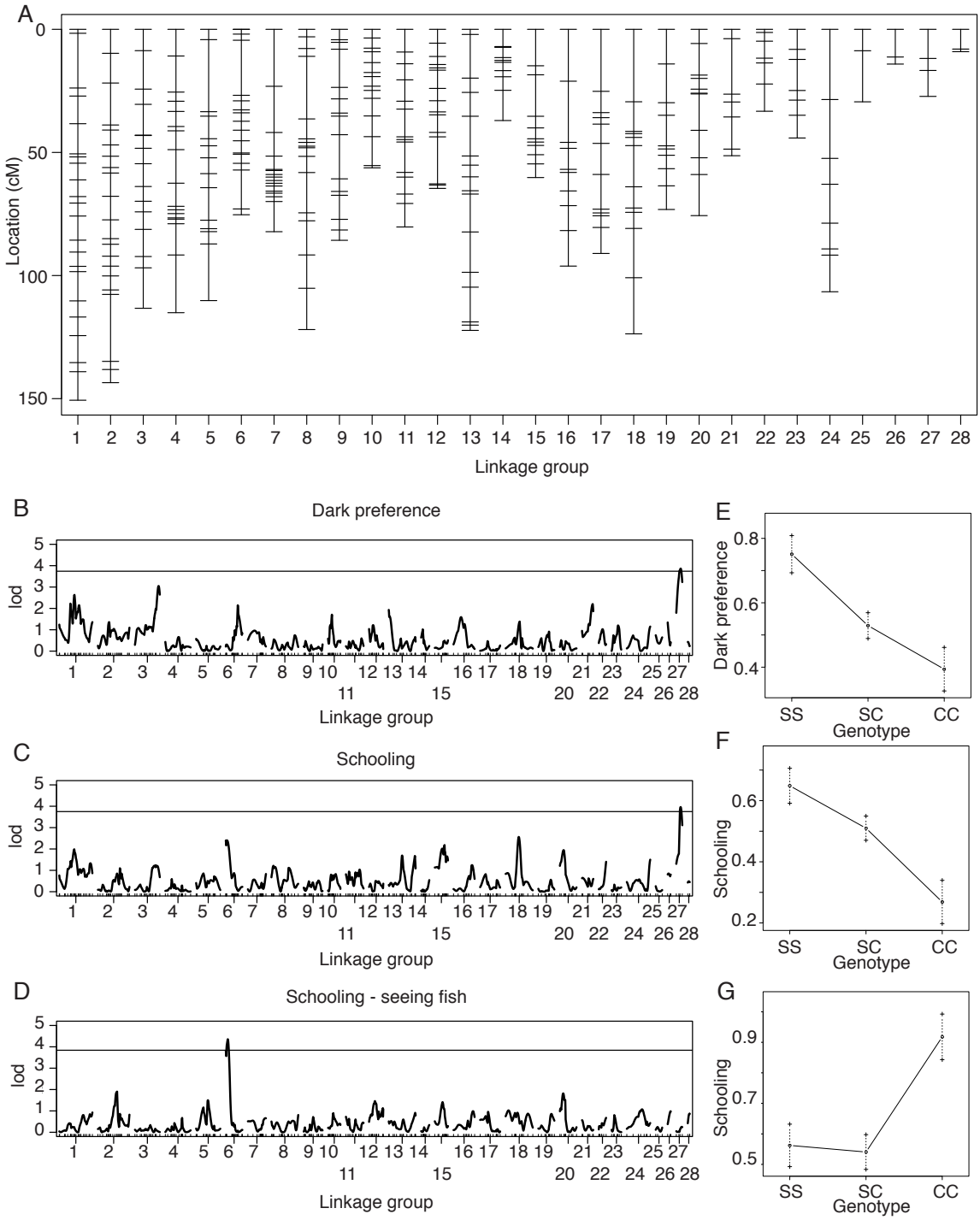
CI = Confidence Interval. PVE = Percent variance explained.

| Trait | LG | cM | LOD | CI | PVE |
|---|---|---|---|---|---|
| Schooling | 27 | 20 | 4.0 | 16-26 cM | 6.4 |
| Dark preference | 27 | 20 | 3.9 | 9-27 cM | 6.4 |
| Schooling – seeing fish | 6 | 9 | 4.4 | 0-14 cM | 12 |
| Eye diameter | 3 | 74.1 | 4.9 | 65-113 cM | 7.9 |
| Pupil diameter | 3 | 74.1 | 4.6 | 67-77 cM | 7.3 |

**Figure 2.11. Visual and non-visual QTL for evolutionary loss of schooling behavior in cavefish.**

A. Linkage map derived from SNPs in a Tinaja/Surface intercross. B. QTL for a binary measure of dark preference (n=267) where fish spending greater than 200 seconds were scored as preferring the dark. The line indicates a significant LOD score for a p-value < 0.05. C. QTL for a binary measure of the tendency to follow the model school (n=276). Fish were scored as schooling if they spent more than 5% of their time following the model on average. The line indicates a genome-wide significance LOD score for a p-value < 0.05. D. QTL for a binary measure of the tendency to follow the model school for the subset of fish that preferred the dark (n=143). E. Plot of the phenotype values by genotype at the QTL for dark preference measured as a binary of time the dark (1=preferring the dark, 0=no dark preference). F. Plot of the phenotype values by genotype at the QTL for schooling, measured as proportion of time following the model schooling and then made into a binary trait (1=schooling, 0=non-schooling). G. Plot of the phenotype values by genotype at the schooling QTL in light-responsive fish, measured as proportion of time following the model schooling and then made into a binary trait (1=schooling, 0=non-schooling). Genotypes are for homozygous surface (SS), heterozygous (SC) or homozygous cave (CC) alleles.

Figure 2.11, cont.



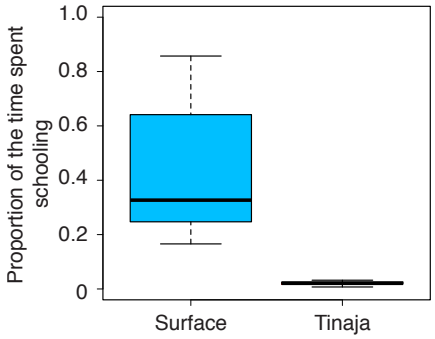



**Figure 2.12. Schooling in surface and Tinaja cavefish tested multiple times each.**

Average proportion of time spent schooling in surface fish (n=11) and Tinaja cave fish (n=9) tested five times each.

using a double digest RADseq technique (Peterson et al., 2012) (Figure 2.11A).

Schooling behavior varied widely across the five trials we tested. To assess the reproducibility for individual fish across multiple trials, we used an intraclass correlation coefficient. The intraclass correlation coefficient for surface fish tested five times each was 0.62, and for F2 fish was 0.51. In addition, there was an effect of schooling trial number in the F2 population ($F_{1,1123}$=101.2, p<0.001) which resulted in an overall trend towards less tendency to school as trial number increased. In order to integrate this data to map the schooling in F2 fish, we used a binary measure of schooling behavior, described in the methods, which would include all surface fish, but exclude all Tinaja cavefish (Figure 2.12).

Using this binary measure of schooling behavior, we mapped a single significant QTL on linkage group 27 that explained 6.4% of the variance (n=276, p<0.05, Figure 2.11C, Table 2.1). Homozygous cave alleles at a marker underlying this QTL result in a decrease in schooling behavior, and a heterozygous genotype result in an intermediate tendency to school (Figure 2.11F). A significant QTL explaining 3.6% of the variance was also observed in this location when schooling was mapped as a continuous trait as the average across five trials.

In addition to schooling behavior, we mapped a binary measure of dark preference, using the cutoff for light-perception as 200 seconds in the dark on average across three trials. Dark preference maps to one significant QTL on

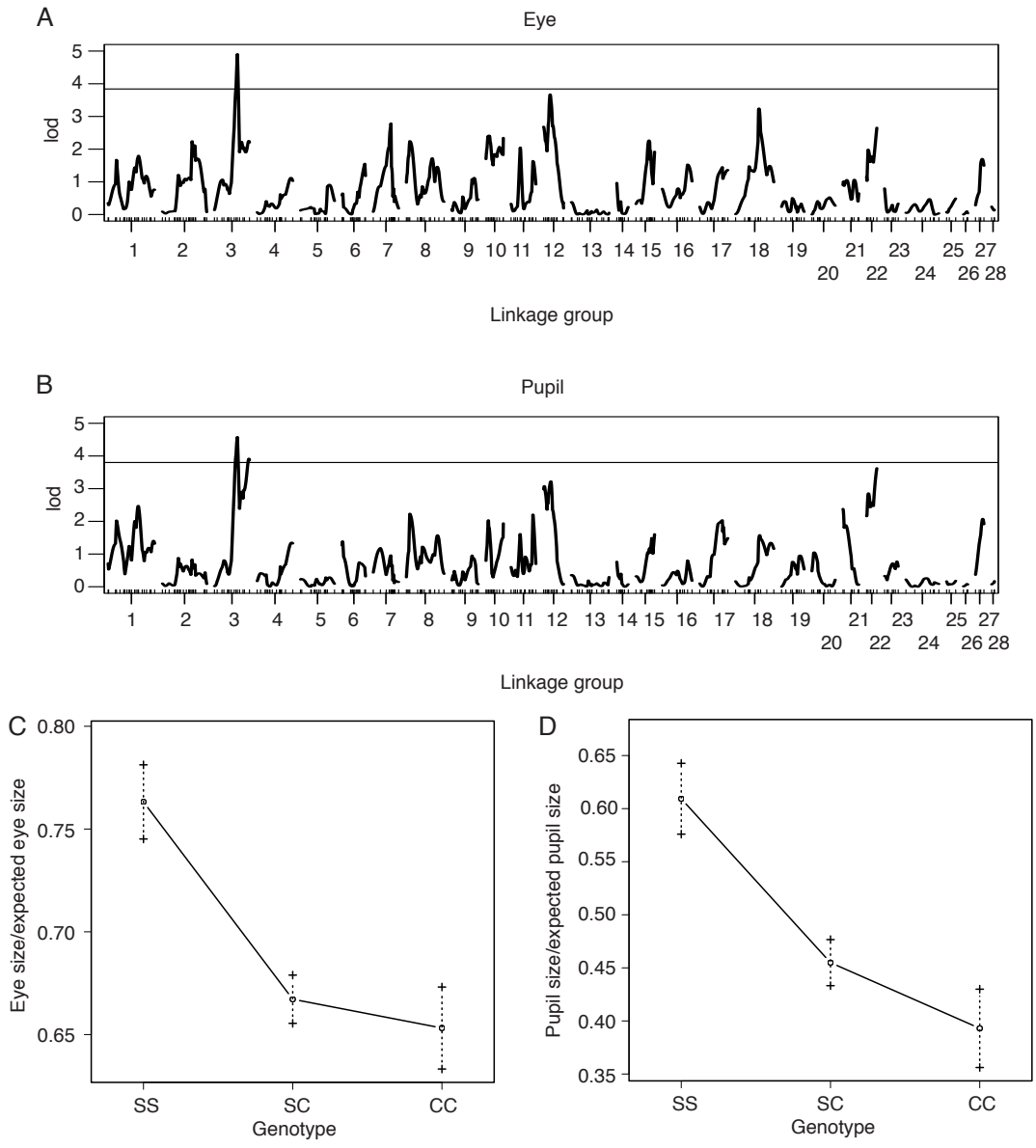
**Figure 2.13. QTL for eye and pupil diameters.**

A. QTL for corrected pupil diameter (n=276). Line is for a LOD score p=0.05.

B. QTL for corrected pupil diameter (n=276). Line is for a LOD score p=0.05.

C. Plot of the phenotype values by genotype at the QTL for corrected eye diameter. D. Plot of the phenotype values by genotype at the QTL for corrected pupil diameter. SS is a homozygous surface genotype, SC is heterozygous, and CC is a homozygous cave genotype.

Figure 2.13, cont.



linkage group 27 that explains 6.4% of the variance (n=267, $p<0.05$, Figure 2.11B, Table 2.1). Homozygous cave alleles at a marker underlying this QTL result in less time spent in the dark, while heterozygous genotypes result in an intermediate percentage of time spent in the dark (Figure 2.11E). This QTL marker mapped to the same location as the schooling QTL.

In order to map the genetic basis of schooling behavior that is independent of visual function, a binary measure of schooling behavior in light-perceiving fish (defined as described above) was mapped. This resulted in one significant QTL on linkage group 6 that explains 12% of the variance (n=143, $p<0.05$, Figure 2.11D, Table 2.1). Somewhat surprisingly, homozygous cave genotypes at a marker underlying this QTL resulted in an increase in schooling behavior, while fish with homozygous surface or heterozygous genotypes schooled a similar amount of time (Figure 2.11G). This QTL does not fall in the same place as the QTL for dark preference, eye size, or pupil size (Figure 2.11B, Figure 2.13 A-D, Table 2.1). Thus this QTL identifies a vision-independent genetic contribution to the evolution of schooling behavior. Although this peak was present as the highest peak when schooling was mapped within these individuals as a continuous trait, it was not significant at a $p=0.05$.

## Discussion

Much is known about the ecology underlying schooling behavior. However, little is known about how this behavior evolves. Here, we use the

loss of schooling in the cavefish *Astyanax mexicanus* to elucidate essential questions about the evolution of schooling. We determine that while both the visual system and the lateral line affect schooling in surface fish to some extent, it is the loss of sight in cavefish that plays the most significant role in the loss of schooling behavior. In contrast, lateral line enhancement in cavefish plays at most a minor role in schooling behavior loss. Our results suggest that loss of schooling evolved by multiple genetic changes, only some of which are vision-dependent.

**The visual system is essential in schooling behavior in surface fish**

Both the lateral line system and the visual system have previously been implicated in schooling behavior in a variety of fish species (Hemmings, 1966; Pitcher et al., 1976; Partridge and Pitcher, 1980). It has been proposed that while the visual system allows fish to swim closer to one another, the lateral line provides a repulsive force (Partridge and Pitcher, 1980). We find that only vision, and not lateral line sensation, played a key role in the evolution of this behavior in the cave population of *Astyanax mexicanus*. Surface *Astyanax* do not school and have reduced shoaling in the dark. Other surface fish behaviors, such as aggression, may involve learned visual cues, which are no longer necessary if fish are raised deprived of vision (Espinasa et al., 2005). However, adult surface fish blinded at 36 hpf also did not school and had a reduction in shoaling. This demonstrates that vision is likely necessary for both schooling and shoaling behavior. However, there remains a possibility that surface fish choose not to school without visual cues.

We also tested the effect of loss of one eye on schooling and shoaling behavior. Interestingly, while fish with one eye could follow the model school, fish with one eye shoaled farther apart from one another. This could result from the importance of two functional eyes in tracking fish swimming in a disorganized manner, while other sensory organs, such as the lateral line, may compensate for the loss of one eye during schooling behavior.

**Loss of vision has a large effect on the evolutionary loss of schooling behavior, while enhancement of the lateral line plays a minor role at most**

Visual function is essential for both schooling and shoaling behavior in surface *Astyanax mexicanus*. Therefore, the ancestral fish would have lost the ability to school immediately upon becoming entrapped in the dark cave environment. Subsequently, the cavefish lost their eyes and also evolved a decreased capacity for schooling, even when returned to a light-exposed environment. By examining the visual system and schooling behavior in the F2 population, we examined the role of visual function in the evolution of schooling behavior. We found that morphological measures of the visual system, eye and pupil size, as well as behavioral measures for vision, were correlated with schooling behavior in the F2 population. Additionally, the QTL for dark preference maps to the same region as a QTL for schooling behavior. This QTL for schooling behavior leads to a decrease in schooling behavior with the homozygous cave genotype, and may explain the proportion of loss of schooling behavior explained by loss of visual function. Alternatively, since

the QTL for dark preference does not fall in the same location as QTL for eye or pupil size, it is also plausible that the behavioral difference in the dark preference assay mapped to this QTL has to do with a loss of dark preference per se, and not to perception of light. However, it could be related to an eye-size independent aspect of visual processing still related to light perception, such as retinal degeneration or lens degeneration. Previous work in Pachón cavefish identified some QTL for retinal degeneration that were not overlapping with eye or lens QTL (O'Quin et al., 2013) and not all lens QTL overlap with eye size QTL (Protas et al., 2007).

While we found a significant correlation between number of neuromasts and schooling in F2 fish, the correlation was weak, and ablation of neuromasts in surface and cavefish was not sufficient to drive fish to swim closer together, or to increase schooling behavior. Therefore, it is unlikely that neuromasts play a large role in the evolution of schooling behavior.

**Potential effects of increase of brain dopamine levels on decrease of schooling**

Both dopamine and serotonin have been implicated in Pachón cavefish evolution (Strickler and Soares, 2011; Elipot et al., 2013). There is an increase in the amount of brain serotonin in Pachón cavefish compared to surface fish, and this increase in serotonin levels may lead to the observed decrease in aggression in Pachón fish (Elipot et al., 2013). In addition, tyrosinase 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon polypeptide 1 (YWHAE), an enzyme involved in dopamine

biosynthesis, is upregulated in Pachón cavefish brains (Strickler and Soares, 2011). Both of these pathways are hypothesized to function in cavefish to change feeding behavior, as cavefish may need to spend an increased amount of time foraging relative to surface fish (Strickler and Soares, 2011; Elipot et al., 2013). Therefore, these pathways may have been selected for in cavefish for other behavior purposes, and have a pleiotropic effect on schooling behavior.

We found that potentially increasing levels of multiple monoamines, presumably including both serotonin and dopamine, with (R)-(-)-Deprenyl hydrochloride, decreased both schooling and shoaling tendency in surface fish. Although (R)-(-)-Deprenyl hydrochloride affects brain levels of serotonin in *Astyanax mexicanus* (Elipot et al., 2013), it is unlikely that changing serotonin levels alone affects schooling behavior given the insignificant effect of fluoxetine. This indicates that another monoamine, not serotonin, plays a role in schooling behavior in *Astyanax mexicanus*. Given the evidence that a molecule involved in the synthesis of dopamine is upregulated in at least one population of cavefish, our results are consistent with an increase in the amount of brain dopamine affecting schooling behavior in cavefish. Thus, brain neurotransmitter levels that have evolved to change adaptive behaviors, such as feeding behavior, may have a secondary, pleiotropic effect on schooling behavior. Testing whether modulation of dopamine specifically affects schooling and shoaling behavior, if R-deprenyl can induce increased levels of dopamine, and whether cavefish have increased amounts of

dopamine compared to surface fish, would be an interesting complement to this work.

**Evolution of schooling behavior independent of loss of vision**

While loss of vision plays an important role in loss of schooling behavior, we also found evidence for a vision-independent loss of schooling behavior. Many F2 fish with a strong response to light still do not follow the model school. This is similar to what was previously seen in shoaling assays in *Astyanax mexicanus,* where many F2 fish found to have visual function did not shoal (Parzefall and Fricke, 1991). Interestingly, when the effects of vision are removed by performing QTL analysis on only those fish that are light responsive, a second QTL, which does not fall in the same location as the vision, eye size, or pupil size QTL, emerges. In addition, we performed QTL analysis for neuromast number, and neither of the schooling QTL fall in the same place as the neuromast QTL (data not shown). This suggests that the second QTL for the loss of schooling is vision and lateral line independent. Markers located under this QTL map to zebrafish chromosome 5 (Table 2.2). Fine scale mapping, combined with detailed analysis of the genes within this interval, will be necessary to identify the specific genetic changes responsible for the schooling QTL.

**Potential evidence for relaxed selection on schooling in the cave**

Once the ancestors of cavefish entered caves, they would not be able to school due to lack of light, and this could relax selection on schooling behavior. In addition, the ecology of the cave habitat suggests that there

**Table 2.2 – Zebrafish location of *Astyanax* markers**

Marker is the RAD-seq marker. LG is the linkage group and cM is the position on that linkage group in centimorgans. Zebrafish chromosome and position refer to the location the marker mapped to in the zebrafish genome.

| Marker | LG | cM | Zebrafish chromosome | Zebrafish position |
|---|---|---|---|---|
| 3777.30r | 1 | 1.61 | 16 | 49352154 |
| 7517.34r | 1 | 27.1 | 24 | 27937870 |
| 1775.14r | 1 | 50.58 | 20 | 10282422 |
| 834.30r | 2 | 56.14 | 7 | 39294283 |
| 1439.29r | 2 | 87.3 | 4 | 23260016 |
| 3863.39r | 3 | 54.58 | 9 | 16877883 |
| 517.28r | 4 | 115.08 | 22 | 748928 |
| 1746.14r | 5 | 4.19 | 23 | 18826367 |
| 2437.18r | 5 | 52.19 | 23 | 20450560 |
| 3652.31r | 6 | 4.44 | 5 | 28800915 |
| 1942.24r | 8 | 45.93 | 21 | 39152588 |
| 5004.36r | 8 | 74.53 | 21 | 44371714 |
| 1720.16r | 9 | 35.26 | 9 | 41231661 |
| 3992.28r | 10 | 9.12 | 24 | 251036 |
| 1056.10r | 10 | 19.2 | 24 | 31446055 |
| 3720.41r | 11 | 20.52 | 14 | 34634976 |
| 2160.11r | 12 | 34.73 | 1 | 29432744 |
| 5928.36r | 12 | 43.67 | 13 | 35581479 |
| 949.12r | 12 | 63.24 | 15 | 1006948 |
| 1627.32r | 21 | 35.59 | 24 | 43047372 |
| 976.33r | 22 | 4.81 | 10 | 25150581 |
| 566.31r | 25 | 8.71 | 19 | 21693166 |
| 4144.36r | 27 | 0 | 5 | 25882088 |

would be no counter-selection to maintain schooling behavior, in spite of the loss of vision. A likely lack of macroscopic predators in the caves removes one major selective pressure for schooling in the cave environment. Although it is speculative, one possibility for the evolution of schooling behavior is that once vision was impaired by the lack of light, schooling was no longer under selection, and alterations in genes affecting this behavior would be neutral in consequence. This could be an explanation for identification of loci where F2 fish show an increase in schooling behavior with a homozygous cave genotype. Since a large percentage of seeing F2 fish still do not school, there are likely other loci where the cave alleles contribute to loss of schooling behavior. We expect that decreased schooling behavior is caused by a variety of genetic changes, and that many of these have effects too small to detect in our current analysis.

**Convergence on a decreased tendency to school in multiple cavefish populations and different fish species**

Here, we demonstrate that multiple, independently evolved cavefish populations have lost the tendency to school. Previous work on *Astyanax mexicanus* also showed a loss of schooling and reduction in shoaling behavior in cave populations (Parzefall, 1983; Parzefall, 1985; Parzefall and Fricke, 1991). Our work corroborates this previous work. Schooling behavior is also lost in other species of cave populations (reviewed in (Parzefall, 1985; Parzefall, 2001)). The importance of the loss of the visual system for loss of schooling behavior in *Astyanax mexicanus* may be general in cave

populations, and it would be interesting to know if other cavefish species have reduced schooling behavior due to lack of visual cues.

In addition to cavefish, benthic threespine sticklebacks (*Gasterosteus aculeatus*) display reduced schooling behavior (Wark et al., 2011). Greenwood and Wark et al. explore the genetics of this loss of schooling behavior in marine versus benthic stickleback populations (cosubmitted). While cave *Astyanax* and benthic sticklebacks both have a reduced tendency to school, the mechanisms that lead to loss of schooling behavior may be different in these two species. Benthic sticklebacks, which have intact visual systems, still show some tendency to follow a model school, but position themselves differently within it. In contrast, cave *Astyanax* have lost all tendency to follow a model school. This may be due to differences in habitats and selective pressures. Once they have entered the cave environment, *Astyanax* could no longer school due to loss of visual cues necessary for this behavior. In addition, cavefish do not encounter the selective pressures usually associated with schooling behavior. For example, within the caves there are probably no macroscopic predators. In contrast, benthic sticklebacks are still confronted with predators, but display a shelter seeking behavior rather than a schooling behavior (Wark et al., 2011).

Interestingly, both cave *Astyanax* and benthic sticklebacks appear to have evolved differences in schooling behavior through modifications of sensory systems. Loss of vision contributes to the evolutionary loss of schooling tendency in cave *Astyanax* and lateral line evolution contributes to

the evolution of schooling position in sticklebacks. Thus, convergent loss of schooling behavior can occur through modulation of different sensory systems and different behavioral components. Together, these studies demonstrate the contribution of sensory system evolution to the evolution of complex behaviors.

This work provides a start towards understanding the genetic basis of the loss of schooling behavior. Identification of the genes underlying these QTL will provide interesting insight into how schooling behavior is controlled, and how it evolves, and will allow us to test whether different species of fish lose schooling behavior through the same genes and pathways.

## Conclusion

In conclusion, we report the results of two behavioral assays for social grouping in surface and three cave populations of *Astyanax mexicanus*. We have shown that the loss of schooling behavior in a cave population of *Astyanax* has a genetic basis and is a complex trait, influenced by at least two loci. We show that vision, but not the lateral line, is important for schooling behavior in surface fish, and that vision is not a learned cue for schooling, but instead is required for this behavior. However, vision is not sufficient for schooling behavior. Loss of schooling behavior in cavefish has a genetic basis independent of eye loss. Additionally, we offer evidence that schooling was likely lost due to relaxed selection, as opposed to selection against schooling behavior in cave populations.

## Materials and Methods

### Animal Husbandry and Crosses

All animal procedures were in accordance with the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee at Harvard Medical School.

Cave and surface populations of fish used in this study were obtained at multiple times. The predecessors for the majority of the fish studied here were collected in either 2001 or 2002. These populations of fish were randomly crossed and all of the fish studied here are from the first generation or subsequent generations of fish bred in the lab. The Molino fish studied here are from a cross between wild caught Molino fish and a lab-maintained Molino fish population. Both surface and cavefish were maintained on a 14 hour light 10 hour dark cycle.

Individual female Tinaja cavefish were crossed to individual male surface fish to generate two families of F1 hybrid fish. F1 hybrid fish were crossed to one another to generate 320 F2 fish. All F2 fish were from the same single Tinaja and surface parent. F2 fish were tagged ventrally using Visual Implant Elastomer tags (Northwest Marine Technology, Inc). All assays and measurements were performed on adult fish, at least four and half months of age.

### Schooling behavior

Schooling behavior was measured as previously described (Wark et al., 2011). Six model fish were created using a surface fish mold. The fish were attached to a round wire, and propelled in a circle at 25 rpm by a motor. Assays were filmed from above using a Basler camera (Basler) at a frame rate of ten frames per second. The assay tank (60cm length x 37cm width x 18.5 cm height) was illuminated from above and to the side to reduce glare on the surface of the water. Light was diffused using photo umbrellas to create a constant light across the surface of the water. Individual fish were introduced to the tank, acclimated for two minutes and then filmed for three minutes. F2 fish and a subset of Tinaja cave and surface fish were assayed five times each, with at least two weeks between each trial. Schooling was scored for each video as the proportion of time spent schooling throughout the three-minute trial. Schooling was defined as a fish being within 1.5 model fish body lengths of the model and oriented in the same direction as the model school for at least ten frames. Schooling videos were scored manually in a program developed in LabVIEW (National Instruments). A small number of fish displayed freezing behavior for more than 1/3 of the time in the assay. These fish were not scored for this trial and were retested. For fish assayed five times, the reported proportion of time spent schooling was the average across all five trials.

Assays performed in the dark were illuminated from below using infrared illumination. Surface and cavefish assayed in the dark were first assayed in the light, and assayed in the dark on the following day. For

assays beyond the initial characterization of schooling behavior we focused on only surface and Tinaja cavefish as these populations were relevant to our genetic studies.

**Shoaling behavior**

Fish were assayed for shoaling behavior in groups of six. Assays were performed in a ten-gallon tank (50.8 cm length x25.4 cm width x30.48 cm height) filled with water to a height of four cm to minimize the effect of height when calculating distances between fish. Assays were filmed and illuminated from above, as described above. Fish groups (6 fish per group) were acclimated to the tank for 10 minutes, and then filmed for 10 minutes. 30 frames, or 3 frames per minute, were extracted from each video. In each frame, the nearest neighbor distance in centimeters was calculated for each individual fish using Fiji (Schindelin et al., 2012). Nearest neighbor distance was measured as the distance from the center of the body of a fish to the center of the body of the fish nearest to it. The average nearest neighbor distance was calculated as the average of all of the nearest neighbor distances for a group of fish over the course of the trial. We also measured shoaling by inter-individual distance, which was assessed by measuring the distance between each fish and all of the other fish in the group, and averaging this measure for across all of the fish in the tank. For shoaling group coherency, distances between pairs of fish were not re-measured for each individual.

To test shoaling in the dark, surface and cavefish assayed in the dark were first assayed in the light, and assayed in the dark on the following day. For the lens ablation experiments, the small number of lens-removed fish available made it impossible to assay more than one group of fish from each category. Therefore, each group was tested multiple times to make sure that results were consistent.

For groups of F2 fish, F2 fish were separated into groups based both on light-perception and the tendency to school. Light-perceiving fish were defined as spending 200 seconds or more on average over three trials in the dark. Non-light-perceiving fish were defined as fish spending less than 200 seconds of their time in the dark. Schooling fish were defined as those that spent at least five percent of their time following the model school, and non-schooling fish as those that spent less than five percent of their time schooling. These numbers were chosen to correspond with our QTL mapping data (see below). Groups of F2 fish were housed together for 5 days before the assay to allow them to acclimate to each other.

For assays beyond the initial characterization of shoaling behavior we focused on only surface and Tinaja cavefish as these populations were relevant to our genetic studies.

**Dark preference assay**

Scototaxis was measured according to (Maximino et al., 2010). Fish were placed in a 10-gallon glass tank with one half covered with aluminum foil to prevent light penetration. The other side was kept uncovered and

illuminated with photo umbrellas to create constant light. Fish were assayed individually for 5 minutes. Trials were repeated three times on consecutive days, with the exception of surface fish, which were assayed one time each. The time the fish spent in the dark was measured and averaged over all trials. We focused on only surface and Tinaja cavefish as these populations were relevant to our genetic studies.

**Quantification of morphological traits**

Standard length was calculated at the end of all behavioral trials. It was measured as the distance from the tip of the nose to the beginning of the tail. Eye size and pupil size were calculated at the same time as body size. The diameter of both eyes and both pupils for each fish were calculated, and averaged together. Measurements were made in Fiji (Schindelin et al., 2012). Eye and pupil size scales with body size in surface fish. For surface and F1 fish, eye and pupil size were corrected for standard length. To correct for eye and pupil size in F2 fish, the expected eye and pupil size for a surface fish was calculated for each fish's standard length. The observed eye or pupil size was divided by the expected size, and numbers were reported as a proportion of the observed over the expected (Protas et al., 2008).

Neuromasts were visualized using (2-(4-(dimethylamino)styryl)-N-Ethylpyridinium iodide, or DASPEI (Invitrogen) (Jørgensen, 1989). Fish were placed in 25 ug/ml for one hour, and then imaged. Staining was imaged under a dissecting scope. Cranial neuromast number was quantified using Fiji (Schindelin et al., 2012) for the left side of the face for each fish, and

included all visible neuromasts from the nose to the gill. Neuromast number scaled with size of the fish. To account for variation in fish size, neuromast number was corrected for the nose to pectoral length taken at the time of imaging neuromasts for each fish. Nose to pectoral length scaled with body size, and was therefore a good proxy for body size. This correction removed all effects of standard length on neuromast number. Neuromast diameter was measured for three suborbital neuromasts per fish, and the average diameter is reported in pixels.

Note that some morphological traits were not measured in all F2 fish, and therefore numbers of F2 fish measured for each trait are variable. We focused on only surface and Tinaja cavefish as these populations were relevant to our genetic studies.

**Neuromast ablation**

Neuromasts were ablated by treating fish with 0.002% gentamicin, which was shown to ablate neuromast function in *Astyanax mexicanus* previously (Yoshizawa et al., 2010). Fish were placed in gentamicin or fish water for 14-20 hours, and then removed to fresh fish water for behavioral assays. Fish were tested first for shoaling, and then a subset of fish were tested for schooling. Control fish were mock-treated with fish water only. We confirmed that the ablations worked by staining the fish using DASPEI after the behavioral assay.

**Lens removal**

Lens removal was conducted as previously described (Yamamoto and Jeffery, 2000; Yamamoto and Jeffery, 2002). Briefly, fish embryos at 36-48 hours post fertilization were treated with a calcium-free medium and embedded in agar. Lenses were removed using a tungsten needle, and embryos were recovered from the agar. Control fish were embedded in agar and recovered, but did not have lenses removed.

**Inhibitor treatment**

Fish were treated with 10 uM of (R)-(-)-Deprenyl hydrochloride (Sigma) or 14 uM of Fluoxetine hydrochloride (Sigma) by administering the drug to their water for 5 hours prior to the experiment. Assays occurred in fresh water. Control fish were placed in fish water only for 5 hours prior to assays. Fish were first assayed for shoaling behavior, and then a subset of these fish were also assayed for schooling behavior.

**SNP identification and genotyping**

SNPs for genotyping and linkage map construction were discovered using double digest RADseq as previously described (Peterson et al., 2012). Tinaja, surface, and F1 hybrid parental fish, as well as all F2 fish were anesthetized in tricaine and fins were clipped for DNA extraction. DNA was digested using MspI and EcoRI (New England BioLabs) overnight. Digested DNA was purified using Agencourt AMPure XP beads (Beckman Coulter) and ligated to 48 unique barcoded adaptors (Peterson et al., 2012). DNA was then pooled, bead purified, and size selected for a size of 224 bp of genomic DNA using the Pippin prep (Sage Science). Size selected DNA was amplified

with Phusion (New England BioLabs) for 10 cycles using Illumina indexed primers. Up to 144 individuals were pooled and sequenced using Hiseq 2000 (Illumina) sequencing. Parental fish were sequenced using 100 bp PE sequencing and F2 fish were sequenced using 50 bp SE reads.

Sequence data were analyzed as described for reference-free clustering in (Peterson et al., 2012). The resulting assembly was used as a reference for mapping using Stampy (Lunter and Goodson, 2011), and realignment and variant calling were performed using the genome analysis toolkit (version 2.1) with indelrealigner and unified genotyper (McKenna et al., 2010; DePristo et al., 2011). Using a QD of 5 and a GQ of 20, genotypes were determined at each locus for each individual. We mapped markers to zebrafish using BLAST as described previously (O'Quin et al., 2013).

**QTL mapping**

Linkage map construction and QTL mapping were performed in R/qtl (Broman et al., 2003). Linkage map construction was performed using the Kosambi map function and an error rate of 0.005. All QTL were mapped using the maximum likelihood and Haley-Knott methods for interval mapping, using the function “scanone”. All methods yielded similar results for the traits reported. Binary traits were mapped using a binary model, while continuous traits were mapped using a normal model (eye and pupil size) or a non-parametric model (schooling). All p-values presented were for $\alpha=0.05$ after 1000 permutations using a genome-wide significance threshold. Percent

variance explained was estimated using “fitqtl.” The confidence interval was established using the “bayesint” function.

For mapping schooling behavior, we used a binary measure of schooling. Fish that schooled 5% of the time or more across five trials were counted as schooling. This cut-off was chosen because no cavefish tested schooled for more than 5% of the time in five trials, while all surface fish (Figure 2.12) and F1 fish (data not shown) schooled for more than 5% of the time. In addition, it included F2 fish that schooled in only one trial, which we did not observe in cavefish. For mapping dark preference, we used a binary cutoff of 200 seconds on average spent in the dark, and for light-perceiving schooling fish, we used only the fish that spent 200 seconds or more in the dark.

**Statistics**

To compare the different traits, we performed ANOVA on traits for which we had measured multiple groups, and then planned-contrast tests and post hoc Games-Howell tests, frequently used post hoc tests for ANOVA, to determine pairwise differences between groups. Comparisons of groups which had non-normal distributions, as measured by a Shapiro-Wilk test, were analyzed using a Kruskal Wallis test, followed by pairwise Mann-Whitney tests corrected for multiple testing using a Bonferroni correction. Comparisons that violated a homogeneity of variance test, using a Levene test, were subjected to a Welch ANOVA, followed by post-hoc Games-Howell tests. Comparisons with only two groups were tested by a Student’s T-test or a Welch test. For

the correlation statistics, we used a Pearson correlation for normally distributed traits and Spearman rank correlations for non-normally distributed traits. To determine repeatability across five trials, we determined the intraclass correlation coefficient. All statistics were performed in R (Team, 2012) or in SPSS.

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# Chapter 3

# Convergence in feeding posture occurs through different genetic loci in independently evolved cave populations of *Astyanax mexicanus*

# Convergence in feeding posture occurs through different genetic loci in independently evolved cave populations of *Astyanax mexicanus*

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This chapter contains a manuscript titled “Convergence in feeding posture occurs through different genetic loci in independently evolved cave populations of *Astyanax mexicanus*” currently under review at PNAS. It has been modified to better fit the style of this dissertation. Johanna Kowalko, Clifford Tabin, Masato Yoshizawa and William Jeffery designed the research. Johanna Kowalko and Masato Yoshizawa and performed the research. Tess Linden aided with the morphological phenotyping. Nicolas Rohner aided with genotyping. Johanna Kowalko and Masato Yoshizawa analyzed the data. Santiago Rompani designed the LabView software. Wesley Warren provided the genome. Richard Borowsky provided fish. Johanna Kowalko, Masato Yoshizawa, Clifford Tabin and William Jeffery wrote the paper.

## Abstract

When an organism colonizes a new environment, it needs to adapt, both morphologically and behaviorally, to survive and thrive. While recent progress has been made in understanding the genetic architecture underlying morphological evolution, behavioral evolution is poorly understood. Here, we use the Mexican cavefish, *Astyanax mexicanus*, to study the genetic basis for convergent evolution of feeding posture. When river-dwelling surface fish became entrapped in the caves they were confronted with dramatic changes in the availability and type of food source and in their ability to perceive it. In this setting, multiple independent populations of cavefish exhibit an altered feeding posture compared to their ancestral surface forms. We determined that this behavioral change in feeding posture did not evolve to accommodate changes in cranial facial morphology, or to take advantage of the expansion in the number of taste buds. Quantitative genetic analysis demonstrates that two different cave populations have evolved similar feeding postures through a small number of genetic changes, some of which appear to be distinct. This work indicates that independently evolved populations of cavefish can evolve the same behavioral traits to adapt to similar environmental challenges by modifying different sets of genes.

## Introduction

The colonization of caves is an extreme example of a species entering a new environment. Unique attributes of caves, relative to the surface

environment, include darkness, high humidity, relatively constant temperature, absence of predators, and scarcity of food. Under these circumstances, many species of cave animals have evolved a suite of similar traits, both constructive, such as heightened sensory systems, and regressive, including loss of pigmentation and reduction in eye morphology (Protas and Jeffery, 2012). To study the evolution of cave-specific traits, we have focused on *Astyanax mexicanus*, the Mexican tetra. *Astyanax mexicanus* exists in two forms, a cave-dwelling form and a river-dwelling surface form. Importantly, in spite of at least 10,000 years of isolation, these forms remain interfertile (Wilkens, 1988), allowing one to take a genetic approach utilizing quantitative trait loci (QTL) analysis for the mapping of cave traits. Furthermore, there are multiple, independently evolved cave populations (Mitchell et al., 1977; Dowling et al., 2002; Strecker et al., 2004; Bradic et al., 2012; Gross, 2012) that in many cases have evolved similar traits, allowing for the study of convergent evolution.

Populations of cave organisms have often been the subjects of studies in convergence. For example, loss of pigmentation evolved via disruptions in the same place in the melanin synthesis pathway in multiple species of cave organisms (Bilandzija et al., 2012). Similarly, a decrease in the levels of melanin synthesis arose in multiple cave populations of *Astyanax mexicanus* through different mutations in the same genes (Protas et al., 2006; Gross et al., 2009). In contrast, crosses between multiple cave populations of *Astyanax mexicanus* result in embryonic hybrid fish with larger, functional

eyes, indicating that evolution of this trait is controlled by different genetic loci in different cave populations (Wilkens, 1988; Borowsky, 2008).

Behavioral traits are among the most intriguing and least understood adaptations to caves. Cave populations of *Astyanax mexicanus* have evolved an enhanced attraction to sources of vibration, loss of schooling behavior, reduction in time spent sleeping, and a loss of aggression (Schemmel, 1974; Burchards et al., 1985; Wilkens, 1988; Teyke, 1990; Parzefall and Fricke, 1991; Yoshizawa et al., 2010; Duboue et al., 2011; Elipot et al., 2013). Little is known about how these behaviors evolve and whether each behavior has evolved through the same or different genes or pathways as an adaptation to cave life.

Here we focus on a convergent aspect of feeding behavior and its potentially related morphological traits found in cave populations of *Astyanax mexicanus*. Surface fish feed at a steep angle relative to the substrate when feeding in the dark. In contrast, multiple cave populations feed at a much lower angle (Schemmel, 1980). We examined feeding posture in three independently evolved cave populations to determine the underlying genetic architecture, including the extent to which this behavior was dependent upon correlated morphological changes, and to determine if multiple cave populations evolved this alternative feeding posture through changes in the same or different genetic loci.

## Results

### Characterizing feeding posture in several independently evolved cave populations

We developed assays to quantify feeding posture by measuring the feeding angle for individual fish, filming them in the dark, from either above or the side, with an infrared camera (Figure 3.1A). We measured feeding posture in surface fish, as well as in three independently evolved populations from different caves, Pachón, Tinaja, and Molino. There were significant differences in feeding angle among groups (ANOVA: $F_{6,194}$=25.4, p<0.001). While surface fish fed at a high angle (average 74°), two populations of cavefish, the Pachón cavefish and the Tinaja cavefish, fed at significantly lower angles on average (38° and 49°; Games-Howell: p<0.001 and p<0.001 respectively; Surface n=43, Pachón n=29, Tinaja n=46; Figure 3.1B-D). However, the third population of cavefish, from the Molino cave, fed at an angle similar to surface fish (66°, Games-Howell: p=0.253; Molino n=24; Figure 3.1D). Thus, cavefish populations can differ from each other in feeding posture and a lower feeding posture has evolved independently in at least two cave populations.

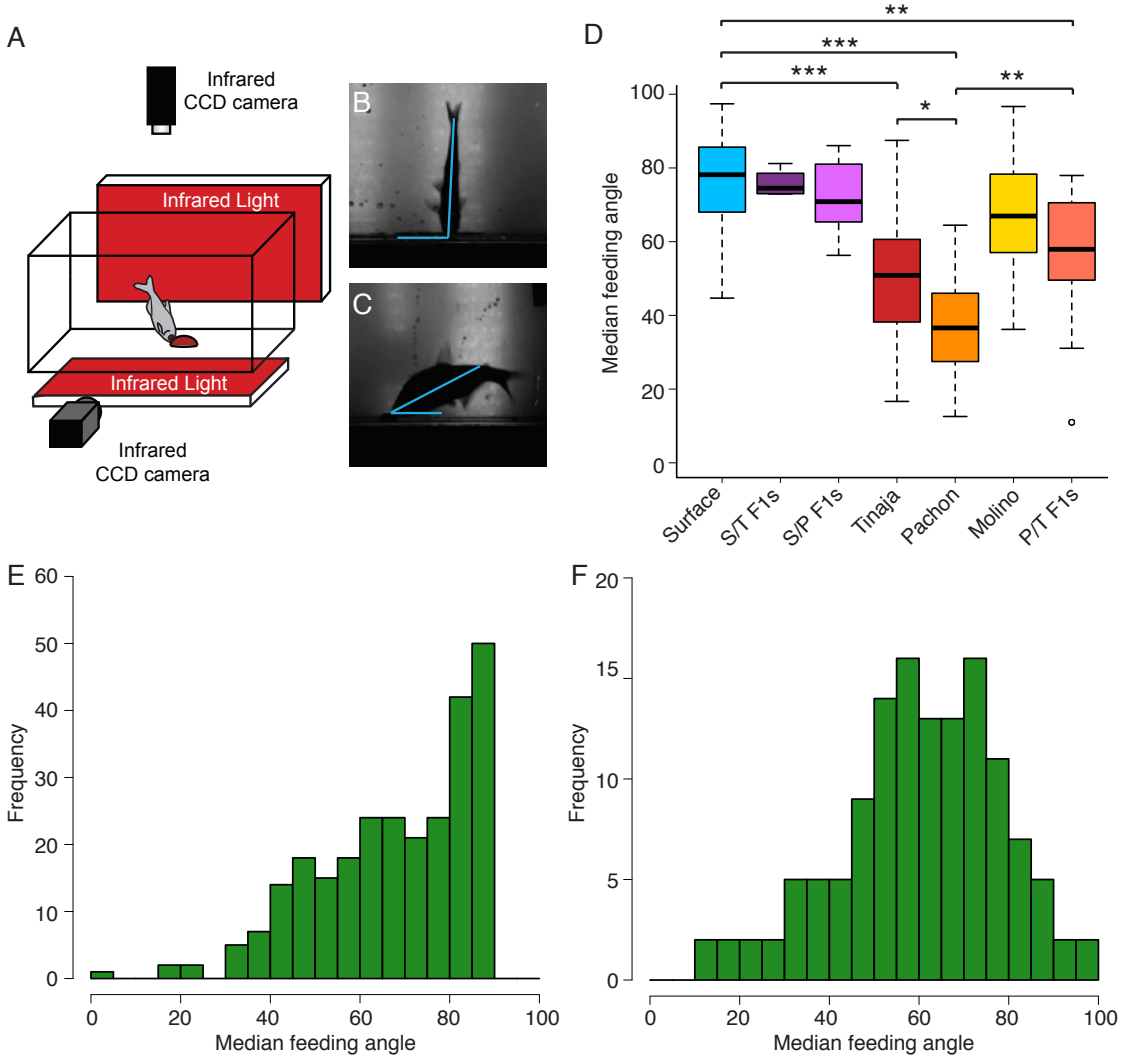
### Genetics of feeding posture

In order to investigate the genetic basis of feeding posture, we performed multiple crosses and measured feeding posture in the resulting progeny. F1 hybrid fish from both surface/Tinaja and surface/Pachón crosses exhibited average feeding angles similar to surface fish, indicating that a

**Figure 3.1. Genetics of feeding angle in multiple, independently evolved cave populations.**

A. Schematic of two methods for filming feeding posture: top or side. The surface/Tinaja cross was filmed from the side, while the surface/Pachón cross was filmed from above. B. Feeding posture in a surface fish. C. Feeding posture in a Tinaja cavefish. D. Distribution of feeding posture in surface fish (n=43), surface/Tinaja F1 hybrid fish (n=4), surface/Pachón F1 hybrid fish (n=30), Tinaja (n=46), Pachón (n=29), Molino (n=24), and Pachón/Tinaja F1 hybrid fish (n=25). E. Distribution of 267 F2 fish from a surface/Pachón F1 intercross. F. Distribution of 226 F2 fish from a surface/Tinaja F1 hybrid intercross. *$p<0.05$, **$p<0.01$, ***$p<0.001$

Figure 3.1, cont.



surface-like phenotype is dominant (76° and 71°; Games-Howell: p=0.998, p=0.958, respectively; surface/Tinaja F1s n=4, surface/Pachón F1s n=30; Figure 3.1D). F1 fish from both of these crosses were significantly different from their parental cave population (Games-Howell: surface/Tinaja F1s p<0.001, surface/Pachon F1s: p<0.001; Figure 3.1D). In addition, feeding posture was similar in surface/Pachón F1 hybrid fish regardless of which parent was a surface fish and which parent was a Pachón cavefish (73° and 68°; independent t-test, $t_{28}$=0.87, p=0.39; Pachón-mothered F1: n=14, Surface-mothered F1: n=16).

F2 fish were generated by intercrossing F1 hybrid fish. F2 population distributions of feeding angle from the surface/Tinaja and surface/Pachón fish both have peaks weighted towards the mean angle observed in surface fish with tails extending over the angle seen in cave populations (surface/Pachón F2s n=267, surface/Tinaja F2s n=226; Figure 3.1E and F). These distributions suggest that this is a multigenic trait in both cave populations. Fish body length had a small contribution to average feeding angle in the surface/Tinaja F2 cross (r=0.25, p<0.001, n=170, Supplemental Figure 1A). Sex of the fish also contributed to feeding posture ($t_{138}$=-2.2, p<0.05, n=170, Figure 3.2B). However, neither of standard length (r=-0.09, p=0.137, n=267) nor sex (Mann-Whitney: U=173, p=0.85, n=39) affected feeding posture in the surface/Pachón F2 population (Figure 3.2C and D).

To determine if feeding posture evolved by the same genes in these two cave populations, we crossed Pachón and Tinaja fish and measured

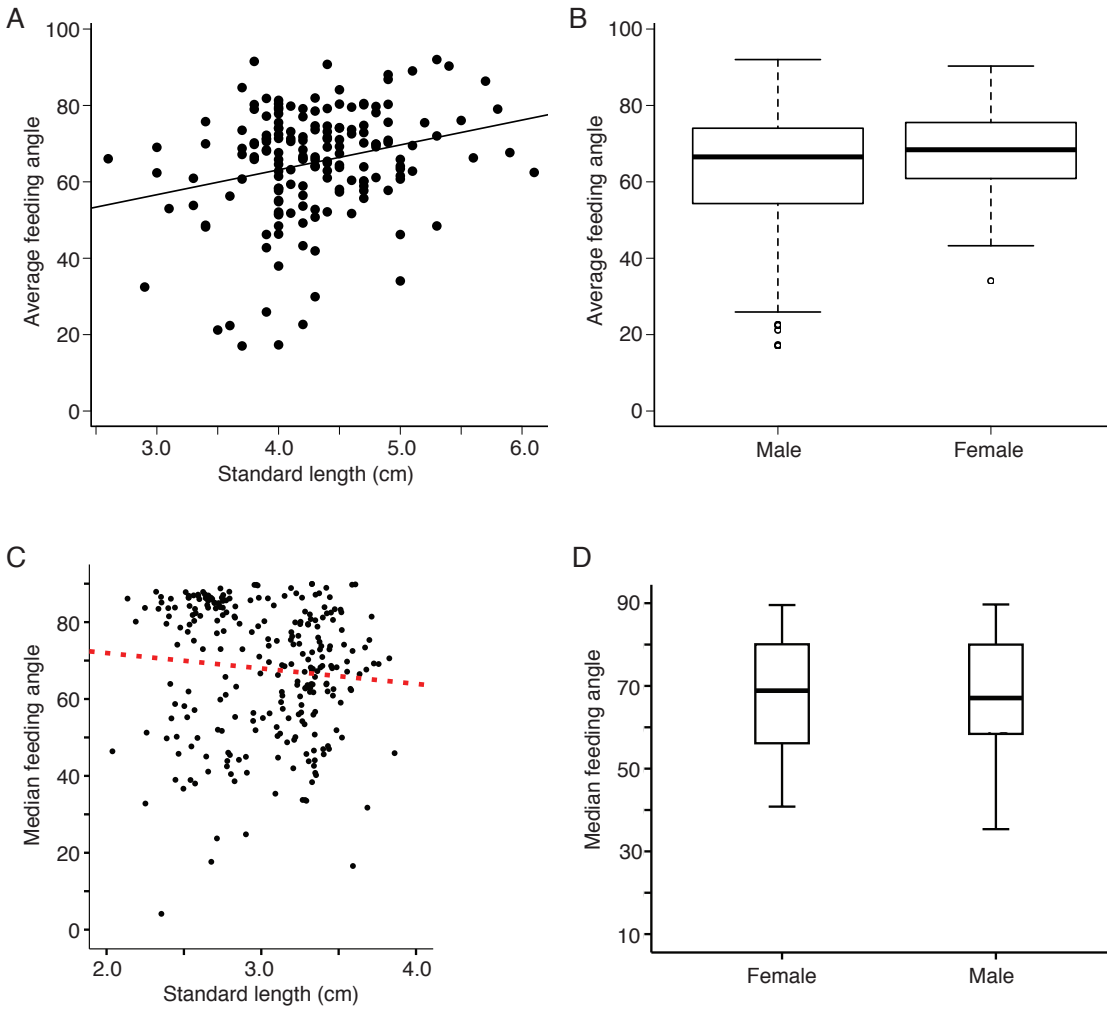



**Figure 3.2. Effects of body length and sex on feeding angle.**

A. Average angle compared to standard length in surface/Tinaja F2 hybrid fish (n=170). B. Average angle in male and female fish from a surface/Tinaja F2 hybrid cross (n=170). C. Median angle compared to standard length in surface/Pachón F2 hybrid fish (n=267). D. Median feeding angle in male (n=15) and female (n=24) fish from a surface/Pachón F2 cross.

feeding angle in F1 hybrid fish. Pachón/Tinaja F1 hybrid fish ranged in their behavior from surface-like to cave-like feeding postures (Figure 3.1D). Furthermore, this F1 population was significantly different from surface (Games-Howell: $p<0.01$, Pachón/Tinaja F1 fish n=25) and Pachón fish (Games-Howell: $p<0.01$), but not significantly different from Tinaja fish (Games-Howell: $p=0.394$). Pachón and Tinaja fish populations were significantly different from one another (Games-Howell: $p<0.05$). Lack of complete complementation in the Pachón/Tinaja F1 hybrid fish data suggests that some of the genetic changes controlling evolution of feeding posture in these two caves were shared, or resulted from independent, but additive genetic changes. However, differences between the Pachón and Tinaja fish populations, and between Pachón/Tinaja F1 hybrid fish and Pachón fish suggest that at least one genetic change resulting in a lower feeding angle was different between these two populations. Furthermore, these data are consistent with previous results demonstrating that these two cave populations evolved independently (Borowsky, 2008; Bradic et al., 2012).

**The evolution of feeding posture is not linked to craniofacial morphological evolution**

In addition to behavioral changes, cavefish have evolved a variety of morphological traits to adapt to cave life. Many of these morphological adaptations are found in multiple, independently evolved cave populations. Therefore, it was possible that feeding posture evolved secondarily, as a result of one or more of these morphological traits. In order to test this

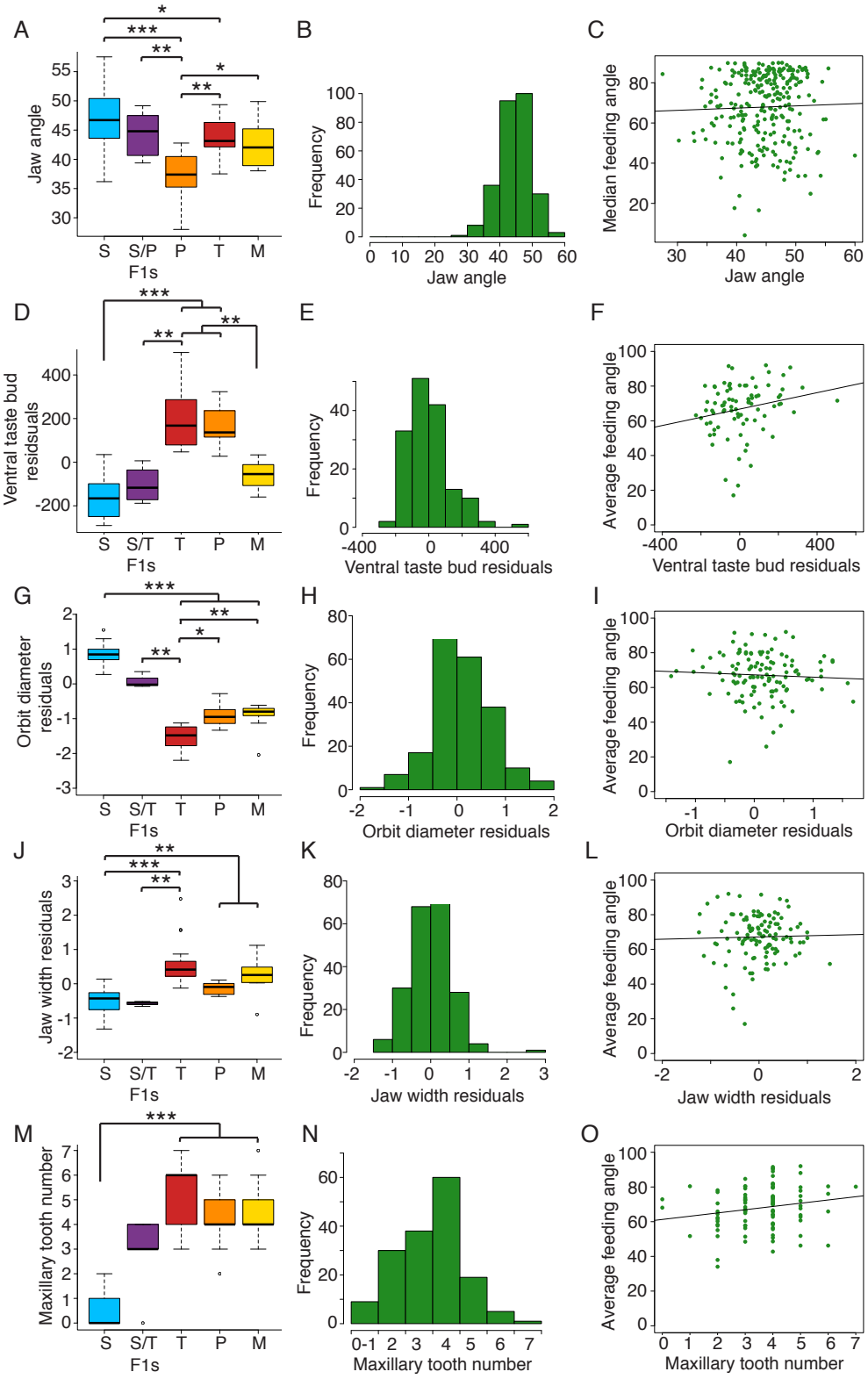
hypothesis, we quantified cranial facial traits that have evolved in cave populations, and compared their inheritance to feeding posture.

The shape of the cavefish face is noticeably different from that of the surface fish (Yamamoto et al., 2003), in particular by having an altered angle of the jaw relative to the body axis. While the adaptive significance of this is unclear, clearly it could relate in a direct manner to feeding behavior. We measured the angle of the jaw by measuring the angle from the tip of the nose to the lower end of the second suborbital bone, relative to a line bisecting the fish from the tip of the nose to the tail, and found significant differences between groups of parental fish (ANOVA: $F_{4,96}=17.4$, $p<0.001$; Surface n=38, surface/Pachón F1s n=9, Pachón n=21, Tinaja n=22, Molino n=10). Surface fish have a larger jaw angle compared to Pachón (Games-Howell: $p<0.001$) and Tinaja (Games-Howell: $p<0.05$) but not Molino cavefish (Games-Howell: $p=0.10$; Figure 3.3A). In addition, Pachón fish have significantly smaller jaw angles than the other cavefish (Games-Howell: Tinaja $p<0.01$ and Molino $p<0.05$). Surface/Pachón F1 hybrid fish looked similar to surface fish (Games-Howell: $p=0.40$) and different from Pachón fish (Games-Howell: $p<0.01$; Figure 3.3A) and the majority of surface/Pachón F2 fish had a jaw angle similar to surface fish, with a subset of these fish having a Pachón-like jaw angle, indicating that this trait is multigenic and that the surface phenotype is largely dominant (Figure 3.3B). However, jaw angle was not significantly correlated with feeding angle in F2 fish ($r=0.03$, $p=0.61$,

**Figure 3.3. Comparison of feeding angle to cranial facial traits.**

A. Jaw angle in surface (S, n=38), surface/Pachón F1 hybrid fish (S/P F1s, n=9), Pachón (P, n=21), Tinaja (T, n=22), and Molino (M, n=10) fish. B. Jaw angle in surface/Pachón F2 fish (n=237). C. Median feeding angle compared by jaw angle in surface/Pachón F2 fish (n=231). D. Ventral taste bud residuals regressed against nose to pectoral length in surface (n=14), surface/Tinaja F1 hybrid fish (S/T F1s, n=4), Tinaja (n=10), Pachón (n=9), and Molino (n=10) fish. E. Ventral taste bud residuals regressed against nose to pectoral length in surface/Tinaja F2 fish (n=154). F. Average feeding angle by ventral taste bud number residuals in surface/Tinaja F2 fish (n=83). G. Orbit diameter residuals regressed against nose to pectoral length in surface (n=25), surface/Tinaja F1 hybrids (n=3), Tinaja (n=18), Pachón (n=9) and Molino (n=10) cavefish. H. Orbit diameter residuals regressed against nose to pectoral length in surface/Tinaja F2 fish (n=213). I. Average feeding angle by orbit diameter residuals in surface/Tinaja F2 fish (n=119). J. Jaw width residuals regressed against nose to pectoral length in surface (n=25), surface/Tinaja F1 hybrids (n=3), Tinaja (n=18), Pachón (n=8) and Molino (n=10) cavefish. K. Jaw width residuals regressed against nose to pectoral length in surface/Tinaja F2 fish (n=212). L. Average feeding angle by jaw width residuals in surface/Tinaja F2 fish (n=118). M. Maxillary tooth number in surface (n=13), surface/Tinaja F1 hybrids (n=5), Tinaja (n=8), Pachón (n=9), and Molino (n=12) cavefish. N. Maxillary tooth number in surface/Tinaja F2 fish (n=162). O. Average feeding angle by maxillary tooth number in surface/Tinaja F2 fish (n=95). *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Figure 3.3, cont.



n=231, Figure 3.3C). Therefore, it is unlikely that feeding posture evolved as a consequence of jaw angle.

Another morphological trait that could potentially affect feeding posture is taste bud number and distribution. Cavefish have more taste buds than surface fish, and unlike surface fish, their taste buds are widely distributed all over their heads (Schemmel, 1974; Varatharasan et al., 2009). In principle, cavefish could have changed their feeding posture in order to allow their expanded ventral taste buds to contact the substrate as they search for food. We visualized taste buds by staining whole fish with an antibody against calretinin, and quantified taste bud number dorsally, ventrally, and on both lips. Number of taste buds scaled with body size, so we corrected for size by calculating the residuals when taste bud number was regressed against nose to pectoral length. There were significantly different numbers of taste buds between groups (ANOVA: $F_{4,42}=24.0$, $p<0.001$; Surface n=14, surface/Tinaja F1s n=4, Tinaja n=10, Pachón n=9, Molino n=10). Surface fish had significantly fewer ventral taste buds than Tinaja (Games-Howell: $p<0.001$) and Pachón (Games-Howell: $p<0.001$) but not Molino cavefish (Games-Howell: $p=0.053$; Figure 3.3D). While Pachón and Tinaja fish do not have significantly different numbers of taste buds from one another (Games-Howell: $p=0.96$), Molino cavefish have significantly fewer taste buds than either of these other caves (Games-Howell: $p<0.01$ and $p<0.01$, respectively). Taste bud number in surface fish was not significantly different than in

surface/Tinaja F1 fish (Games-Howell: p=0.85), suggesting that the surface phenotype is dominant (Figure 3.3D).

Surface/Tinaja F2 fish range in their number of taste buds, with the majority of F2 fish having an intermediate phenotype (n=154, Figure 3.3E). However, ventral taste bud number was not significantly correlated with feeding angle in F2 fish (r=0.21, p=0.051, n=83, Figure 3.3F). Therefore, it is unlikely that increased number of taste buds affected the evolution of feeding posture.

To determine if other aspects of cranial facial morphology affected feeding posture, we measured cranial skeletal traits previously described to be different between cave and surface fish, including orbital opening diameter, jaw width and maxillary tooth number (Wilkens, 1988; Jeffery, 2001).

Orbital diameter scaled with body size, and was corrected for body size as described above. There were significant differences in orbit diameter in the different fish populations (ANOVA: $F_{4,60}$=159.6, p<0.001; Surface n=25, surface/Tinaja F1s n=3, Tinaja n=18, Pachón n=9, Molino n=10). Surface fish have larger orbital diameters than all three cave populations (Games-Howell: Tinaja p<0.001, Pachón p<0.001, Molino p<0.001, Figure 3.3G). Molino and Pachón cavefish do not have significantly different orbital diameters (Games-Howell: p=0.99), however, Tinaja fish have smaller orbits than fish from the other two caves (Games-Howell: p<0.01 and p<0.05, respectively). Surface/Tinaja F1 hybrid fish have an intermediate orbit size,

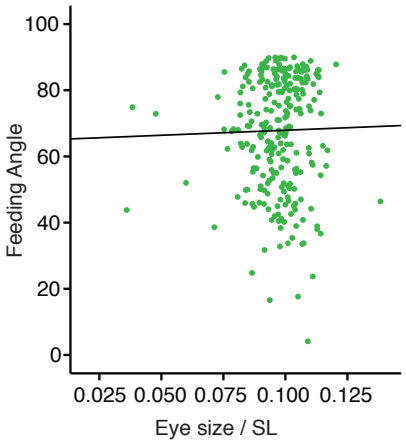



**Figure 3.4. Effect of eye size on feeding angle in a surface/Pachón cross.**

Median angle compared to eye size in surface/Pachón F2 hybrid fish.

significantly different from Tinaja fish (Games-Howell: $p<0.01$) but not significantly different from surface fish (Games-Howell: $p=0.056$, Figure 3.3G). Surface/Tinaja F2 fish ranged in orbit size, with the majority of fish having orbits similar to F1 hybrid fish (n=213, Figure 3.3H). Orbit size did not correlate with feeding angle ($r=-0.06$, $p=0.54$, n=119, Figure 3.3I). In addition, eye size was not correlated with feeding angle in the surface/Pachón cross ($r=0.02$, $p=0.75$, Figure 3.4). Thus, we found no evidence for orbit size affecting feeding posture.

Jaw width was measured at the jaw joint, and corrected for body size as described above. There were significant differences between groups of fish (ANOVA: $F_{4,60}=15.9$, $p<0.001$; Surface n=25, surface/Tinaja F1s n=3, Tinaja n=18, Pachón n=8, Molino n=10). Surface fish have significantly narrower jaws than cave populations (Games-Howell test: Tinaja $p<0.001$, Pachón $p<0.01$, and Molino $p<0.01$; Figure 3.3J). Surface/Tinaja F1s have jaw widths similar to surface fish (Games-Howell: $p=0.909$) and significantly different from Tinaja fish (Games-Howell: $p<0.01$), indicating that the surface phenotype is dominant (Figure 3.3J). The majority of surface/Tinaja F2 fish have an intermediate jaw width (n=212, Figure 3.3K). However, as with the other morphological traits, jaw width was not correlated with feeding angle ($r=0.03$, $p=0.77$, n=118, Figure 3.3L).

Finally, fish groups have significantly different numbers of total maxillary teeth (ANOVA: $F_{4,38}=19.7$, $p<0.001$; Surface n=13, surface/Tinaja F1s n=5, Tinaja n=8, Pachón n=9, Molino n=12). Surface fish have

significantly fewer maxillary teeth than all cavefish (Games-Howell: Tinaja $p<0.001$, Pachón $p<0.001$, Molino $p<0.001$; Figure 3.3M). Surface/Tinaja F1 hybrid fish are intermediate in phenotype, not significantly different from surface fish (Games-Howell: $p=0.18$) or from Tinaja cavefish (Games-Howell: $p=0.13$). The majority of F2 fish look like F1 hybrid fish, with an intermediate phenotype between Tinaja and surface fish ($n=162$, Figure 3.3N). Tooth number is also not correlated with feeding angle in F2 fish ($r=0.19$, $p=0.06$, $n=95$, Figure 3.3O). Together, these morphological data suggest that it is unlikely that differences in feeding posture among surface fish, Tinaja and Pachón cavefish are secondary consequences of differences in any of these morphological traits.

**QTL mapping of feeding posture**

We performed QTL mapping on F2 fish from a surface/Pachón F1 intercross and from a surface/Tinaja F1 intercross. For the surface/Pachón cross, 382 individuals were genotyped for 698 markers, from RAD-seq, microsatellites, and candidate genes, and a linkage map of 25 linkage groups was generated as previously described (O'Quin et al., 2013) and was used for mapping. For the surface/Tinaja cross, a linkage map was generated from 330 SNPs and 292 individuals, as previously described (Kowalko et al., submitted), and eight microsatellites mapped in the surface/Pachón cross were included in this map. In addition, 47 individuals were added to the surface/Tinaja cross. Together, these additional data resolved the linkage map to 27 linkage groups.

We found two significant QTL at a p-value of 0.05 in the surface/Pachón cross, one on LG 6 and one on LG 17 that explained 7 and 5.4 percent of the variance in feeding angle, respectively (Figure 3.5A,D-G, Table 3.1). Homozygous cave genotypes at markers under both QTL resulted in a decrease in feeding angle, with heterozygous genotypes resulting in an intermediate feeding phenotype (Figure 3.5E and G).

We found one significant QTL at a p-value of 0.05 in the surface/Tinaja cross on LG 24 that explained 10.8 percent of the variance in feeding angle (Figure 3.6A and C, Table 3.1). Homozygous cave genotypes at the marker under this QTL resulted in an increase in feeding angle (Figure 3.6D). The heterozygous genotype had a similar feeding angle to the homozygous cave genotype.

In order to determine if the QTL were present in the same or different locations in these two crosses, we performed alignment analyses using the latest *Astyanax mexicanus* genomic assembly (Assembly: Astyanax_mexicanus-1.0.2). In addition, we genotyped microsatellites under the QTL peaks of the surface/Pachón cross in the surface/Tinaja cross, and placed these markers onto the linkage map. In total, 194 genomic markers of both surface/Pachón and surface/Tinaja crosses were successfully aligned to 101 genomic scaffolds indicating 101 syntenic regions between Pachón and Tinaja linkage maps. We confirmed that we were matching the appropriate linkage groups by determining that the eight microsatellites we genotyped in the surface/Tinaja cross mapped to the appropriate linkage groups in the

**Figure 3.5. QTL mapping of feeding angle, jaw angle and eye size in a surface/Pachón cross.**

A. Genome-wide LOD scores from a scanone QTL for feeding angle (red) and jaw angle (blue) and eye size (green). The scales on the left are for feeding posture and jaw angle, and on the right for eye size. Dotted line indicates a p-value cutoff of 0.05 with the color corresponding the color for the trait. The X-axis indicates genetic distance in each linkage group. B. Jaw angle QTL at LG 3. Blue shaded area indicates the confidence interval using Bayesian credible intervals with probability coverage as 0.95 for the QTL. C. Effect plot of phenotypic values of jaw angle against each genotype (mean ± s.e.m.) at the peak locus at LG 3. D. Feeding angle QTL at LG 6. Red area indicates the confidence interval. E. Effect plot of feeding angle QTL at LG 6. F. Feeding angle QTL at LG 17. Red shaded areas indicate confidence intervals. G. Effect plot of feeding angle QTL at LG 17. SS, surface fish homozygote. SC. heterozygote, and CC, cavefish homozygote.

Figure 3.5, cont.

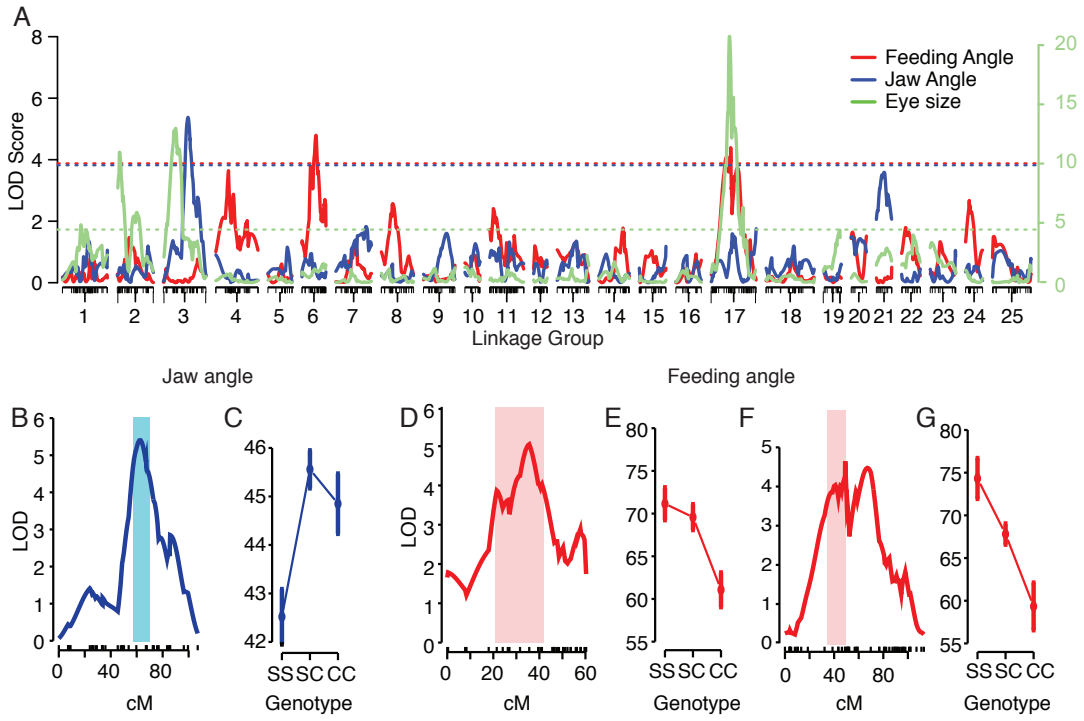


**Table 3.1 – QTL analysis.**

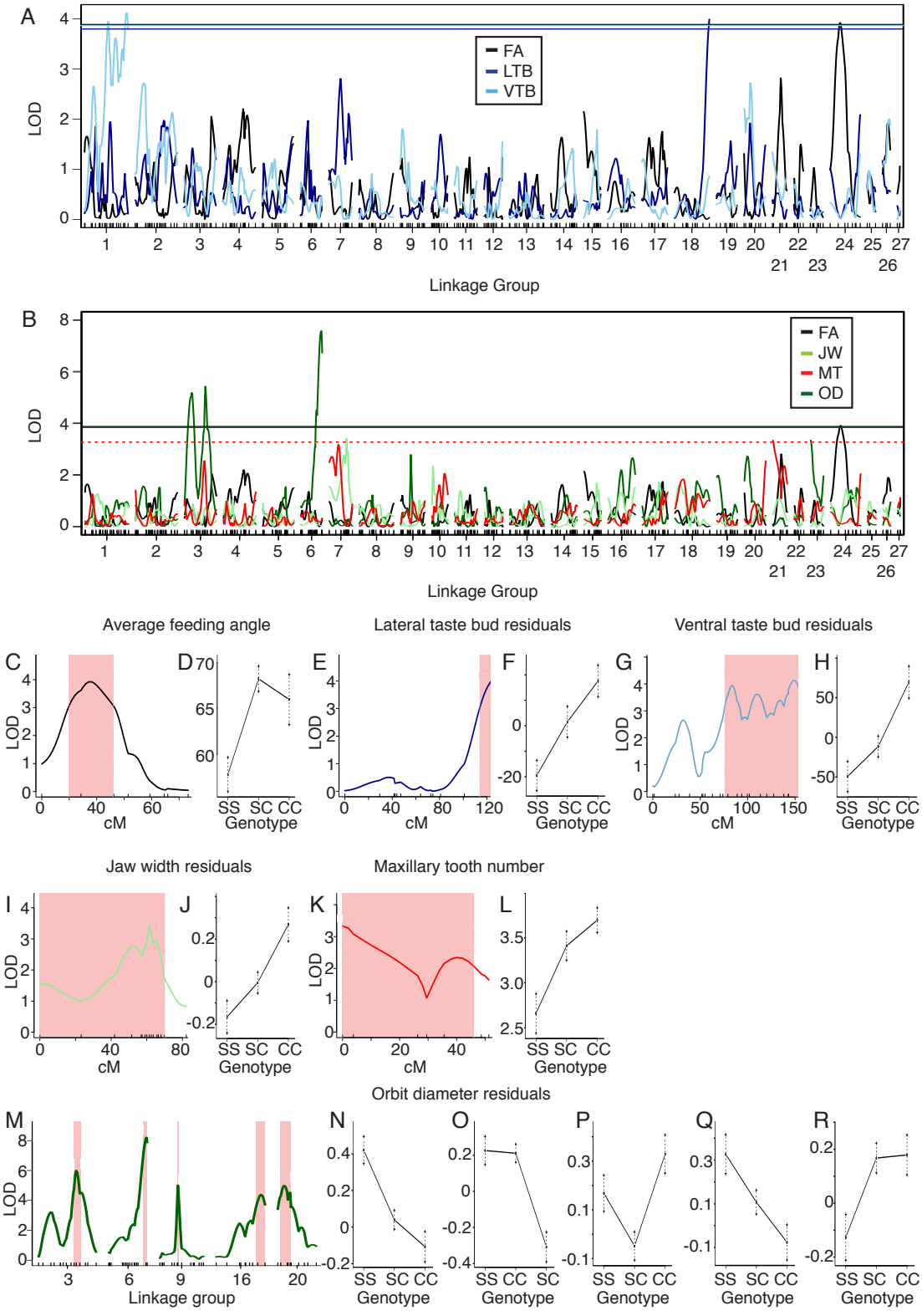
LG-P: Linkage groups of the surface/Pachón cross. LG-T: Linkage groups of the surface/Tinaja cross. cM: centimorgan. CI: Confidence intervals for the position of the QTL using a 95% Bayesian credible interval. PVE: percentage of the phenotypic variance explained. S/P: Surface/Pachón cross. S/T: Surface/Tinaja cross.

| Trait | Cross | LG-P | LG-T | cM | CI | PVE | alpha |
|---|---|---|---|---|---|---|---|
| Feeding angle | S/P | 6 | 11 | 35.6 | 21-42 | 7 | 0.05 |
| | | 17 | 13 | 49 | 34-73 | 5.4 | |
| Jaw angle | S/P | 3 | 2 | 63 | 55-72 | 8.3 | 0.05 |
| Feeding angle | S/T | 20 | 24 | 36 | 20-52 | 10.8 | 0.05 |
| Tastebuds – lateral | S/T | 25 | 18 | 123 | 114-124 | 10.9 | 0.05 |
| Tastebuds – ventral | S/T | 2 | 1 | 148 | 76-154 | 11.6 | 0.05 |
| Orbit | S/T | 11 | 3 | 74 | 70-84 | 7.8 | 0.05 |
| | | 19 | 6 | 74 | 68-75 | 11.2 | |
| | | 15 | 9 | 35 | 34-36 | 7.0 | |
| | | 13 | 16 | 88 | 78-96 | 5.6 | |
| | | 12 | 20 | 12 | 4-24 | 6.6 | |
| Jaw width | S/T | 22 | 7 | 61 | 0-70 | 7.2 | 0.15 |
| Maxillary tooth number | S/T | 1 | 21 | 0 | 0-46 | 9.1 | 0.15 |

**Figure 3.6. QTL mapping of feeding angle, taste buds, orbit diameter, jaw width, and maxillary tooth number in a surface/Tinaja cross.**

A. Genome-wide LOD score from scanone QTL for feeding angle (FA - black), lateral taste bud number residuals (LTB - dark blue) and ventral taste bud number residuals (VTB -light blue). Lines indicates p-value cutoff of 0.05 for each trait. B. Genome-wide LOD score from scanone for feeding angle (FA - black), jaw width residuals (JW - light green), maxillary tooth number (MT - red), and orbit diameter residuals (OD – dark green). Lines indicate p-value cutoff of 0.05 (solid) or 0.15 (dotted) for each trait. C. Feeding angle QTL at LG 24. D. Effect plot for feeding angle QTL. E. Lateral taste bud residuals QTL at LG 18. F. Effect plot for lateral taste bud residuals at LG 18. G. Ventral taste bud residuals QTL at LG 1. H. Effect plot for ventral taste bud residuals at LG 1. I. Jaw width residuals QTL at LG 7. J. Effect plot for jaw width residuals at LG 7. K. Maxillary tooth number QTL at LG 21. L. Effect plot for maxillary tooth number QTL at LG 21. M. Orbital diameter residuals QTL at LGs 3, 6, 9, 16, and 20. N. Effect plot for orbital diameter residuals at LG 3. O. Effect plot for orbital diameter residuals at LG 6. P. Effect plot for orbital diameter residuals at LG 9. Q. Effect plot for orbital diameter residuals at LG 16. R. Effect plot for orbital diameter residuals at LG 20. For D,F,H,J,L,N-R, CC stands for homozygous cave genotypes, SS for homozygous surface genotypes, and SC for heterozygous genotypes. For C,E,G,I,K,M, red shaded area indicates the 95% confidence interval.

Figure 3.6, cont.



surface/Pachón cross. Pachón LG 6 displayed synteny with Tinaja LG 11, and Pachón LG 17 displayed synteny with Tinaja LG 13 (Figures 3.7A and B). In addition, Tinaja LG 24 showed synteny with Pachón LG 20 (Figure 3.8). None of these linkage groups shared QTL, even at a lenient p-value of 0.1. Thus, it appears that we identified distinct QTL, representing different loci regulating feeding angle in each cavefish population.

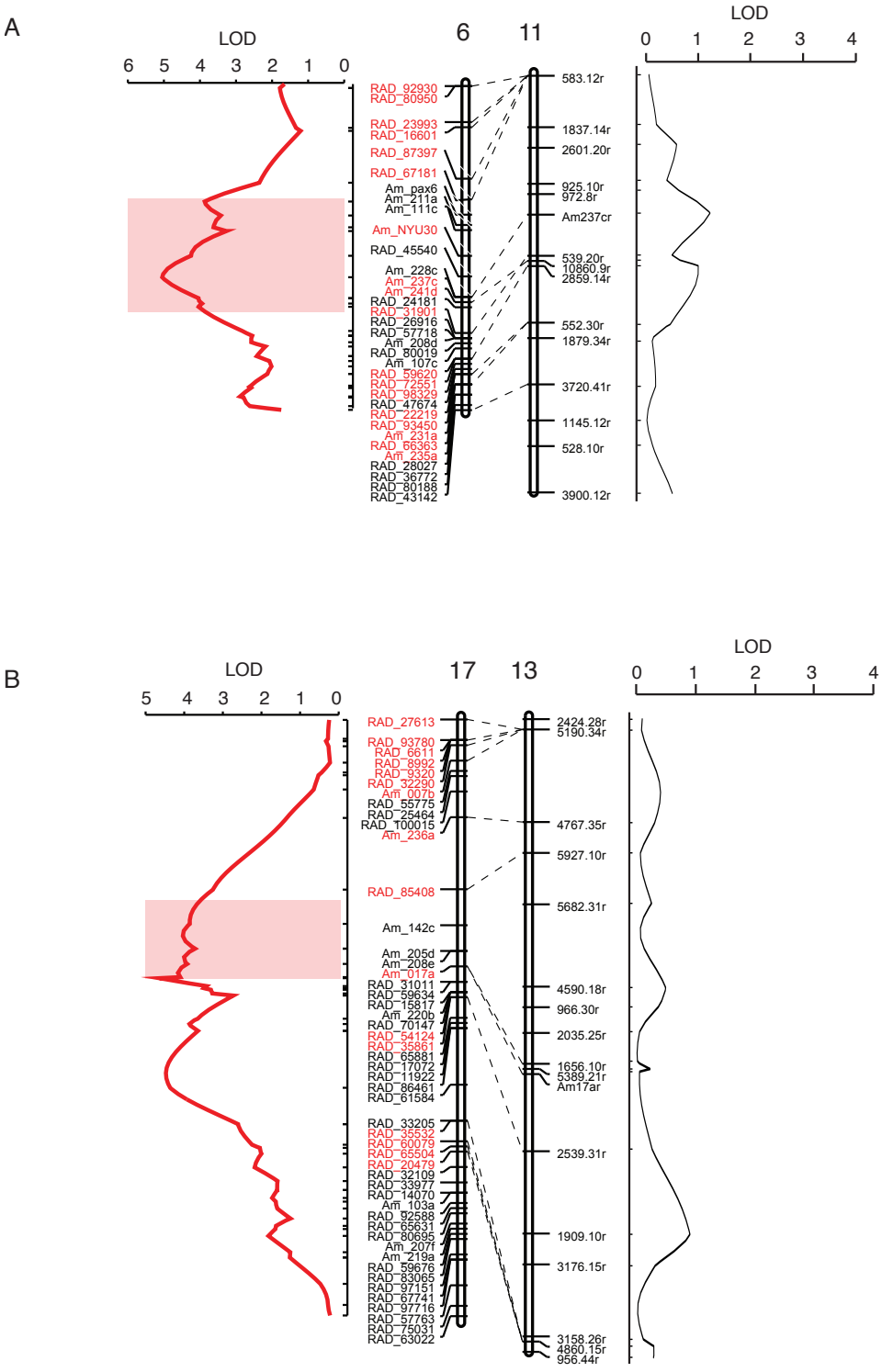
**Morphological trait QTL do not overlap with behavioral QTL**

We also mapped the morphological traits described above to determine if there was overlap with the behavioral QTL we identified. QTL mapping of jaw angle in the surface/Pachón cross resulted in one significant QTL on LG 3 at a p-value of 0.05 (Figure 3.5A-B). Both heterozygous and homozygous cave alleles resulted in a larger jaw angle (Figure 3.5C). QTL mapping of taste bud number in the surface/Tinaja cross resulted in two significant QTL, one for ventral taste bud number, and one for the number of taste buds on the lateral left side of the face (Figure 3.6A, E, G). The lateral taste bud QTL explained 10.9 percent of the variance of this trait, while the ventral taste bud QTL explained 11.6 percent of the variance (Table 3.1). Both of these QTL resulted in more taste buds in individuals with homozygous cave alleles, and an intermediate number of taste buds in heterozygous individuals (Figure 3.6F and H). Orbit diameter QTL mapped to two QTL on LG3 and LG6 using a one QTL model of mapping, and an additional 3 QTL on LGs 9,16, and 20 using a step-wise mapping method ($p<0.05$, Figure 3.6B

**Figure 3.7 Aligning Pachón and Tinaja linkage maps reveals a lack of overlap of the surface/Pachón feeding angle QTL regions with surface/Tinaja QTL.**

A. Feeding angle QTL on LG 6 from the surface/Pachón cross in red. Red area indicates the confidence interval. The LOD trace for feeding angle on the corresponding linkage group, LG 11, from the surface/Tinaja cross in black. B. Feeding angle QTL on LG 17 from the surface/Pachón cross in red. Red area indicates the confidence interval. The LOD trace for feeding angle on the corresponding linkage group, LG 13, from the surface/Tinaja cross in black. Red characters indicate genomic markers of the surface/Pachón cross, which were successfully anchored on the shared genomic scaffolds between the surface/Pachón and the surface/Tinaja crosses.

Figure 3.7, cont.



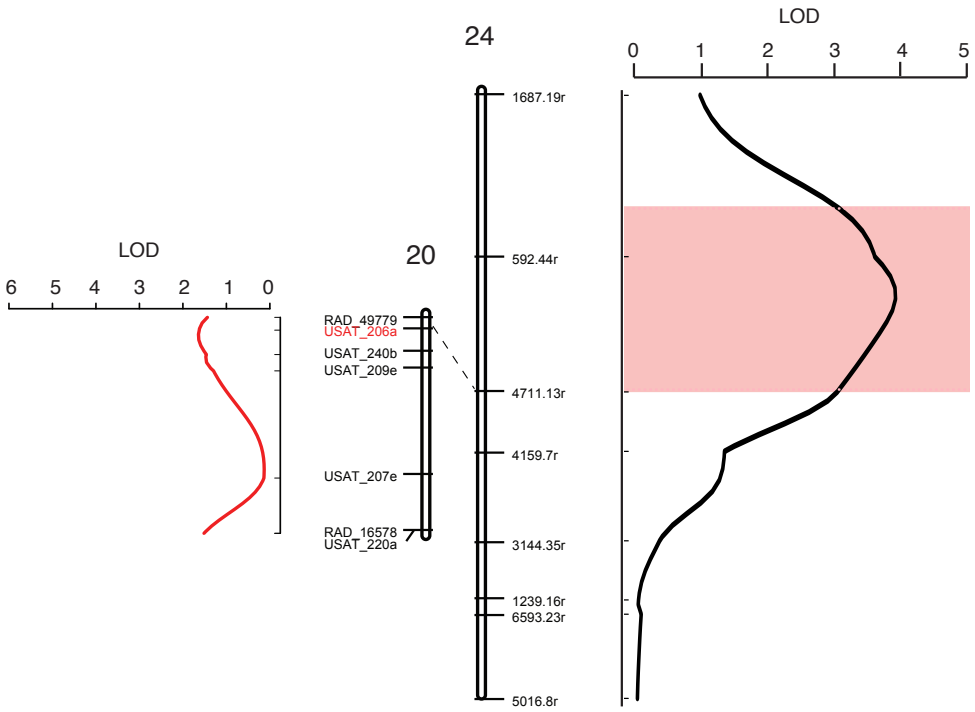



**Figure 3.8 Aligning Pachón and Tinaja linkage maps reveals a lack of overlap of the surface/Tinaja feeding angle QTL regions with the surface/ Pachón QTL.**

Feeding angle QTL on LG 24 from the surface/Tinaja cross in black. Red area indicates the confidence interval. The LOD trace for feeding angle on the corresponding linkage group, LG 20, from the surface/ Pachón cross in red.

and M). These QTL explained 7.8, 11.2, 7.0, 5.6, and 6.6 percent of the variance of orbit size, respectively (Table 3.1). Cave alleles resulted in smaller orbit size in three of the five of these QTL (Figure 3.6N-R). Jaw width mapped to one suggestive QTL on LG 7 that explained 7.2 percent of the variance in jaw width ($p<0.15$, Figure 3.6B and I, Table 3.1). Cave alleles resulted in a larger jaw width at this QTL (Figure 3.6J). Maxillary tooth number mapped to one suggestive QTL on LG 21 that explained 9.1 percent of the variance of this trait ($p<0.15$, Figure 3.6B and K, Table 3.1). Cave alleles of this QTL resulted in more maxillary teeth (Figure 3.6L).

We compared the QTL from the Tinaja cross with the morphological QTL described here and elsewhere ((Protas et al., 2007; Protas et al., 2008) and Figure 3.5A) from a Pachón cross. We found that the Tinaja taste bud and maxillary tooth QTL were not in the same regions as those in Pachón fish (Protas et al., 2007; Protas et al., 2008). We found that only one eye size QTL, the Tinaja LG 6 QTL for orbit diameter, was located at the syntenic region of the Pachón eye size QTL at LG 19 (Figure 3.5A and Figure 3.6L). This is consistent with the idea that Pachón and Tinaja fish share only a small number of eye loci (Borowsky, 2008).

None of the QTL for morphological traits mapped to the same location as the QTL for feeding angle in the surface/Tinaja cross, or to the locations of the QTL for feeding angle in the surface/Pachón cross (Table 3.1). While the one QTL for eye size maps to the same region as a QTL for feeding angle in the surface/Pachón cross, it is unlikely that eye size plays a large role in the

evolution of feeding angle in Pachón fish given the lack of correlation between these traits in the F2 fish from this cross (Figure 3.7, Figure 3.5A). These data suggest that evolutionary changes in cranial facial morphology and sensory systems are not responsible for the differences in feeding posture observed in some cavefish populations and surface fish.

## Discussion

We find that a similar feeding posture has evolved in multiple, independently evolved cave populations. F2 fish generated from crosses between surface fish and fish from two of the caves had a range of feeding postures, indicating that multiple genes control the evolution of this behavior. Furthermore, a complementation test indicates that at least some different loci control the reduction in feeding angle in these cave populations. These conclusions are consistent with QTL mapping, in which we find multiple QTL for feeding posture, and different loci in each cavefish cross. Last, we measure a variety of craniofacial morphological traits, and find that none of them correlate with feeding posture, and, moreover, that the majority of QTL for these traits do not fall in the same locations as feeding posture QTL, indicating a small, if any, contribution of morphological traits to the evolution of feeding posture.

**Convergence in feeding posture in two cave populations is controlled by at least some different genetic loci**

QTL analysis indicates that evolution of feeding posture is controlled by multiple genetic loci, which are not shared between Pachón and Tinaja cave populations. Consistent with this, hybrid individuals in a Pachón/Tinaja cross have an intermediate phenotype, significantly different from the Pachón parental population.

It is likely, however, that there are loci that are the same between the two cave populations, which result in Pachón/Tinaja hybrid fish with a low feeding angle. We may not have identified loci that are similar between these populations in our QTL mapping because they control only a small amount of the variance of this trait. With more individuals one might identify overlapping genetic loci and hence detect direct evidence for parallel evolution of this trait.

**Feeding posture is not controlled solely by evolution of morphological traits**

There is evidence in *Astyanax mexicanus* that some morphological traits evolved through changes in the same genes in multiple cave populations (Protas et al., 2006; Gross et al., 2009). In addition, there is evidence for co-evolution of behavioral and morphological traits through the same genetic loci, for example, neuromast number and vibration response (Yoshizawa et al., 2012b). Morphological changes can, in principle, affect behavioral traits, either because morphological traits are themselves important for the behavior, or through pleiotropic effects. However, in the case of feeding posture we did not find evidence for a large effect of morphological traits, such as altered craniofacial morphology and distribution

and number of taste buds, either through correlations in F2 fish or from QTL mapping.

We did find overlapping QTL for eye size and feeding posture in the surface/Pachón cross. While this may be the result of a single loci that contributed to the evolution of both feeding posture and eye size, loss of the eyes in cavefish alone cannot explain the change in feeding posture we observe here. First, the feeding posture QTL located at the eye size QTL explains a small amount of the variation in this trait. Second, we see no correlation or overlap with orbit QTL in feeding posture in the surface/Tinaja cross. Furthermore, Molino cavefish also have lost eyes, yet feed similarly to surface fish. In addition to eye size, many of the morphological traits we quantified were shared between all three of the cave populations we studied. This provides additional evidence that these morphological traits are not, in and of themselves, sufficient to alter feeding posture. While it is possible that other morphological changes, not quantified here, played a role in the evolution of feeding posture, the most parsimonious model is that feeding angle evolved through changes in the nervous system itself.

**Evidence for adaptive nature of feeding posture in the cave environment**

Two of the three independently evolved cave populations we tested fed at a lower angle compared to surface fish. The Molino cave population, which displayed a feeding posture similar to surface fish, is an evolutionarily young population of cavefish (Bradic et al., 2012), which retains some surface-like traits that have subsequently been lost in older cave populations, as shown

here and previously (Espinasa et al., 2005). Some ecological factors, such as possible differences in feeding requirements, may play a role in preventing the evolution of feeding posture. However, if there is an adaptive advantage for an altered feeding posture, Molino fish may not have had enough time to evolve this posture.

Although Pachón and Tinaja fish evolved independently, they have converged on similar feeding postures. Moreover, this behavioral change does not appear to be a mere pleiotropic consequence of morphological alterations that arise for some other reason. Together, this evidence suggests some adaptive reason to evolve feeding posture.

Cavefish must forage in caves, many of which have a limited amount of food. Furthermore, they must identify food in an environment independent of sight. That they have successfully adapted to these challenges is indicated by experiments where surface fish are reintroduced to cave conditions. These experiments indicate that cavefish are better at finding food in the dark than surface fish (Hüppop, 1987). In addition, surface fish found trapped in the cave show signs of starvation (Mitchell et al., 1977). Therefore, it is likely that an altered feeding posture evolved as a result of selection for improved foraging. For example, tactile cues resulting from more contact with the substrate may aid in successful foraging in the dark. Other behavioral and morphological traits have been implicated in the evolution of foraging and finding food in the dark (Yoshizawa et al., 2010; Elipot et al., 2013).

Together, these highlight the complexity of the evolutionary response of *Astyanax mexicanus* to the entrapment in the extreme cave environment.

## Materials and methods

### Animal Care

All animal procedures were in accordance with the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee at Harvard Medical School or the University of Maryland Animal Care and Use Committee.

### Crosses

Female Tinaja cavefish were crossed to male surface fish to generate two families of F1 fish. F1 hybrid fish from one Tinaja and one surface parent were interbred to generate a large population of F2 fish for phenotypic analysis and mapping.

For the Pachón cross, we generated all hybrid progeny from an original mating between one pair of cavefish and surface fish (Jeffery and Yamamoto, 2000; Yoshizawa and Jeffery, 2008; Yamamoto et al., 2009; Yoshizawa et al., 2010; Yoshizawa et al., 2012a; Yoshizawa et al., 2012b). One pair of F1 hybrids from this cross was mated to generate 384 F2 individuals for phenotypic and genetic analysis.

### Feeding posture assays

Feeding posture was tested by placing individual fish into tanks and starving them for six days. Fish were placed in the dark on the sixth night,

and assayed on the seventh day. For the assay, fish were placed on the filming platform, allowed to acclimate for 3 minutes, and filmed for 2 minutes (surface/Pachón fish) or 3 minutes (surface/Tinaja fish). Fish were fed one piece of food once filming had begun, and fish that fed less than 3 times in a trial were retested. Fish that were tested for multiple trials were given at least 1 week to recover between trials. Fish were either filmed from above (surface/Pachón fish) or from the side (surface/Tinaja fish), and were visualized by utilizing infrared illumination.

For fish filmed from above, feeding angles were calculated with the following equation:

$$\text{feeding angle} = \cos^{-1}\frac{FdL}{SL}$$

where FdL is the fish length when fish struck the food and SL is the standard length. The assay was duplicated if the fish showed more than 40˚ of angle differences among their strikes per assay. Standard length (SL) was measured by immersion in ice-cold 66.7 mg/ml Ethyl 3-aminobenzoate methanesulfonate salt (MS222, Sigma) in conditioned water and imaged on the left side using a SteREO Discovery V.20 with Achromat S lens (0.3 ×) equipped with AxioCam HRc (Zeiss, Göttingen, Germany). When imaged, each fish standard length was determined by digitally measuring the length of the body from the tip of the snout to the base of the caudal fin using AxioVision software (Release 4.8.2, Zeiss).

For measurements from the side, the angle of feeding posture was measured manually using software developed in LabVIEW (National

Instruments). Angle was measured during the highest angle feed from each feeding bout, and from the side by drawing a line through the head to the nose, and measured relative to the bottom of the tank. SL was measured immediately after the first trial using a ruler.

All fish were measured once for feeding posture, and the median feeding angle is reported, with the exception of the F2 fish from the surface/Tinaja cross, which were assayed 3 times to obtain appropriate reproducibility. The feeding angle for each trial was averaged across all three trials for these fish only.

Fish were fed either beef liver food (beef liver, carrots, spinach, oatmeal, and water, homogenized and frozen) or ground brine shrimp flake (O.S.I. marine lab, Burlingame, CA) mixed into 40˚C preheated and liquidized 1% agar (Sigma-Aldrich, St. Louis, MO) in fish conditioned water (pH 6.8; conductivity approximately 600 mS), and then poured into 35 mm dish (Fisher Scientific, Pittsburg, PA). After the solidification of the food, it was cut into small pieces (approximately 5 × 5 × 5 mm cubes).

**Quantification of morphological traits**

Sacrificed fish were antibody stained to detect taste buds, and then stained to image the skeletons. For anti-calretinin antibody staining, fish were placed into 4% paraformaldehyde for 2 hours at room temperature. They were washed 5 times with PBS + 1% triton, and then moved into block (PBST + 5% HINGS) for one hour, and then incubated in antibody (monoclonal anti-calretinin, SWANT) overnight for 2 nights at room temperature while rocking.

The following day fish were placed in 5 1-hour washes in PBST, and then placed into secondary overnight, rocking at room temperature. After imaging, fish were placed into formaldehyde. After fixing, fish were placed into alizarin red in 1% KOH to stain their skeletons, washed in 1% KOH, and imaged in 25% glycerol.

Taste bud quantification and skeletal measurements were performed in Fiji (Schindelin et al., 2012). Taste buds were quantified from the dorsal and ventral sides of the head, as well as laterally on each lip. Orbital size was measured as the diameter of the orbit. Jaw width was measured ventrally at the posterior tip of the dentary bone. Maxillary tooth number was the number of teeth on both maxillary bones. Taste bud number, orbital size, and jaw width were all corrected for size of the fish using the distance between the pectoral fin and the tip of the fish nose as a proxy for fish length. The measurements were regressed against nose to pectoral length, and the residuals from this line were calculated and used as the trait value for each of these traits. Residuals were calculated by pooling all fish.

Eye size was determined as previously described (Yoshizawa et al., 2012b) by measuring the diameter of each eyeball along its rostral-caudal axis. We standardized these measurements by dividing them by each fish's standard length.

**Statistics**

When groups of 3 or more were compared, a one-way ANOVA analysis was performed, followed by post hoc Games-Howell tests to obtain

pairwise comparisons. A Pearson correlation coefficient was calculated to compare feeding angle to the morphological traits. All statistics were performed in R (Ihaka, 1996) or in IBM SPSS 20.0.0 software (IBM, Somers, NY, USA).

**Genotyping and QTL analysis**

Tinaja cross

Fish from the surface/Tinaja cross were genotyped for SNPs and a linkage map was built as described previously (Kowalko et al. (submitted)). 46 additional individuals were added to this analysis. In addition, microsatellites from the surface/Pachon cross were genotyped in the surface/Tinaja cross by identifying size differences on a gel after PCR. These markers were added to the map in R/qtl (Broman et al., 2003), which resolved two separate linkage groups from the previous analysis into one.

Pachón cross

We isolated genomic DNA from fin-clips using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) or the quick extraction protocol (Nusslein-Volhard and Dahm, 202). Genotyping methods were as described previously (Yoshizawa et al., 2012b; O'Quin et al., 2013). Briefly, we genotyped 382 $F_2$ fish for 235 genome-wide polymorphic microsatellite loci and 117 of 382 $F_2$ fish for a conservative set of 463 single nucleotide polymorphisms (SNPs) identified by sequenced restriction-site associated DNA tags (RAD-seq) method. We performed genetic linkage in the program R/qtl (Broman et al., 2003) following the protocols described in Broman and Sen (Broman et al., 2003; Yoshizawa

et al., 2012b; O'Quin et al., 2013). Briefly, for genetic linkage mapping, we grouped the RAD-seq and microsatellite markers into linkage groups by specifying a maximum recombination distance of 0.35 and minimum LOD threshold of 6. We then ordered markers along each linkage group using "ripple" function of R/qtl or by manually, and then estimated genetic distances using the Kosambi map function.

For both crosses, following linkage mapping, we scanned the genome for QTL associated with phenotypes in R/qtl by using both the scanone function, and using stepwiseqtl, a model selection algorithm for multiple QTL mapping (Broman et al., 2003; Manichaikul et al., 2009). For the surface/Pachón cross, standard length and age at sampling (1 or 2 years old) were used as covariates for all of the traits mapped. For the surface/Tinaja cross, standard length and sex were used as covariates for mapping feeding angle, and no covariates were used for mapping the other traits, as effects of standard length had already been taken into account. We assessed the statistical significance of the resulting LOD scores by calculating the 95th percentile of genome-wide maximum penalized LOD scores for each trait using 1,200 random permutations of the genotypic and phenotypic data. We defined confidence intervals for the position of the final QTL using 95% Bayesian credible intervals.

**Alignment analysis**

To identify syntenic region between the linkage maps generated from surface/Pachón and surface/Tinaja crosses, we anchored Pachón linkage

map (O'Quin et al., 2013) to the latest Astyanax genome assembly (Assembly: Astyanax_mexicanus-1.0.2; BioProject: PRJNA89115 at NCBI Genbank). To do this, we first downloaded the assembly Astyanax_mexicanus-1.0.2 from the NCBI Genome resource (http://www.ncbi.nlm.nih.gov/genome, accessed at April 30, 2013). We then built searchable databases of this genome and searched the consensus sequences of each RAD-seq and microsatellite locus to these databases using the default parameters of the Burrows-Wheeler Aligner (BWA version 0.6.2-r126, (Li and Durbin, 2009)) the blastn option of the program blast v2.2.27+ (Camacho et al., 2009), respectively. We retained the top hit (96M for BWA, as for blastn, an expectation value (E) as 0.00001 or less), and used all anchored sites to identify regions of synteny between the maps from surface/Pachón and surface/Tinaja crosses.

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# Chapter 4

# Final Discussion

At the beginning of this dissertation, I raised a number of questions about how behaviors evolve. Using the cavefish *Astyanax mexicanus*, I have answered some of these questions. This work will add to the growing body of literature on the genetics of behavioral evolution and will play a role in supporting general theories of how behaviors evolve. Thus, it is worth discussing how this work answers these questions of evolutionary interest, as well as what remains to be learned about the evolution of schooling and feeding posture in *Astyanax mexicanus*.

*Do behaviors evolve through changes in one gene of large effect, or multiple genes of small effect?*

In both of the behaviors studied here, multiple QTL were identified. Additionally, all of these QTL are of small effect. This result is similar to what has been found for other behavioral QTL in natural populations; for example, QTL for burrowing behavior in *Peromyscus* (Weber et al., 2013) and QTL for vibration attraction behavior in *Astyanax* (Yoshizawa et al., 2012), as well as the majority of QTL for behavior found in laboratory mice and rats (reviewed in Bendesky and Bargmann, 2011). However, it is also possible for natural variation in one gene to have a large effect on behavior in natural populations. For example, different alleles of the *foraging* gene in *Drosophila* affect feeding behavior and metabolism in both larvae and adults (Sokolowski, 1980; Pereira and Sololowski, 1993; Kent et al., 2009).

It is interesting to speculate that the complexity of the behavior may play a role in the number of genes involved in natural variation of the behavior. For instance, if schooling behavior could be parsed into discrete behavioral components would we then find one gene of large effect modulating each of these behavioral components? Modeling of collective behaviors, including schooling and shoaling in fish, suggests that large group patterns can be explained by a relatively simple set of rules followed by each individual (reviewed in Couzin and Krause, 2003; Sumpter, 2006). If individuals are given three rules to obey in relation to the individuals closest to them (to want to be closer to other individuals until they are too close, to be repulsed at closer distances, and to want to orient to neighboring individuals) group patterns including schooling and shoaling emerge (Couzin et al., 2002). These parameters have been determined for actual groups of fish by observing and measuring individuals in groups (Katz et al., 2011). It may be possible, therefore, to determine these rules for surface and cavefish, and then measure and QTL map additional, potentially less complex parameters for schooling behavior in F2 fish. This parsing of a complex behavior may lead to identification of genes of larger effect modifying this behavior. Alternatively, the same set of genes may govern different aspects of the same behavior. Thus, in addition to effect size of individual genes, it would be informative to know if the same or different genes and loci play a role in regulating different parameters involved in schooling behavior.

*What are the contributions of the evolution of morphological traits, such as sensory systems, to the evolution of behavioral traits?*

In this dissertation, I found that the visual system plays an important role in schooling behavior and that a locus responsible for loss of schooling may also play a role in the visual system. That evolving sensory systems have an effect on behavior has been shown elsewhere. An enhanced lateral line in cave *Astyanax mexicanus* plays a role in the evolution of vibration attraction behavior (Yoshizawa et al., 2010, 2012). The importance of sensory system evolution in the evolution of behaviors might be a universal principle. It has been argued that sensory system genes may be preferred targets for natural selection on behavioral adaptations (Bendesky and Bargmann, 2011).

The drastic differences in morphology between cave and surface fish make *Astyanax mexicanus* an excellent system to study the contributions of morphological traits to behavioral evolution. However, this question is of interest in organisms beyond cavefish. Understanding how an organism senses its environment, and then how these environmental cues are processed, is essential for the study of any behavior. Studying the contributions of the evolution of sensory systems to behavioral evolution is another way of determining how organisms sense their environment and process this sensory information into behavioral outputs.

Furthermore, studying behavioral evolution in the context of evolving morphological traits may aid in finding the genetic changes responsible for

evolution of both behavioral and morphological traits. For example, a single locus that affects both a behavior and a sensory system trait may result from the gene responsible for the locus acting in that sensory system, or in regions of the brain responsible for processing information from that sensory system. This information could provide clues as to the identity of the gene underlying behavioral evolution, as well as to how the gene is functioning. For example, in the case of the schooling QTL that overlaps with the dark preference QTL, we can first look for candidate genes that are involved in visual processing, as changes in these genes could affect both of these traits.

Finding that behaviors rely on the evolution of morphological traits, or contribute to the evolution of these morphologies, raises interesting questions about how this occurs during evolution. If, for example, a change in a behavioral trait requires a change in morphology, this morphological change would have to occur before or at the same time as the behavioral change. Another interesting question is what it means when QTL for a sensory system change and a behavioral change overlap. If the QTL overlap due to changes in the same gene affecting both of these traits, this could be due to the requirement for the sensory system modification for the behavioral change, or because the same genetic change affects both of these processes separately. If the dark-preference QTL identified here does indeed modify the visual system, then it is possible that the genetic change that affects the visual system affects schooling behavior only secondarily, as a result of visual system changes. This would be consistent with the observation that surface

fish do not school when they no longer have the capacity to see. However, further work needs to be done, to first identify this genetic change, and then to test it functionally, to determine the meaning of the overlapping QTL.

*When a behavior evolves multiple times, does it evolve through the same genes?*

Convergence occurs when two populations evolve a similar trait independently. Convergence can occur through changes in the same gene, or through changes in different genes that result in the same phenotype. For example, in the evolution of coloration, similar morphologies evolve through changes in different genes as well as through changes in the same gene (reviewed in Manceau et al., 2010). In *Astyanax mexicanus* cavefish, reduction in pigmentation and albinism have evolved independently through changes in the same gene in multiple cave populations (Protas et al., 2006; Gross et al., 2009), but reduction of the eyes has occurred through at least some different genetic changes (Wilkens and Strecker, 2003; Borowsky, 2008).

Convergence in behavioral traits is of particular interest, since apparently similar behaviors evolve over and over again. For example, while the majority of mammals are polygamous, monogamy has likely evolved in mammals independently multiple times (Turner et al., 2010). In voles, male pair bonding, a component of monogamy, is controlled by changes in expression in the arginine vasopressin 1a receptor (Winslow et al. 1993; Lui

et al. 2001; reviewed in (Donaldson and Young, 2008)). However, some species of monogamous *Peromyscus* do not show changes in expression of this receptor, correlations between microsatellite number variation and mating system, nor do they have coding differences between monogamous and polygamous populations that change the receptors signaling activity, making it unlikely that monogamous *Peromyscus* evolved this behavior through the same gene as voles (Turner et al., 2010). Furthermore, variation in the promoter of this locus cannot explain variation in mating systems across all mammals (Fink et al., 2006). In other cases, variation in one gene is responsible for differences in behavior across species. An example is the *foraging* gene identified in *Drosophila*. Natural variation in this gene is responsible for variation in behavior in both flies and nematodes (reviewed in Bendesky and Bargmann, 2011).

Convergence of feeding posture has evolved in multiple, independently evolved populations of *Astyanax mexicanus* cavefish (Schemmel, 1980). We found that two of these populations appear to have evolved similar feeding postures through at least some independent genetic changes. It will be interesting to identify the actual genetic changes responsible for feeding posture in each of these populations in the future and to determine how each is acting to modify this behavior. This will allow us to determine exactly how different genes and genetic changes can produce similar behavioral changes. For instance, are these mutations acting on genes in the same biochemical

pathway or a common genetic network? Are they active in the same populations of neurons, but in different ways?

We also found that schooling behavior is lost in multiple, independently evolved populations of cavefish. This result is unsurprising in the context of convergence on similar morphological traits, in this case, loss of eyes. Future work identifying the location of loci important for loss of schooling in other cavefish populations will be necessary to find out if the same or different genes are responsible for reduction in schooling behavior in these populations. Because regression of eyes was lost by at least some different genetic changes in these populations (Wilkens and Strecker, 2003; Borowsky, 2008), it is likely that the visual system dependent loss of schooling behavior also occurred through changes in different loci. It would be of particular interest, therefore, to find out if the Molino and Pachón cavefish populations have a visual system independent loss of schooling behavior, and, if so, if this component of the behavior is controlled by the same gene or genes as in the Tinaja population.

Schooling behavior has been lost in other, more distantly related fish as well, such as benthic sticklebacks. Comparing Pachón and Tinaja linkage groups to the early version of the *Astyanax mexicanus* genome as described in Chapter 3 allowed me to take advantage of the existing, more extensive synteny maps created between Pachón linkage maps and the zebrafish genome (Gross et al., 2008; O'Quin et al., 2013). Interestingly, a marker from Tinaja LG 27, which contains one of the QTL for schooling, maps to Pachón

LG 2. This linkage group is syntenic with the zebrafish genomic region containing Ectodysplasin (Masato Yoshizawa, personal communication), a gene that falls under a QTL for reduction of schooling in benthic sticklebacks. The current state of the *Astyanax mexicanus* genome, along with a relatively sparse linkage map, unfortunately, did not allow us to confirm this result (Jessica Lehoczky, unpublished results). Once the *Astyanax mexicanus* genome is fully assembled and annotated, it should be straightforward to test the hypothesis that the QTL for reduction in schooling in these two species share some of the same genes.

*What is the contribution of natural selection to behavioral evolution?*

The longstanding debate about evolution of morphological traits in cave organisms – whether they evolve traits through neutral mutation, because they no longer require the surface trait, or through natural selection, because it is advantageous to lose the surface trait or gain a cave trait within the cave, can also be applied to the evolution of behavior. For the traits studied here, it is possible that both situations occurred. Once within the caves, fish could no longer school without visual cues in the dark, and no longer needed schooling behavior to avoid predators. Therefore, loss of schooling behavior could have evolved through an accumulation of mutations affecting a trait that was no longer necessary. Alternatively, the behavior could be adaptive, if there is better allocation of scarce food resources in the absence of aggregation.

A lower feeding posture may be adaptive in the cave. Multiple cave populations evolved feeding posture independently, and there is no evidence that feeding posture evolved secondarily to candidate morphological traits. Furthermore, feeding posture may be important for foraging behavior. For example, feeding at a lower angle may increase the tactile cues a cavefish has for locating food along the ground. On the other hand, it is possible that feeding posture arose by genetic drift, due to a lack of selection to maintain a higher feeding posture. For example, a high feeding posture in surface fish may be less likely to expose the fish to predators while feeding. Within the cave, a lack of predators would release selective pressures to maintain a higher feeding posture. In order to test whether a lower feeding posture is adaptive in the cave, future work is needed to determine if having a lower feeding posture does indeed give fish an advantage when trying to find food in the dark. Similar experiments could be performed to those which found that cavefish are more successful at finding food in the dark than surface fish (Hüppop, 1987), by feeding groups of high and low feeding posture F2s in the dark, and determining which are more successful at obtaining food. Initial experiments performed by an undergraduate in the laboratory, Tess Linden, did not reveal significant differences between these populations. However, in these experiments, we did not control for differences in other morphological traits, such as number of neuromasts, which likely play a role in the ability to find food in the dark. Controlling for morphological traits, along with increasing the number of groups tested, could reveal differences in food

finding ability based on feeding posture. Additionally, identification of the genes underlying the QTL for these traits would allow for tests of signatures of selection at these locations, which would provide further evidence for the adaptive or non-adaptive nature of these traits.

*What are the developmental and genetic constraints on behavioral evolution?*

Constraints on behavioral evolution will limit the number of possible behaviors existing in a population of animals that can be used to respond to a new environmental challenge, in this case, the cave environment. There are genetic constraints, for example, the genetic underpinnings of the behavioral repertoire in the current, invasive population, which have been influenced by the past adaptations to the old environment. This history of adaptation will have limited the potential behavioral responses available to the current population (Kappeler et al., 2013). Other constraints may be developmental. For example, the overrepresentation of neurotransmitters and their receptors identified in cases where behaviors vary naturally could be due to developmental constraints on rewiring neural circuitry, such that changes in behavior are more likely to occur through changes in expression of neuromodulators, rather than changes in neural circuitry (Bendesky and Bargmann, 2011). Pleiotropy could act as an additional constraint on evolution of behaviors. Cavefish may lose their eyes because the same genes responsible for eye degradation result in an increase in constructive traits, and changes to more adaptive behaviors (Protas et al., 2007;

Yamamoto et al., 2009; Yoshizawa et al., 2012). The genes responsible for the evolution of behavioral traits would also have to either have few or no pleiotropic effects, or have effects that are neutral or outweighed by the benefits of evolving an adaptive behavior.

To fully understand the constraints on the evolution of schooling and feeding behaviors, we would need to have a much more extensive knowledge of the genes underlying these behaviors, of which genes are responsible for the evolutionary change, and of how these genes function during development. However, our analysis of morphological traits has given us some insight into constraints on the evolution of these behaviors. First, we did not identify morphological differences between cave and surface fish that constrained feeding posture. It is possible, however, that other morphological traits not studied here, such as the shape of profile of the face, constrain this behavior (see Appendix 2). Further investigation into the contribution of these morphological traits is needed to fully understand how this behavior evolved. Finally, we found that the visual system plays a large role in the evolution of schooling behavior. Surface fish do not school in the dark. Therefore, it is likely that not having a functional visual system, first because it was dark, and secondarily because of the regression of the eyes, played a large role in constraining social behavior in cave *Astyanax mexicanus*, and was a constraint on behavioral evolution.

## Summary

In this dissertation, I use the Mexican cavefish, *Astyanax mexicanus*, to explore the evolution of two behaviors. I found that both schooling and shoaling behaviors were lost in multiple cave populations of *Astyanax mexicanus.* Surface *Astyanax* cannot school in the dark or when they are blinded during development, underscoring the importance of a functional visual system for schooling behavior in this species. Reduction of the visual system plays a large role in the evolutionary loss of schooling behavior in cave *Astyanax*. While the lateral line may play a small role in the loss of schooling behavior, ablation of the lateral line does not have a large effect on schooling behavior in surface *Astyanax*. QTL analysis revealed that the loss of schooling behavior is complex, occurring through multiple genetic loci. Furthermore, QTL analysis revealed both visual system-dependent, and visual system-independent contributions to loss of schooling behavior.

Feeding posture has also been modified in multiple populations of cavefish. The candidate morphological traits examined here do not appear to have played a large role in the evolution of feeding posture. The evolution of this trait is also controlled by multiple genetic loci. At least some of these loci appear to be different between two independently evolved populations of cavefish that have evolved similar feeding postures.

Together, this work has provided insight into how behavioral traits evolve, elucidating the genetic architecture underlying behavioral evolution, and providing insight into the contributions of evolving morphological traits to the evolution of behavioral traits.

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# Appendix 1

# QTL mapping of neuromast number and size

# QTL mapping of neuromast number and size

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This appendix contains material that will be contributed to a manuscript currently in preparation by Dr. Joshua Gross. This manuscript will include QTL mapping data collected by Dr. Gross's laboratory for neuromasts and taste buds in a surface/Pachón cross.

## Results and Discussion

Multiple, independently evolved populations of cavefish (Mitchell et al., 1977; Dowling et al., 2002; Strecker et al., 2003; Strecker et al., 2004; Bradic et al., 2012) have an increased number of neuromasts relative to surface fish (Kruskal-Wallis: $H_4$=57.2, p<0.001; Mann-Whitney compared to surface: Tinaja: U<0.001, z=-5.5, p<0.001; Pachón: U<0.001, z=-4.1, p<0.001; Molino: U<0.001, z=-4.8, p<0.001; Surface: n=21, surface/Tinaja F1s: n=7, Tinaja: n=21, Pachón: n=8, Molino: n=13; Figure A1.1A). Furthermore, Molino cavefish have significantly fewer neuromasts than Tinaja (U=51, z=-3, p<0.05) and Pachón (U=4, z=-3.5, p<0.001) cavefish. Tinaja cavefish also have significantly larger neuromasts compared to surface fish ($t_{30}$=14.7, p<0.001; Surface: n=9, Tinaja: n=7; Figure A1.1B).

QTL mapping of neuromast number results in three significant QTL at a p<0.05 (Figure A1.2A). The QTL at LG 18 explained 14.2 percent of the variance, and a homozygous cave genotype at this locus results in an increase in the number of neuromasts relative to a homozygous surface genotype (Figure A1.2C). The QTL at LG 10 and 24 explained 7.6 and 3 percent of the variance, respectively, and a homozygous cave genotype at either of these loci results in decreased numbers of neuromasts relative to the homozygous surface genotype (Figures A1.2B and D). QTL mapping of neuromast size results in one significant QTL on LG 10 at a p<0.05 (Figure A1.2A). This QTL accounts for 11.2 percent of the variance of the trait, and a

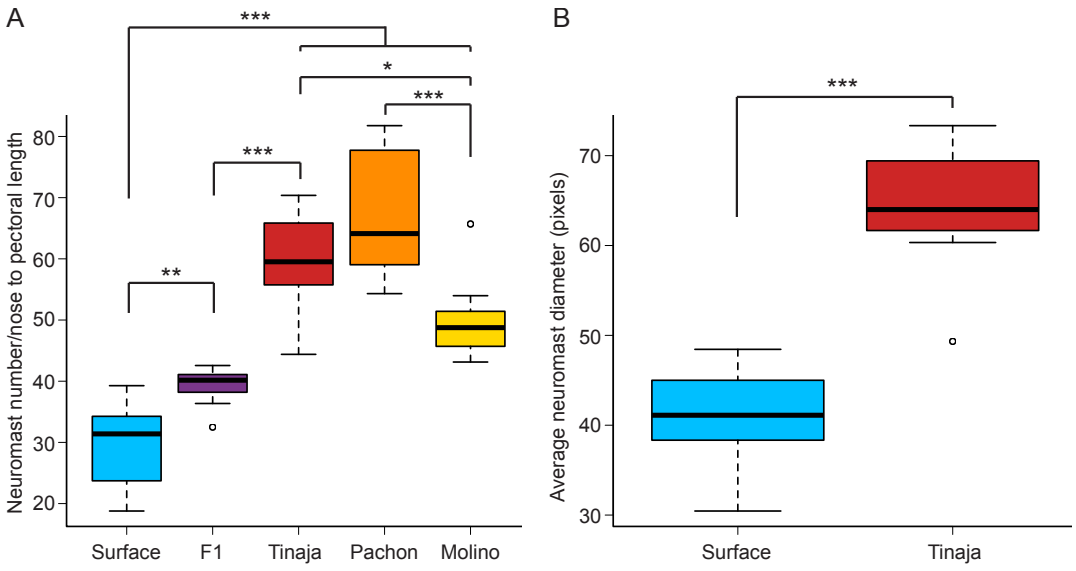


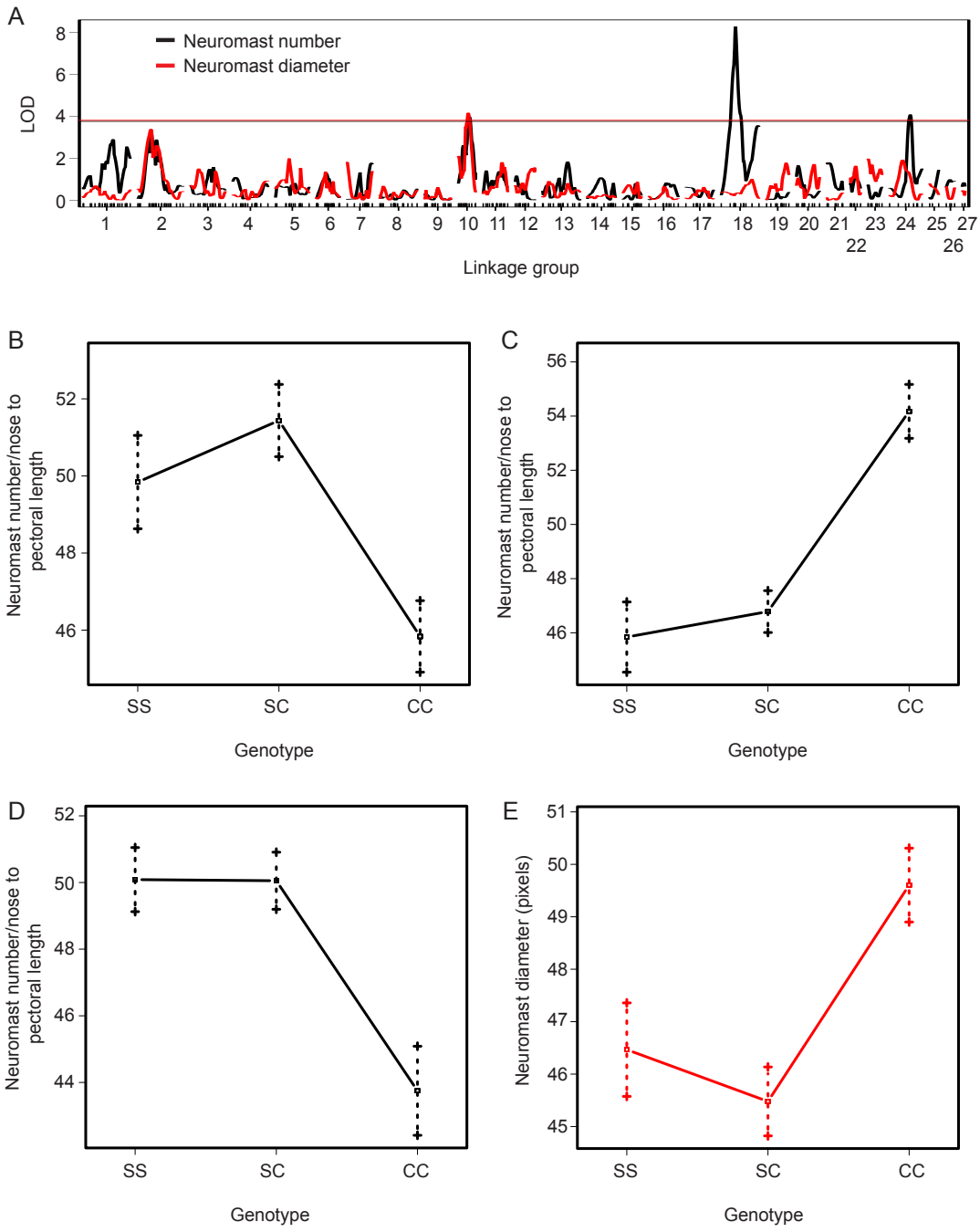

**Figure A1.1. Cavefish have an increased number and size of neuromasts relative to surface fish.**

A. Number of neuromasts corrected for nose to pectoral length in surface fish (n=21), surface/Tinaja F1 hybrid fish (n=7), Tinaja (n=21), Pachon (n=8), and Molino (n=13) cavefish. B. Average neuromast diameter (in pixels) in surface fish (n=9) and Tinaja cavefish (n=7). *p<0.05, **p<0.01, ***p<0.001.

**Figure A1.2. QTL for neuromast number and diameter.**

A. QTL for neuromast number corrected for nose to pectoral length (black) and neuromast diameter (red). B. Neuromast number by genotype at a significant marker on LG 10. C. Neuromast number by genotype at a significant marker on LG 18. D. Neuromast number by genotype at a significant marker on LG 24. E. Neuromast diameter at a significant marker on LG 10. B-E. SS = homozygous surface, SC = heterozygous, CC = homozygous cave.

Figure A1.2, cont.



homozygous cave genotype at this locus results in larger neuromasts relative to the homozygous surface genotype (Figure A1.2E). This QTL overlaps with one of the neuromast number QTL, which may indicate that the same gene is responsible for both neuromast number and neuromast size.

The QTL for neuromasts do not overlap with the QTL for the behavioral traits presented earlier in this dissertation, schooling, which has QTL on LG 6 and LG 26, and feeding angle, which has one QTL on LG 24. Although the QTL for feeding angle and neuromasts are on the same linkage group, these QTL, and their confidence intervals, are not overlapping.

## Materials and Methods

Neuromast number and diameter were measured by staining each fish with DASPEI. Neuromast number is the number of cranial superficial neuromasts on the right side of the face, from the gill to the nose. Neuromast number was corrected for body length of the fish by dividing by the nose to pectoral length of each fish, which scales with body size. Neuromast diameter was measured for cranial superficial neuromasts in the suborbital region of the face. Neuromast diameter did not scale with body size, and was measured at the same magnification for each fish. Three neuromasts were measured for each fish, and the average diameter of these three neuromasts is reported.

QTL mapping was performed as described in Chapters 2 and 3. The linkage map used for this analysis was the linkage map in Chapter 3, which

had additional markers and individuals compared to the map in Chapter 2, and one less linkage group.

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# Appendix 2

# Morphometric analysis of cavefish craniofacial skeletons

# Morphometric analysis of cavefish craniofacial skeletons

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This appendix contains a preliminary morphometric analysis. Johanna Kowalko, Tess Linden, and Clifford Tabin designed the research. Tess Linden performed the research under the supervision of Johanna Kowalko. Craig Albertson contributed to the design of the analysis, and gave advice on setting up morphometric analysis. Johanna Kowalko wrote the text.

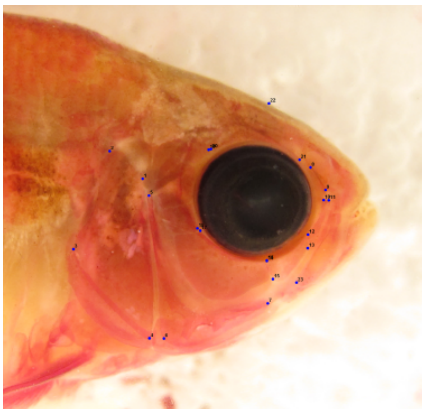
Chapter 3 of this dissertation contains linear measurements of different aspects of the cranial skeleton for comparison with feeding posture. In order to get a better idea of if changes in the form of the cranial skeleton, rather than just discrete linear measurements, contributed to evolution of feeding posture, we performed morphometric analysis of Tinaja cave, surface, and F2 fish.

## Results and Discussion

Landmarks that were shared between both Tinaja cavefish and surface fish on the lateral profile of the face (Figure A2.1A) and the ventral jaw (Figure A2.1B) were placed on Tinaja cavefish (n=26), surface fish (n=29) and F2 fish (n=187). Principal component analysis (PCA) was performed to determine the differences in shape. For the lateral analysis, cave and surface fish were significantly different along PC 1 ($p<0.001$) but not PC 2 (Figure A2.2). PC 1 explained 35.5 percent of the variance of the shape, and PC 2 explained 10.1 percent of the variance. All of the remaining principal components explained less than 10 percent of the variance. The ventral PCA analysis resulted in both PC 1 ($p<0.001$) and PC 2 ($p<0.001$) being significantly different between cave and surface fish (Figure A2.3). PC 1 explained the majority of the variance (84%) while PC 2 explained only 6 percent of the variance.

To determine if the shape of the face is associated with changes in feeding posture, we looked at the relationship between each major principal

A



B

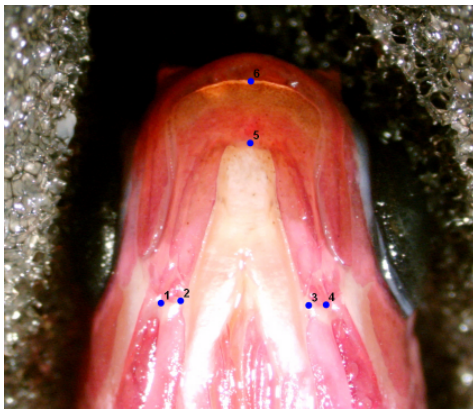



**Figure A2.1. Landmarks for morphometric analysis.**

A. Landmarks used for the lateral profile view. B. Landmarks used for the ventral jaw view.

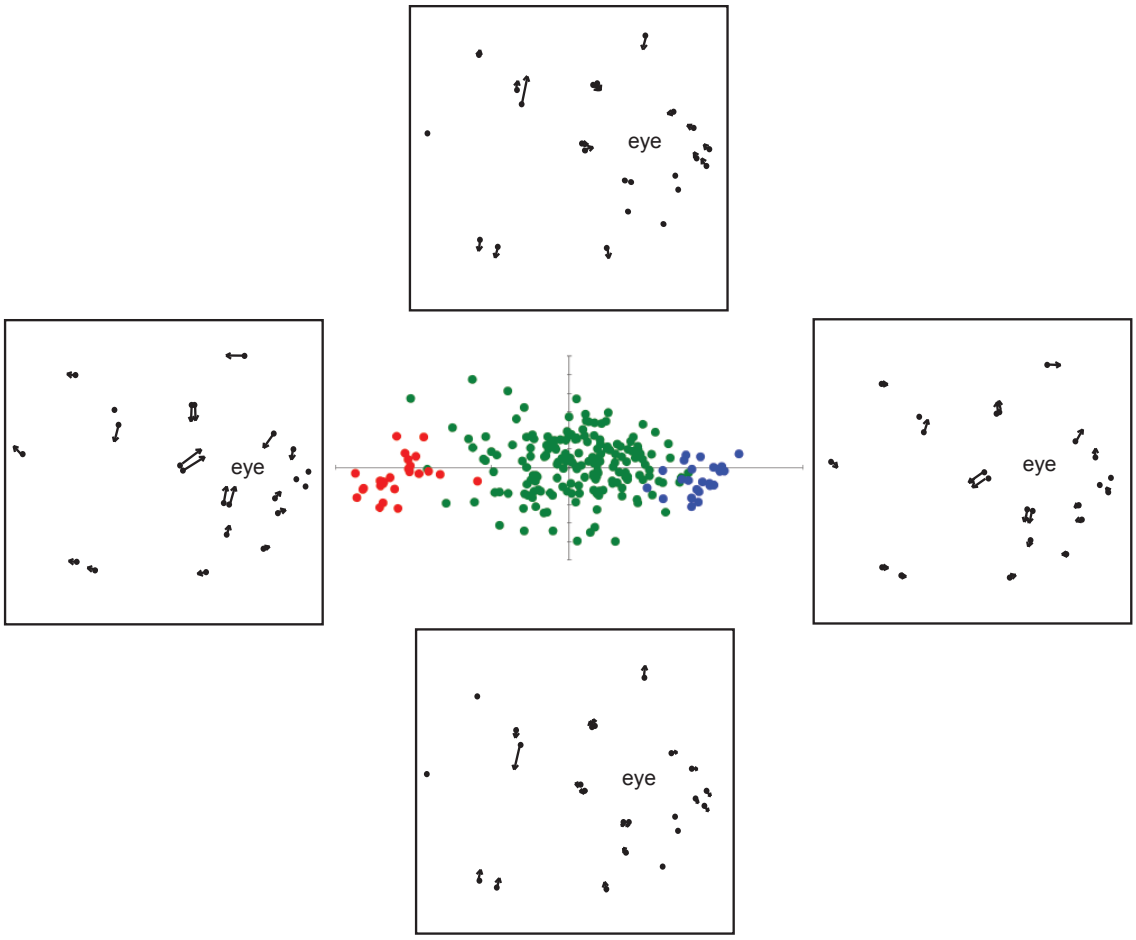



**Figure A2.2. Lateral PCA.**

Surface = blue, Tinaja = red, F2 = green. X-axis is PC 1 and Y-axis is PC 2.

Boxes are warps from the consensus for the extreme of each axis.

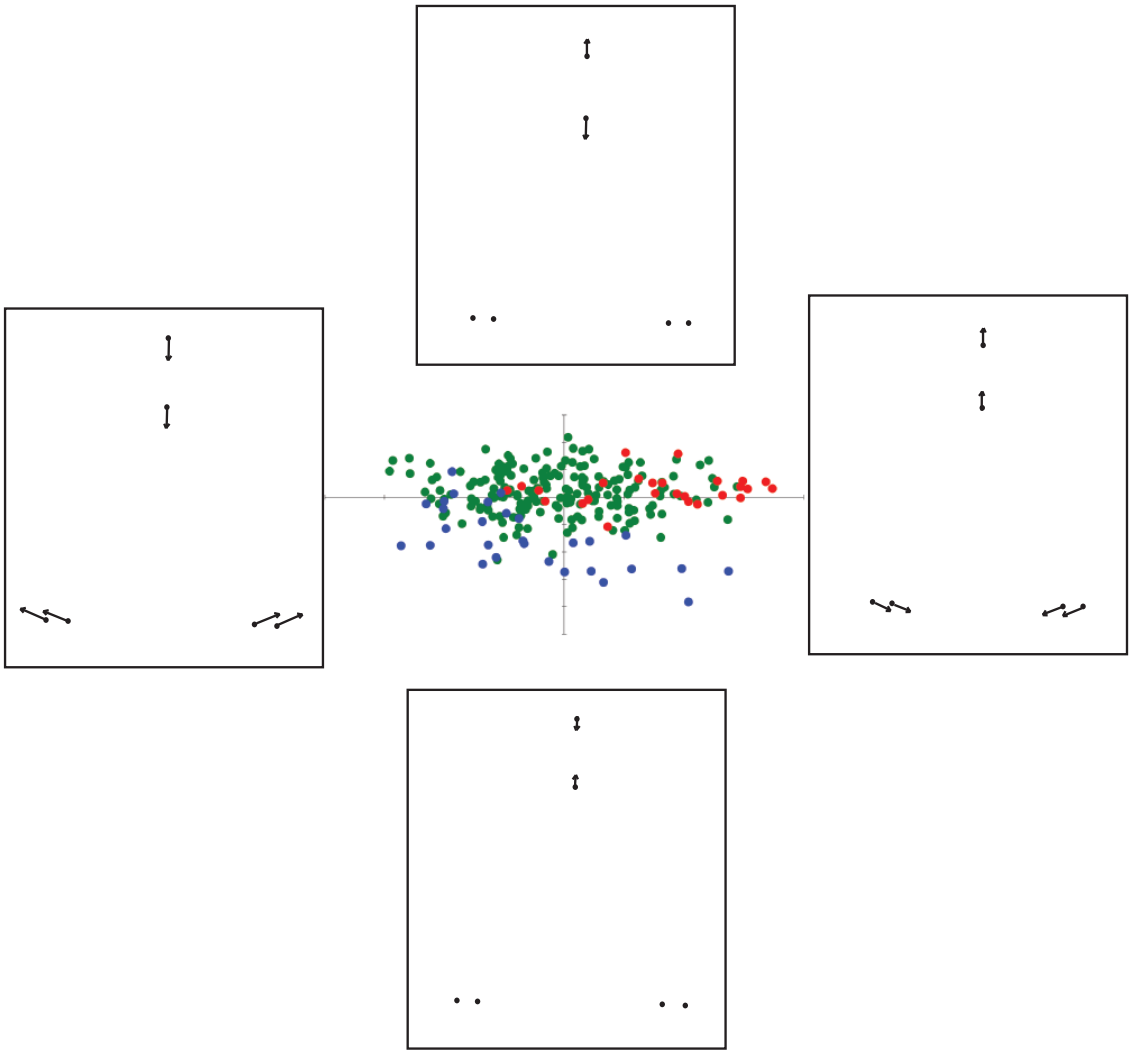


**Figure A2.3. Ventral PCA.**

Surface = blue, Tinaja = red, F2 = green. X-axis is PC 1 and Y-axis is PC 2.

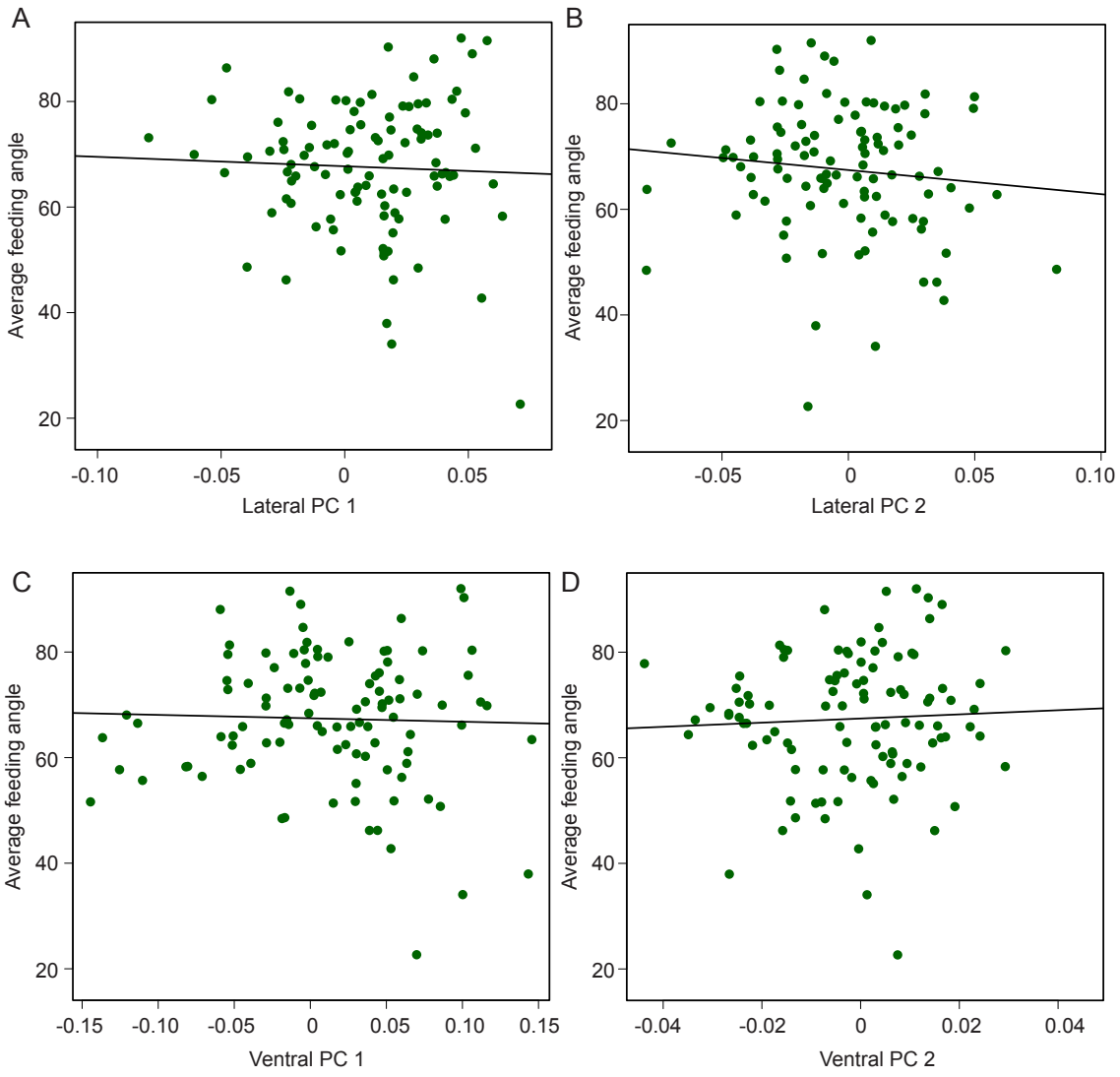



**Figure A2.4. Principal components do not correlate with feeding angle.**

A. Feeding angle compared to lateral PC 1 in F2 fish. B. Feeding angle compared to lateral PC 2 in F2 fish. Feeding angle compared to ventral PC 1 in F2 fish. Feeding angle compared to ventral PC 2 in F2 fish.

component and feeding posture in the F2 fish. Lateral PC 1 (R=-0.04, p=0.67) and PC 2 (R=-0.11, p=0.28) were not significantly correlated with feeding angle (Figures A2.3A and B). Ventral PC 1 (R=-0.03, p=0.75) and PC 2 (R=0.05, p=0.62) were also not significantly correlated with feeding angle (Figures A2.3C and D).

Last, we attempted to QTL map the principal components to determine the genetic basis of the shape changes in cavefish. Unfortunately, none of the principal components had QTL significant at a p-value of 0.05. For future analyses, we will try to add additional landmarks to the PCA analysis to try to gain information about the shape of the face. Additionally, we will include cavefish from the other caves in the analysis, to determine if cavefish which have evolved independently have converged upon similar shapes. Last, we will compare fish with one or two lenses removed to surface and cavefish to determine the contribution of lens degeneration to evolution of the shape of the face.

## Materials and Methods

Fish were stained with alizarin red to identify the bones. All images were taken in 25% glycerol. Landmarks were placed on each fish using tpsDIG2 (Morphometrics at SUNY Stony Brook). Size correction was performed using Coordgen6f and Standard6 (Morphometrics at SUNY Stony Brook), correcting for standard length. The PCA analysis was run in PCAGen6n (Morphometrics at SUNY Stony Brook) and plotted in Excel. Correlations were performed in R .

# Appendix 3

# Identification of mouse perichondrium-specific genes

# Identification of mouse perichondrium-specific genes

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This appendix contains preliminary work identifying perichondrium-specific markers in the mouse performed as a side project during the dissertation work presented here.

## Introduction

Endochondral ossification, the process by which most bones develop, begins when mesenchymal cells in the limb bud condense in areas that will become the future bones (Hall and Miyake 2000). The cells in these condensations differentiate into chondrocytes, cells that make up the cartilage. Adjacent to the condensations, cells differentiate into the perichondrium, a flattened layer of cells that surrounds the cartilage. After a period of proliferative growth, the centrally located chondrocytes stop dividing and enlarge, becoming hypertrophic and mineralizing the matrix surrounding them. At this time, the perichondrium adjacent to the hypertrophic chondrocytes becomes the periosteum, a portion of which differentiates into osteoblasts. Osteoblasts are bone progenitor cells, and the osteoblasts originating from the perichondrium form both cortical bone collar and trabecular bone. As hypertrophic chondrocytes undergo apoptosis, osteoblasts from the perichondrium invade along with the vasculature, forming the tissue that will become the trabecular portion of the bone (Kronenberg, 2003, 2007).

Interactions between the cartilage, the perichondrium, and the vasculature regulate both cartilage and bone development. Determining the contributions of each of these tissues to skeletal development is challenging because many genes important in this process are expressed in multiple tissues (Colnot 2005). The perichondrium is the least well studied of these tissues. It consists of two morphologically and molecularly distinct layers of

cells that have multiple functions in skeletal development. The outer layer is believed to play a structural role by interacting with connective tissue such as the tendons and ligaments (Scott-Savage and Hall 1980). The inner layer is thought to be important for the growth of cartilage and the bone (Bairati et al. 1996; Pathi et al. 1999). In addition to these roles, some perichondrium cells are believed to be the progenitors for some osteoblasts (Bairaiti et al. 1996; Colnot et al. 2004).

Signals from the perichondrium are essential for bone growth. When the perichondrium is removed, chondrocyte proliferation and hypertrophy are affected and the cartilage increases in length (Pathi et al. 1999; Long and Linsenmayer 1998; Di Nino et al. 2002; Colnot et al. 2004). In addition, perichondrium removal inhibits vascular invasion and ossification (Colnot et al. 2004). Some of the signaling molecules generated by the perichondrium that are critical for bone development have been identified. These molecules include parathyroid hormone-related peptide (PTHrP), which is regulated by Indian hedgehog, and multiple fibroblast growth factors (FGFs) and bone morphogenetic proteins (BMPs) (Kronenberg 2003; Pathi et al. 1999; Vortkamp et al. 1996). In addition, Hox genes, which are important in patterning and growth of skeletal elements, are expressed in the outer perichondrium layer. This is the major site of Hox expression in the limb after the early limb bud stages. However, Hox gene function in this layer is not fully understood. Further understanding of the role(s) of genes expressed in

the perichondrium during bone development will lead to a better understanding of the tissue interactions necessary for skeletal development.

In order to further understand the role that the perichondrium plays in bone development, Bandyopadhyay et al. (2008) identified genes specifically expressed in the perichondrium and the periosteum relative to other skeletal tissues. They performed a microarray screen comparing gene expression in the perichondrium, the periosteum, bone tissue, and non-hypertrophic cartilage from chicken tibio-tarsi, and looked for genes upregulated in the perichondrium or periosteum compared to the rest of the tissues. They found 20 genes expressed only in the periskeletal tissue, and confirmed the tissue expression of these genes by in situ hybridization. Through the identification of these new marker genes, the authors were able to conclude that the perichondrium and the periosteum are molecularly distinct, and that the perichondrium is composed of two molecularly distinguishable layers. The genes identified in this study can be used to further determine the role of the perichondrium in skeletal development.

Hox genes encode transcription factors that pattern the body axes. Mammalian Hox genes evolved by 2 sequential duplications of an ancestral cluster which is shared with invertebrates, to create four clusters of *Hox* genes, HoxA, HoxB, HoxC, and HoxD, containing a total of 39 genes. Each cluster has up to thirteen paralogues, or corresponding genes on separate clusters, and these paralogues are the most closely related to each other. Along the main body axis Hox gene clusters are expressed from 3' to 5' both

temporally and spatially, with 3' genes expressed earlier, and in a more anterior position than the 5' genes in the same cluster (Kmita and Duboule 2003). The anterior expression boundary of Hox gene expression maps axial morphology across vertebrates (Burke et al. 1995). In addition to patterning the body axis, Hox genes pattern secondary body axes, including the limb. While all four clusters of Hox genes are expressed in the limb, the HoxA and the HoxD clusters are the most important. Loss of both HoxA and HoxD clusters in the forelimbs results in truncations of the distal skeletal elements due to an arrest in early patterning of the limb (Kmita et al. 2005). Paralogues from these two groups have both overlapping and specific functions, and synergistic phenotypes are observed in double mutants of HoxA and HoxD paralogues in the limb, relative to individual phenotypes for mutants of the same genes (Davis et al. 1995; Favier et al. 1996; Fromental-Ramain et al. 1996). In contrast, expression of genes from the HoxB and HoxC clusters can be eliminated with little effect on limb phenotypes (Medina-Martinez et al. 2000; Suemori and Noguchi 2000).

The 5' HoxA and HoxD genes are initially expressed in nested domains along the anterior-posterior limb axis, with more 5' genes expressed in the posterior portion of the limb (Tarchini and Duboule 2006). Later, these genes are expressed in a dynamic fashion, including a late phase of expression in which their spatial order is reversed, with 5' Hox genes extending more anteriorly (Nelson et al 1996). They are expressed in the precartilagous condensations (Dolle and Duboule 1989). After cartilage

condensation, the Hox genes become restricted to the cells adjacent to the to cartilage, in the mesenchyme and the perichondrium (Favier et al. 1996; Dolle and Duboule 1989; Dolle et al. 1993). They are expressed in the outer layer of the perichondrium, and do not overlap with the inner layer, as defined by Bmp4 and Patched expression (Suzuki and Kuroiwa 2002).

Hox gene expression is important for both patterning and growth of the developing skeletal elements (Dolle et al. 1993; Davis et al. 1995). Patterning takes place before and during the formation of cartilage condensations (Mariani and Martin 2003). Early Hox gene expression, in concert with sonic hedgehog (Shh) expression, is responsible for patterning the digits (Knezevic et al. 1997; Zakany et al. 2004; Tarchini et al. 2006). Hox genes are also important for bone condensation. For example, Hoxd-11 misexpression results in extra condensations in digit I in the hindlimb (Goff and Tabin 1997). Hox genes affect the bone growth. For example, removal of all $11^{th}$ paralogue activity (Hoxa11/Hoxd11 compound null mutant) does not affect initial condensations but results in an almost complete subsequent lack of growth of the forearm elements (Davis et al. 1995). Similarly, through a presumed dominant-negative effect on the $11^{th}$ paralogues, Hoxd-13 misexpression results in a shortening of the femur, tibia, fibula and the tarsometatarsals (Goff and Tabin 1997). This could be a consequence of the action of *Hox* genes within the cartilage progenitors at early stages of limb development, or alternatively the continued activity of Hox genes at later stages, influencing growth signals produced in the perichondrium. Because Hox genes are

important over multiple stages of limb development, discerning the importance of Hox expression in specific tissues and at particular times in limb development is difficult. Generating inducible, tissue-specific loss of Hox genes in the perichondrium will help to understand the mechanism by which Hox expression ultimately affects the growth phase of bone development.

## Results and Discussion

To perturb Hox genes specifically in the perichondrium, we needed a perichondrium-specific marker to generate a perichondrium-specific Cre line. This line could then be used to remove the HoxA locus using a floxed HoxA line (Kmita et al. 2005). To identify markers appropriate for this line, I performed in situ hybridizations in mouse for candidate genes that were perichondrium-specific in the chicken (Bandyopadhyay et al. 2008).

I looked at expression patterns of genes at about E13.5, when the perichondrium is formed and there is a minimal amount of bone in the limb skeleton. Galectin-1 is not expressed in the cartilage, but is expressed in most of the other tissues (Figure A3.1). Stromal cell-derived factor 1 (SDF1) is expressed in what appears to be the outer layer of the perichondrium, along with other non-cartilage tissues (Figure A3.2). Thrombospondin 2 (Tsp2) is expressed in the perichondrium, next to the cartilage (Figure A3.3). ABI family, member 3 (ABI3BP) is expressed most strongly around the cartilage, and in additional non-cartilage tissue (Figure A3.4). V-maf musculoaponeurotic fibrosarcoma oncogene homolog B (MafB) is expressed

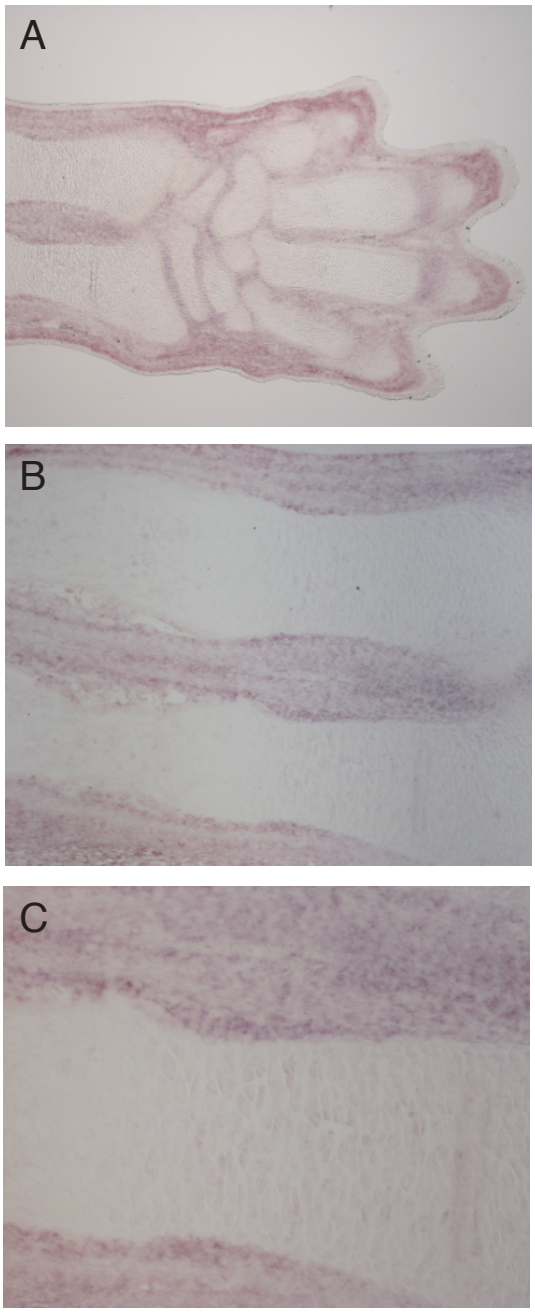



**Figure A3.1. Galectin-1 expression.**

Galectin-1 expression in an E13.5 forelimb at A. 5x B. 10x and C. 20x.

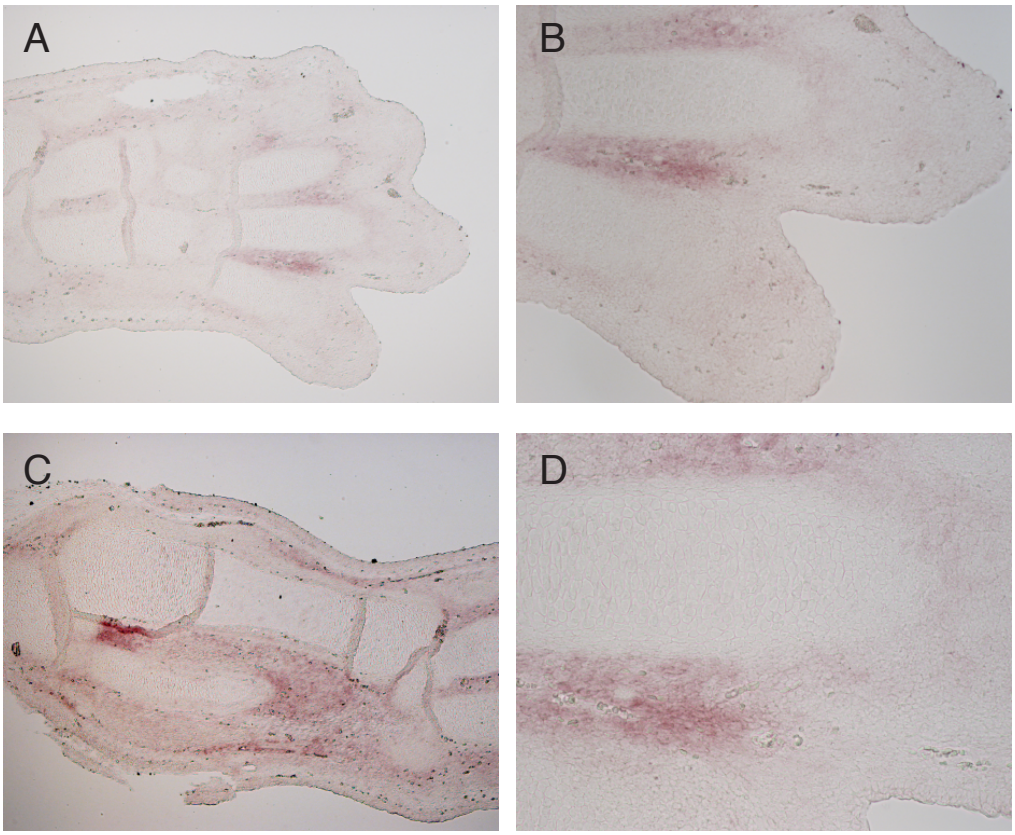



**Figure A3.2. SDF-1 expression.**

SDF-1 expression in an E13.5 hindlimb at A. 5x B. 10x C. 5x and D. 20x.

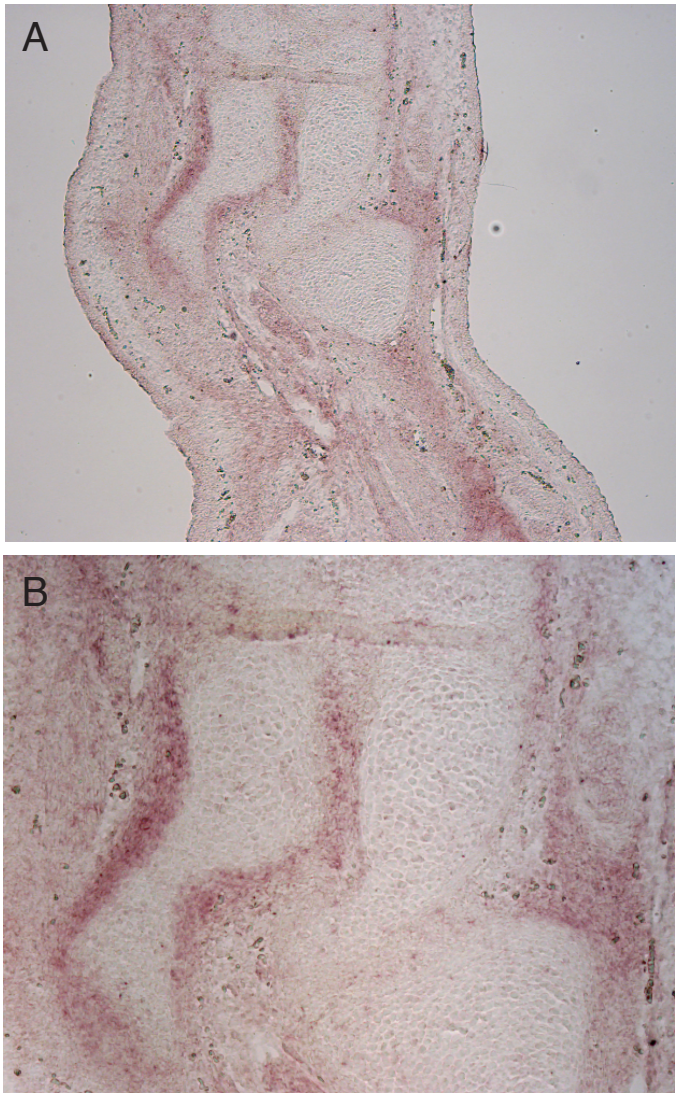



**Figure A3.3. Tsp2 expression.**

Tsp2 expression in an E13.5 hindlimb at A. 5x and B. 10x.

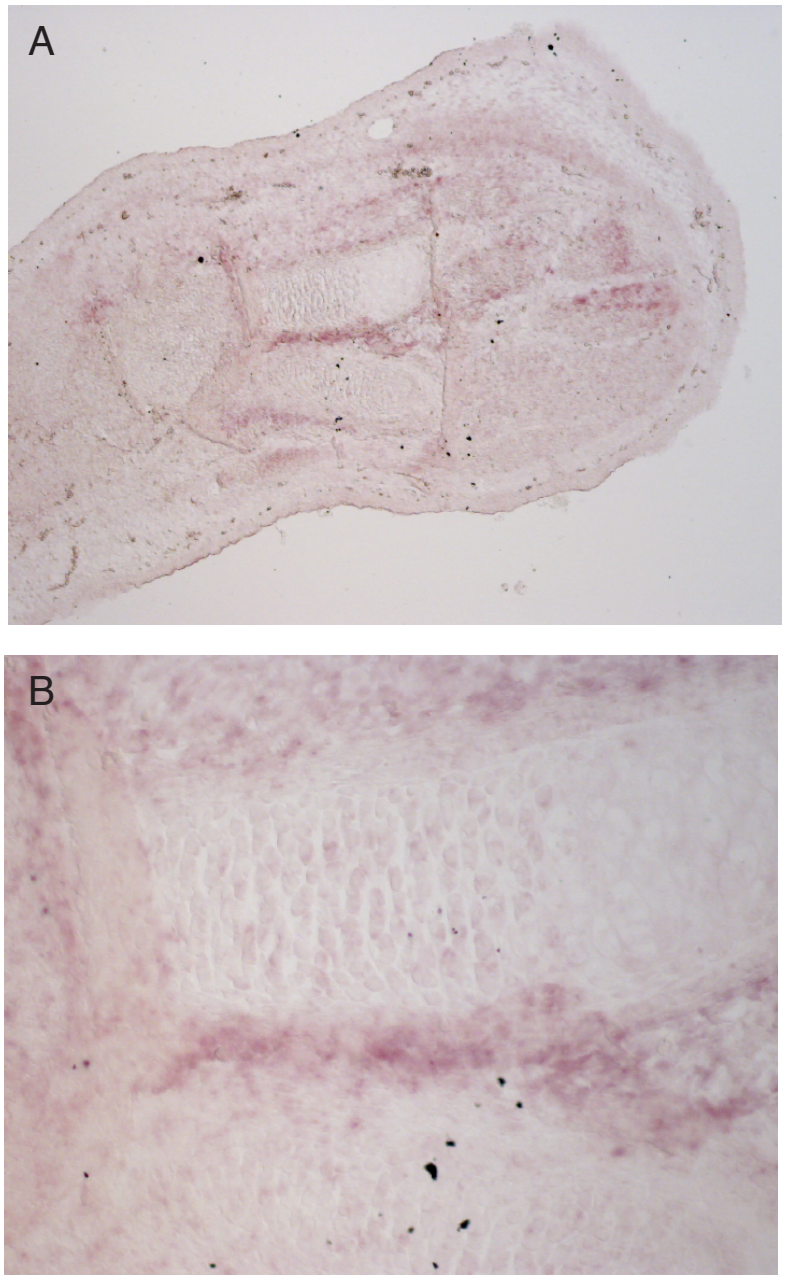



**Figure A3.4. ABI3BP expression.**

ABI3BP expression at E13.5 in at A. 5x and B. 20x.

around the digits, in the outer layer of the perichondrium (Figure A3.5). Dickkopf-related protein 3 (Dkk3) is expressed in the perichondrium, and most strongly around the joints (Figure A3.6). Cellular retinoic acid-binding protein 1 (CRABP1) is expressed in the outer layer of the perichondrium (Figure A3.7).

I next looked at promising markers by whole mount in situ, to get an idea of the expression patterns throughout the body, over developmental time. I looked at CRABP1 and Dkk3 at multiple time points in development (Figures A3.8 and A3.9).

From these analyses, it appeared that CRABP1 was a good candidate for an outer layer perichondrial marker, and that it could be used to make a Cre line to ablate Hox gene expression in the perichondrium. To test whether Hox genes and CRABP1 were coexpressed, I performed double fluorescent in situs on Hoxd11 and CRABP1. Hoxd11 and CRABP1 are coexpressed around the digits of the forelimbs, but not in the wrist area at about E13.5 (Figure A3.10). However, given the broad limb expression of CRABP1 early in development (Figure A3.8) it may be necessary to make an inducible Cre line in order to have perichondrium-specific expression. Thus, a CRABP1 inducible Cre line would be a useful tool for studying the outer layer of the perichondrium, and for removing Hox gene expression from the perichondrium.

We are also interested in having a Cre line that is active in the inner perichondrial layer. Dkk3, from both the literature and from the

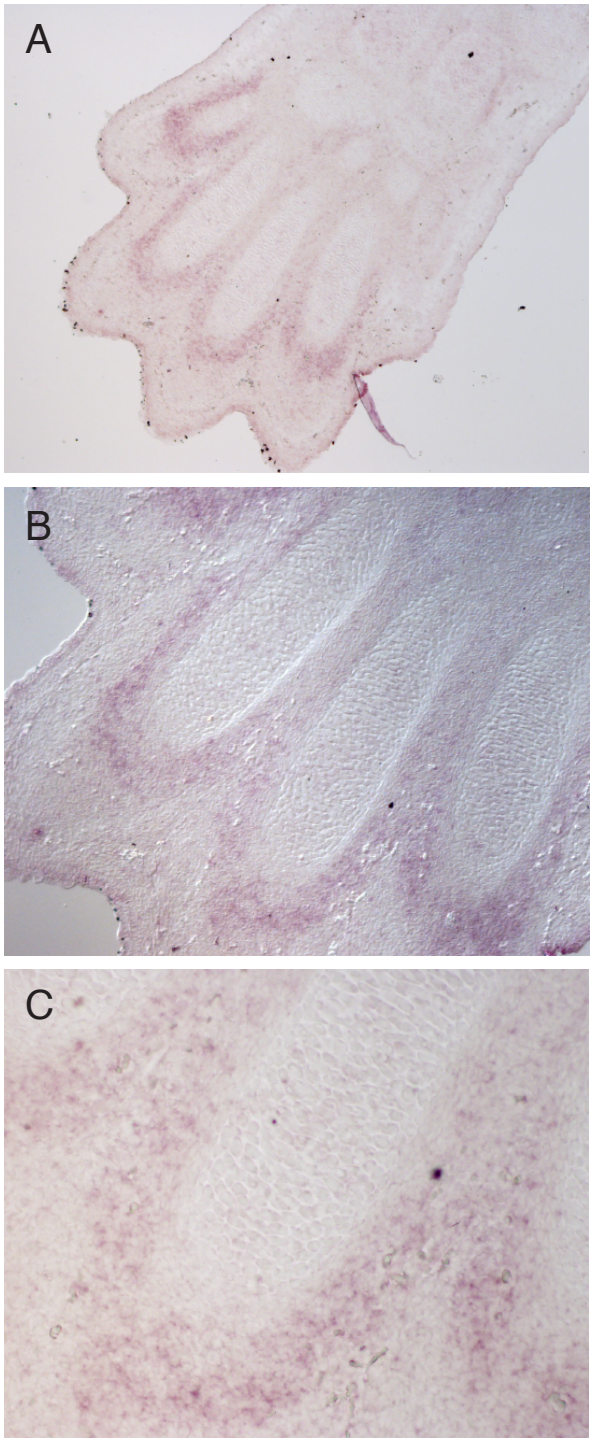



**Figure A3.5. MafB expression.**

MafB expression in E13.5 forelimbs at A. 5x B. 10x and C. 20x.

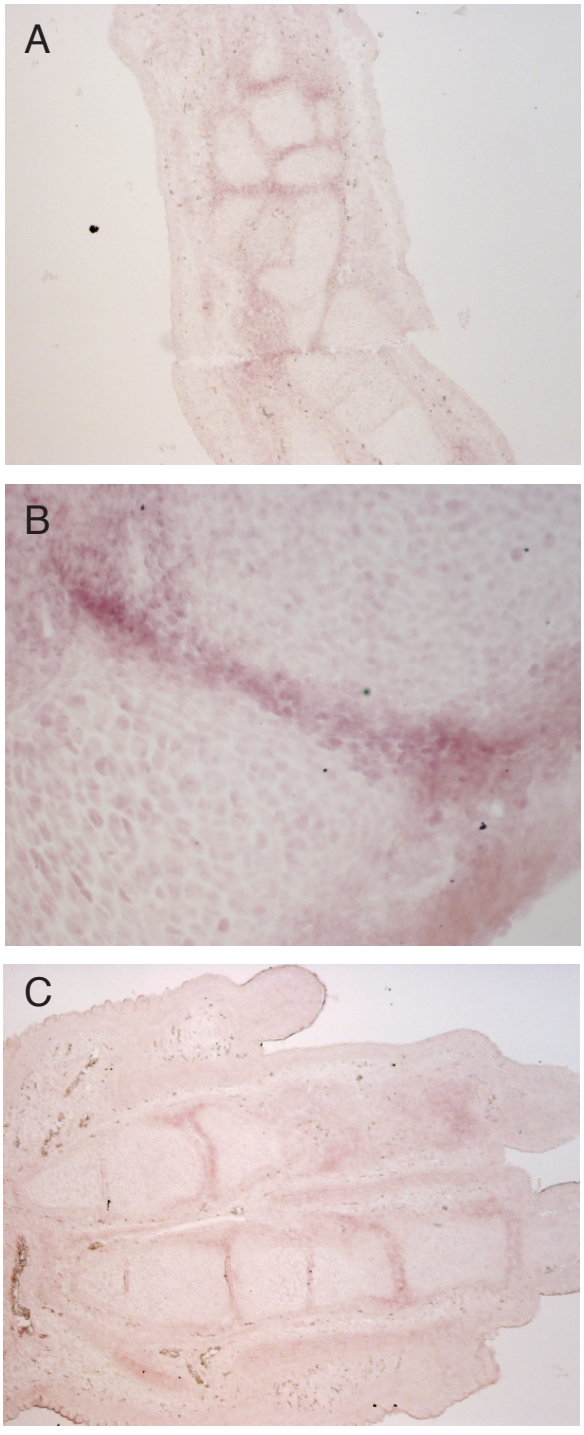



**Figure A3.6. Dkk3 expression.**

Dkk3 expression in E13.5 limbs at A. 5x and B. 20x and at C. 5x E17.5.

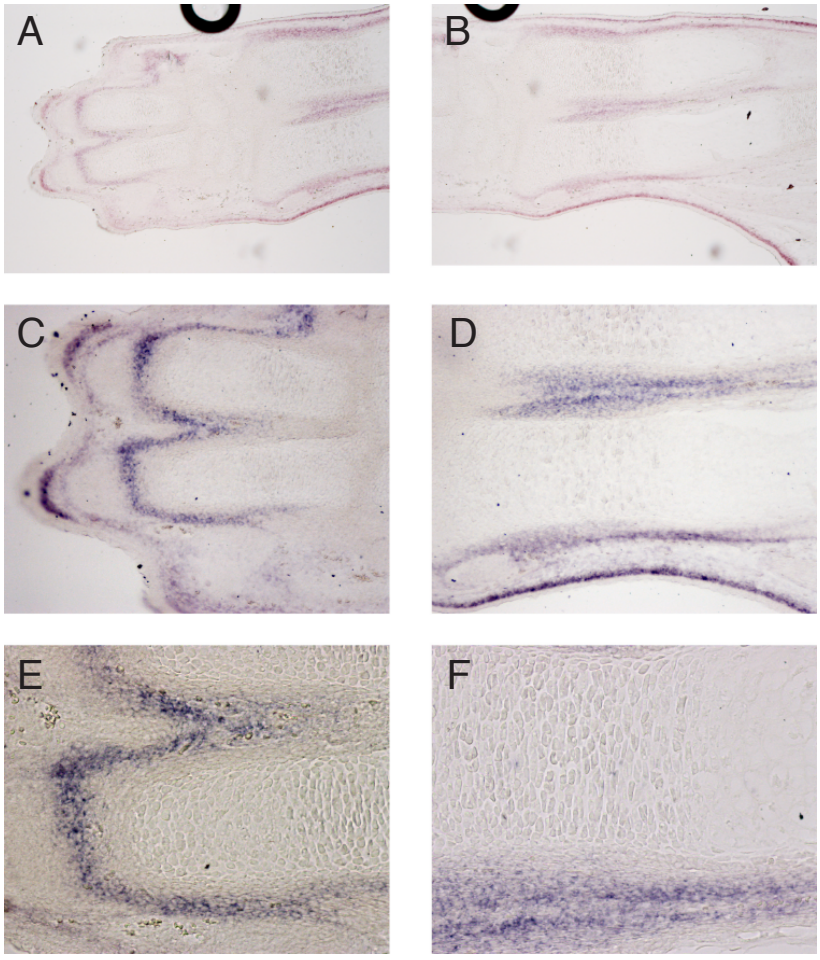
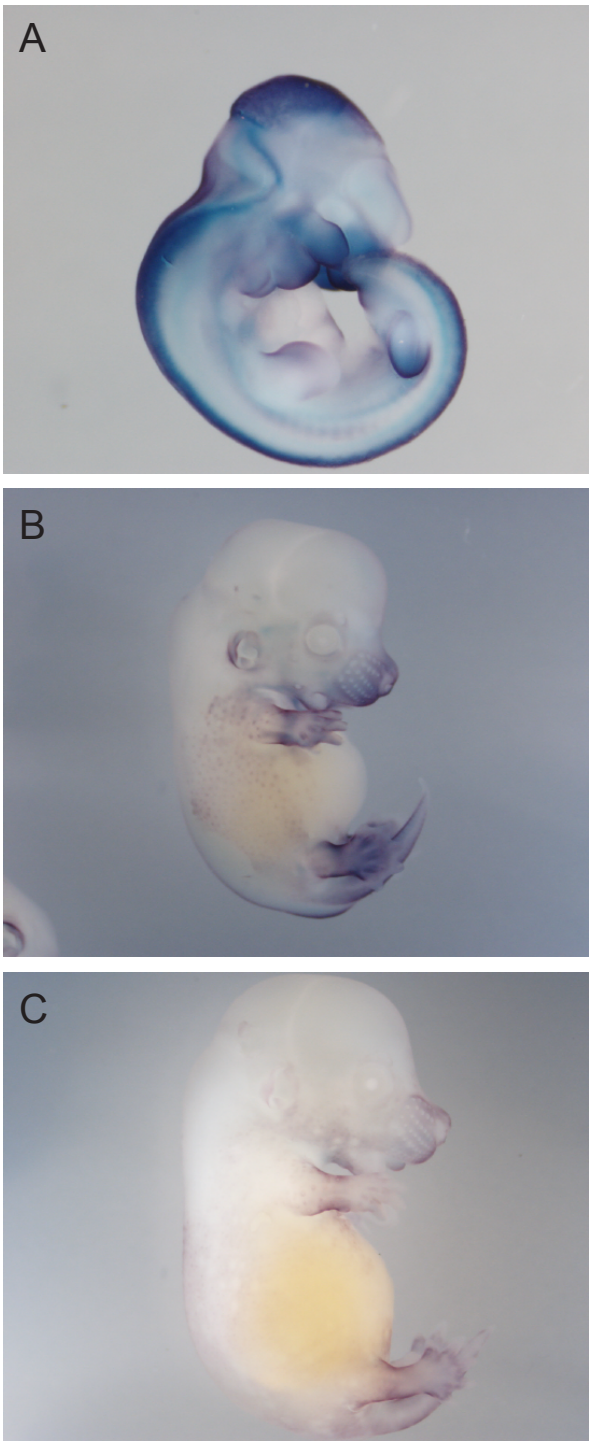



**Figure A3.7. CRABP1 expression.**

CRABP1 expression in E13.5 forelimbs in A. the autopod at 5x B. the zeugopod at 5x C. the autopod at 10x D. the zeugopod at 10x E. the autopod at 20x and F. the zeugopod at 20x.




**A3.8. CRABP1 expression by whole mount in situ.**

Expression at approximately A. E11 B. E13.5 and C. E14.5.

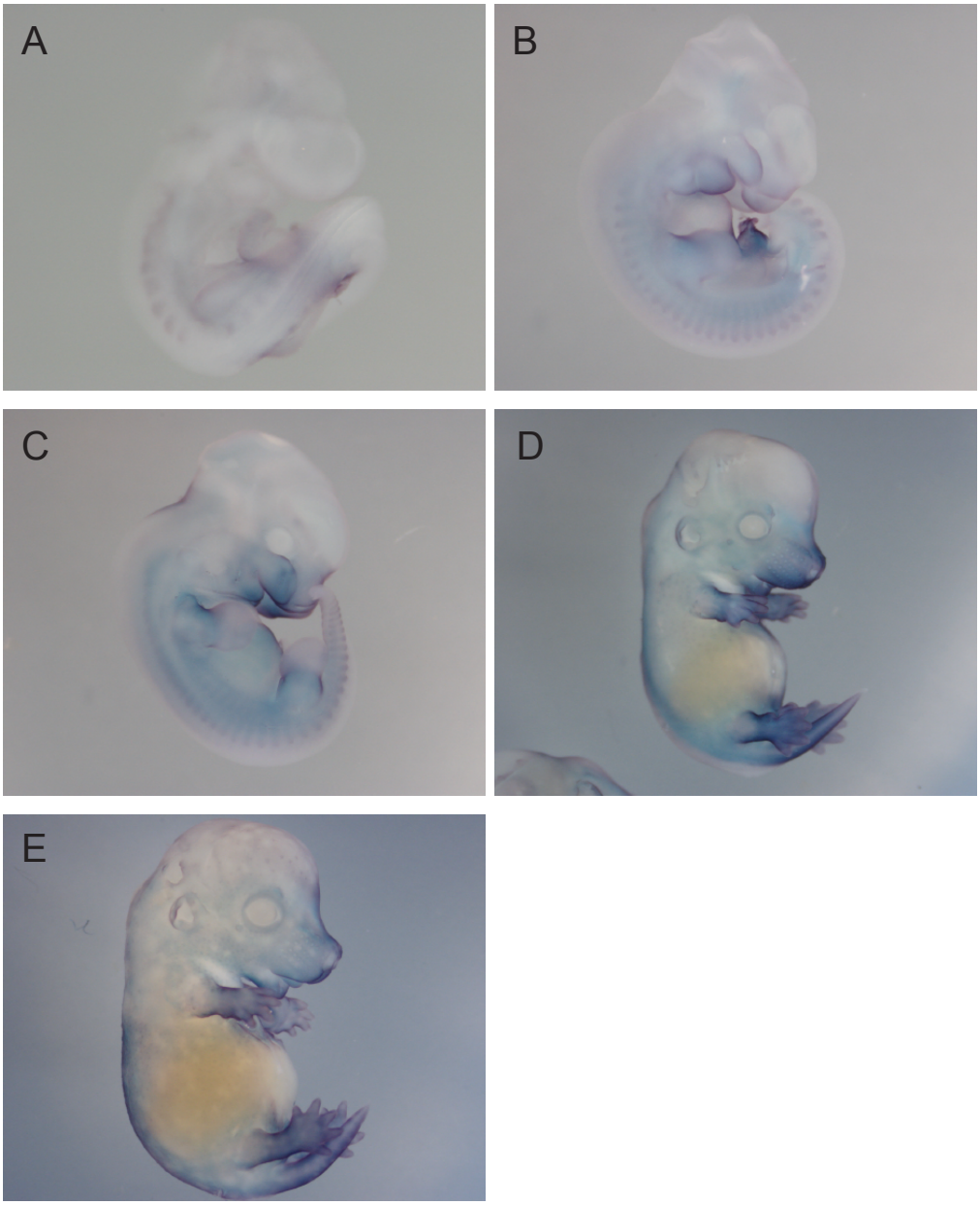



**Figure A3.9. Dkk3 expression by whole mount in situ.**

Expression at approximately A. E9.5 B. E11 C. E12 D. E13.5 and E. E14.5.

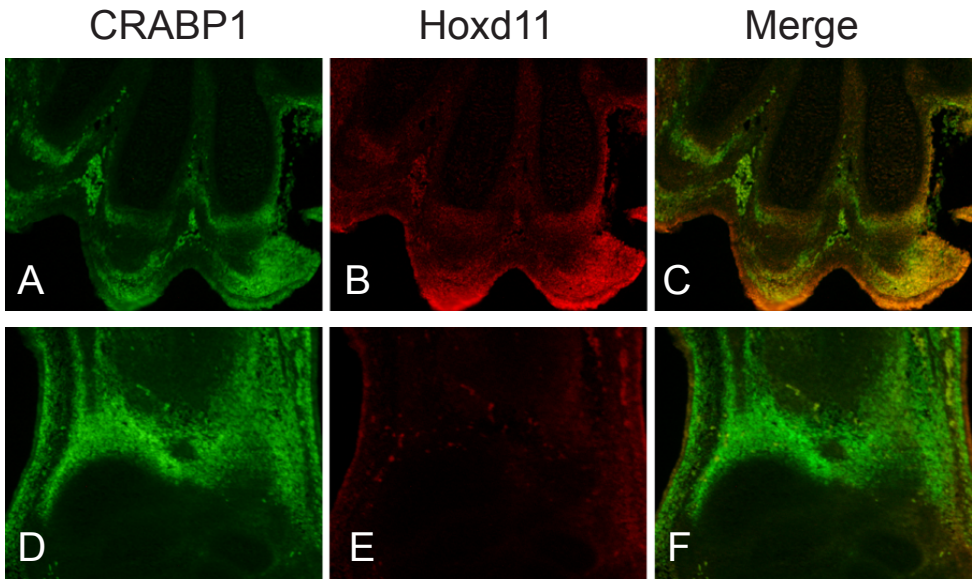



**Figure A3.10. Double fluorescent in situs of CRABP1 and Hoxd11.**

A. CRABP1 in green. B. Hoxd11 in red. C. Merge of A and B. D. CRABP1 in green. E. Hoxd11 in red. F. Merge of D and E.

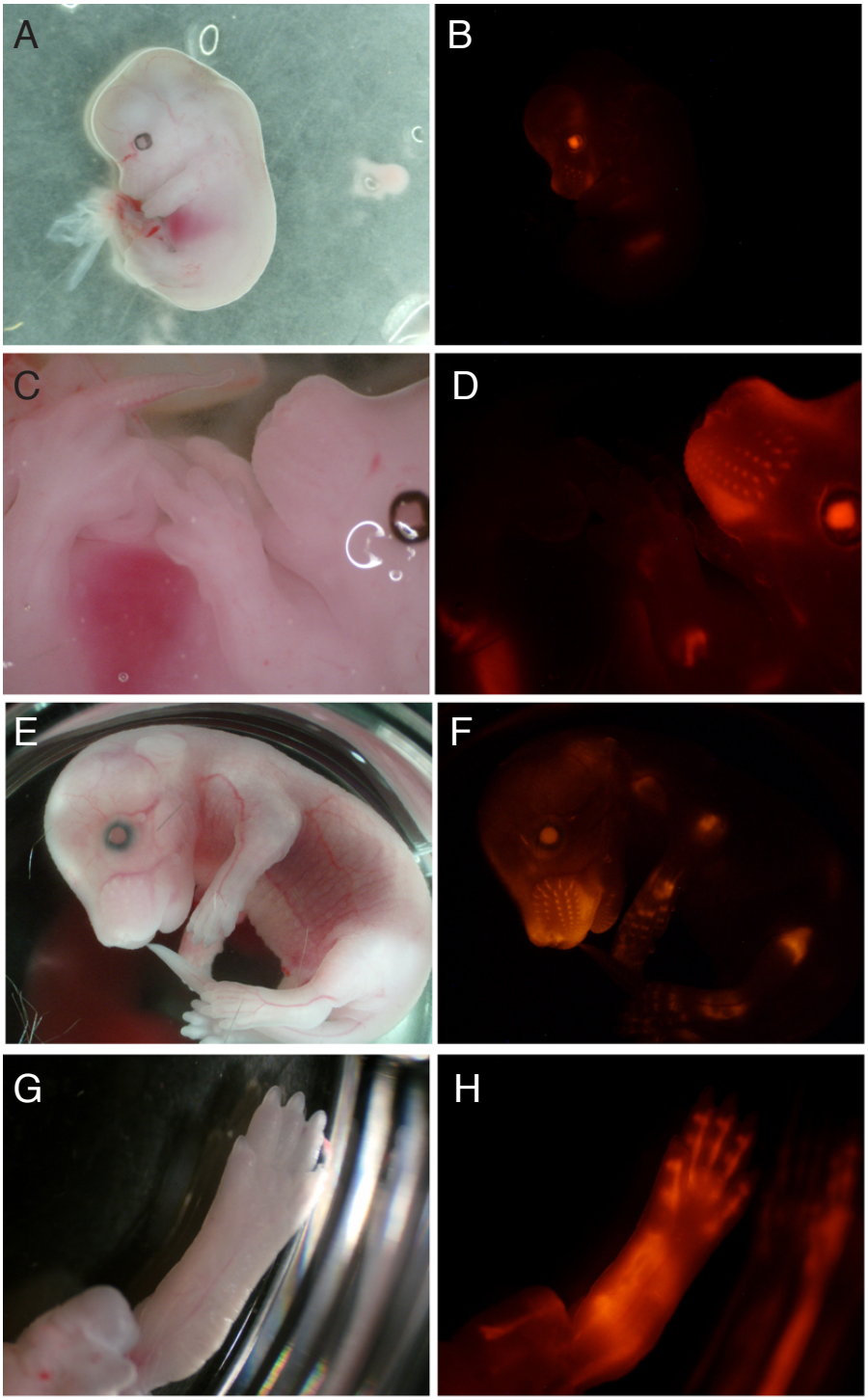
expression studies here, was a promising candidate for this. In addition to being expressed in the joints, Dkk3 is expressed in the periarticular perichondrium (Witte et al. 2009). Additionally, a BAC transgenic Cre for Dkk3 exists (Sato al. 2007). We obtained this line and crossed it to a reporter line with Tdtomato in the Rosa locus. Tdtomato could be seen without antibody staining in the limb starting at E13.5. As the limb matured, cells with a history of Dkk3 were located in the joints, and in the perichondrium adjacent to the joints. There appeared to be some Cre activity in connective tissue as well (Figures A3.11-13). This preliminary fate map sets the groundwork for future more extensive fate mapping, and identifies a potentially useful marker of at least a portion of the inner layer of the perichondrium.

Last, we were interested in determining the role of Hox genes expressed in the joints. Removal of the 5' Hox genes from the limb results in disruption of growth differentiation factor 5 (GDF5) expression and joint fusions (Villavicencio-Lorini et al. 2010). However, at least some of the HoxD genes are also expressed later in the joints (Suzuki and Kuroiwa 2002). Thus, Hox genes might be playing a role later in joint development. GDF5 is a marker expressed early in joint development (Storm et al. 1994) and Figure A3.14). A GDF5-cre line that expresses Cre in the developing joints exists (Koyama et al. 2008). We obtained this line from the Kingsley lab to cross to HoxA conditional mice. However, a subset of the individuals in each cross expressed Cre in tissues beyond the joints (Figure A3.15). Future work on the role of Hox genes in the developing joints will have to be performed in

**Figure A3.11. Fate mapping of Dkk3-cre in embryonic mice.**

A-C. E13.5 mice. E-H. Approximately E18.5. A, C, E, G – photographs of Embryos. B, D, F, H – photographs of embryos under the fluorescence.

Figure A3.11, cont.



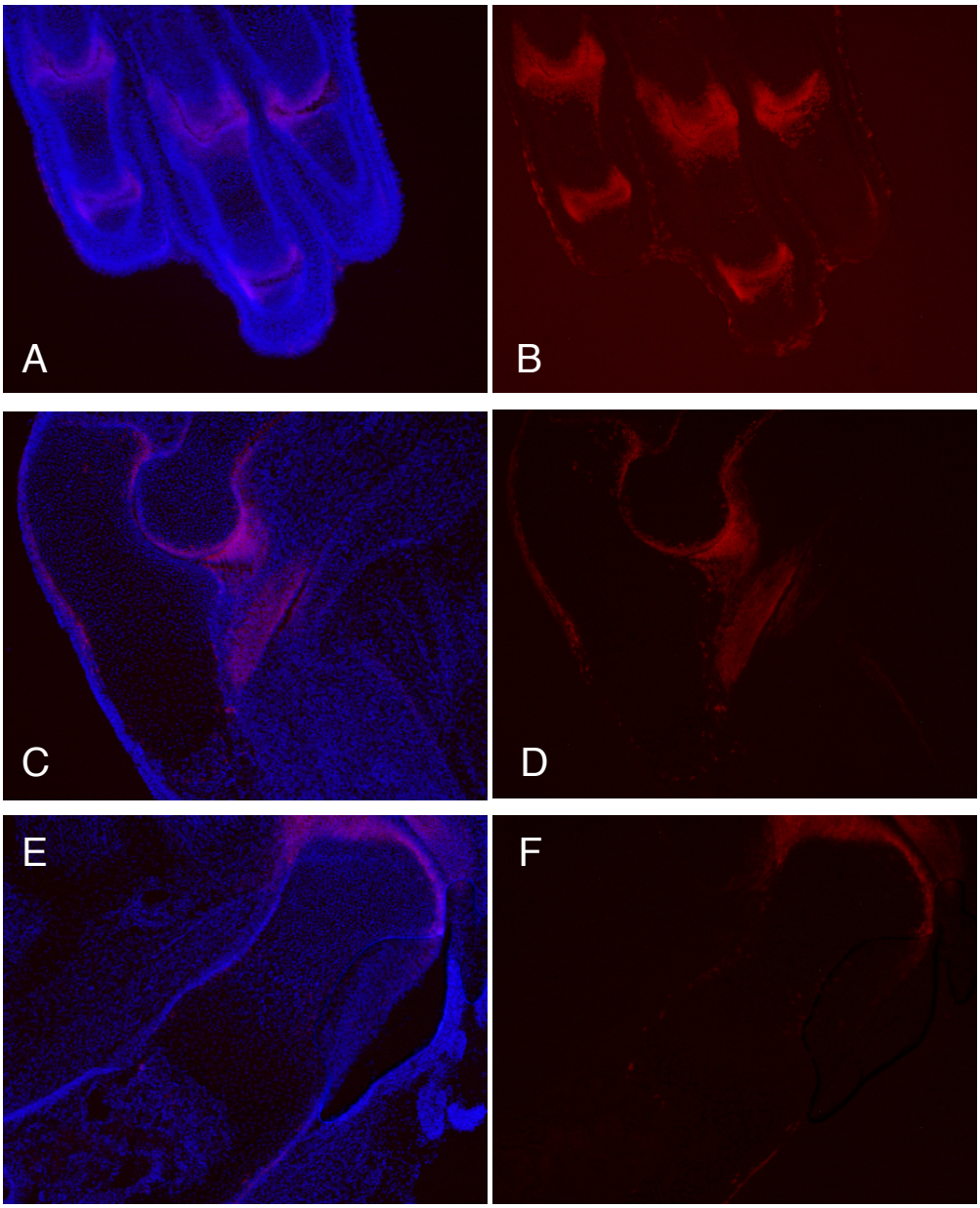



**Figure A3.12. Dkk3-cre fate map at E18.5.**

Sections of Dkk3-cre Tdt mice at E18.5. B, D, F – Tdtomato. A, C, E - Merge of Tdtomato and DAPI.

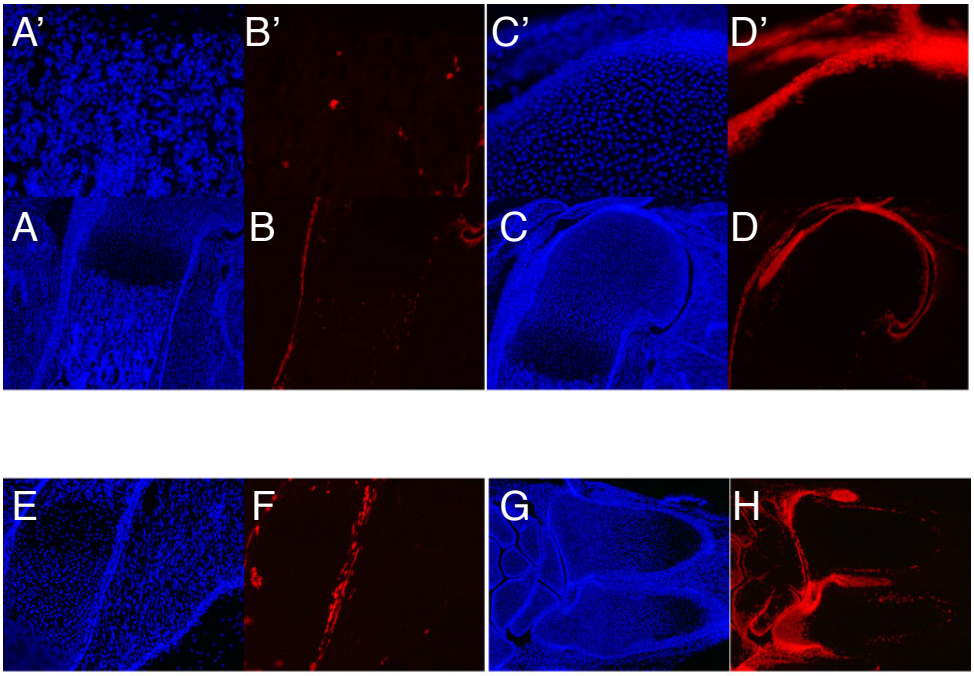



**Figure 3.13. Dkk3-cre fate mapping at P0.**

Sections of Dkk3-cre Tdt mice at P0. A’-D’ are magnifications of A-D. A, C, E, G – DAPI stain. B, D, F, H – Tdtomato.

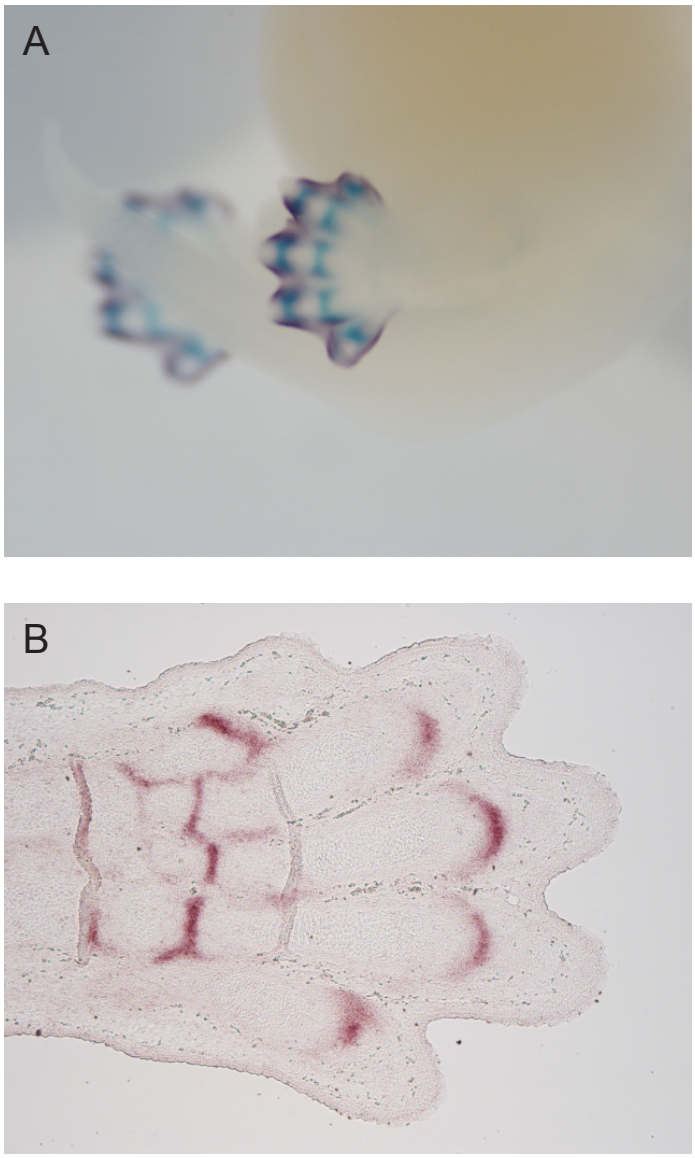



**Figure 3.14. GDF5 expression.**

A. Whole mount in situ of GDF5 at approximate E13.5. B. Section in situ of GDF5 at approximately E13.5.

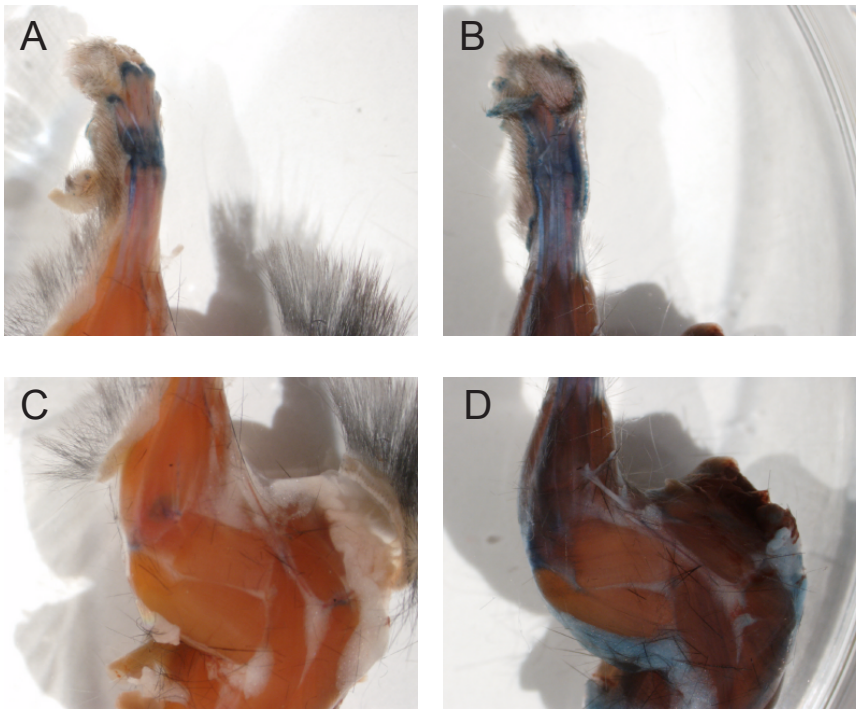



**Figure 3.15. GDF5-cre fate mapping.**

X-gal stained adult mouse limbs that are GDF5-cre LacZ. A and C – Joint specific GDF5-cre. B and D – Non-joint specific GDF5-cre.

mice that have both Cre and a reporter crossed into the background to determine which individuals are expressing a joint-specific Cre for the analysis of loss of Hox genes in the joints.

## Materials and Methods

All in situs were performed following the standard Cepko/Tabin lab in situ protocols for either whole mount or section in situs. Fluorescent in situs were performed using a modified version of the section in situ protocol using a tyramide amplification step (Trimarchi et al. 2007).

For the fate mapping work, the Dkk3-cre fate mapping was performed by crossing male mice heterozygous for Dkk3-cre to female mice homozygous for Tdtomato in the Rosa locus. All of the images shown here were from whole mice or embryos, or sections that were not antibody stained. Sections were stained with DAPI. GDF5-cre fate mapping was performed by crossing male mice heterozygous for GDF5-cre to female mice homozygous for the R26R allele, with LacZ expressed from the Rosa locus, and then performing X-gal staining on whole limbs. Note that the number of mice expressing Cre outside of the joints may be affected by the mouse line backgrounds, and that determining which background gives the cleanest expression may aid in future studies.

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# Appendix 4

# Micromasses: More than just a tool to study cartilage

# Micromasses: More than just a tool to study cartilage

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This appendix is preliminary work for a manuscript titled “Micromasses: More than just a tool to study cartilage.” This work was performed as a side project during graduate school. It is the result of a collaboration between Jessica Whited and Johanna Kowalko, who contributed equally to this work. Jessica Whited, Johanna Kowalko, and Clifford Tabin designed the experiments and contributed to the writing. Johanna Kowalko, Jessica Whited, and Stephanie Tsai did the experiments. Scott Stadler contributed reagents.

## Results and Discussion

Micromasses have classically been thought of as an assay for cartilage formation. Any patterning that has been studied in micromasses has been limited to patterns occurring between cartilage nodules and non-cartilage spaces. However, a previous study identified digit-like cartilage projections forming radially around the edge of the micromass (Stadler et al., 2001). We confirmed that micromasses form a digit-like pattern around their edges, and that these digit-like structures stain with alcian blue (Figure A4.1A and B).

We next investigated whether the non-cartilage spaces in our micromass cultures express markers of different cell types. We performed in situ hybridizations for genes expressed in different cell types in the limb bud during cartilage formation. Multiple cell-type-specific markers showed restricted patterns in the micromass culture system reminiscent of their expression in the limb bud. Sox9, which marks cartilage condensations, was expressed in the cartilaginous nodules (Figure A4.2A). Msx2, a marker for the interdigital space, was expressed between cartilage nodules (Figure A4.2B). In addition, perichondrial markers CRABP1 and Tsp2 were expressed in space between cartilaginous nodules, as was Bmp2 (Figure A4.2C-E). Bmp4 was expressed in both the cartilage and the cells surrounding the cartilage nodules (Figure A4.2F). GDF5, a joint marker, was expressed in between cartilage nodules (Figure A4.2G).

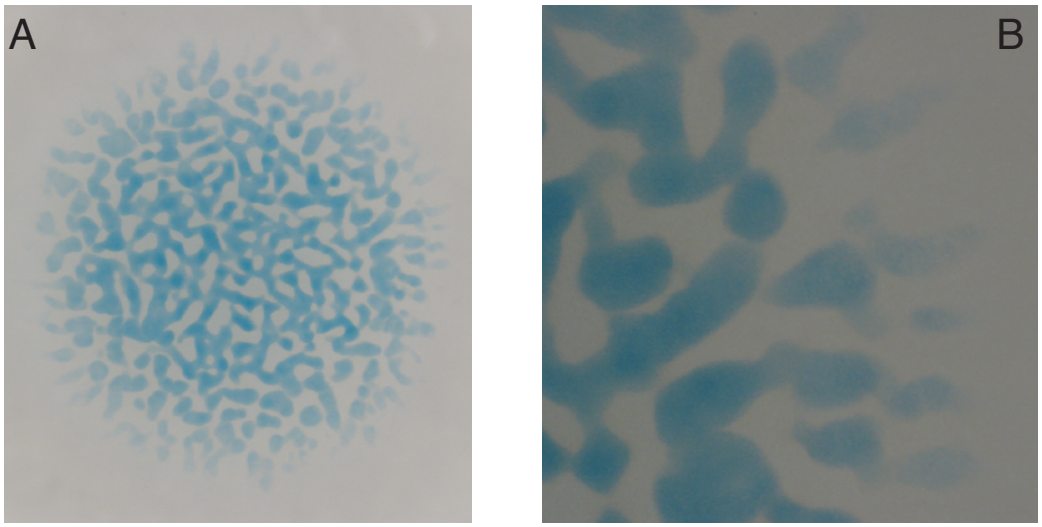


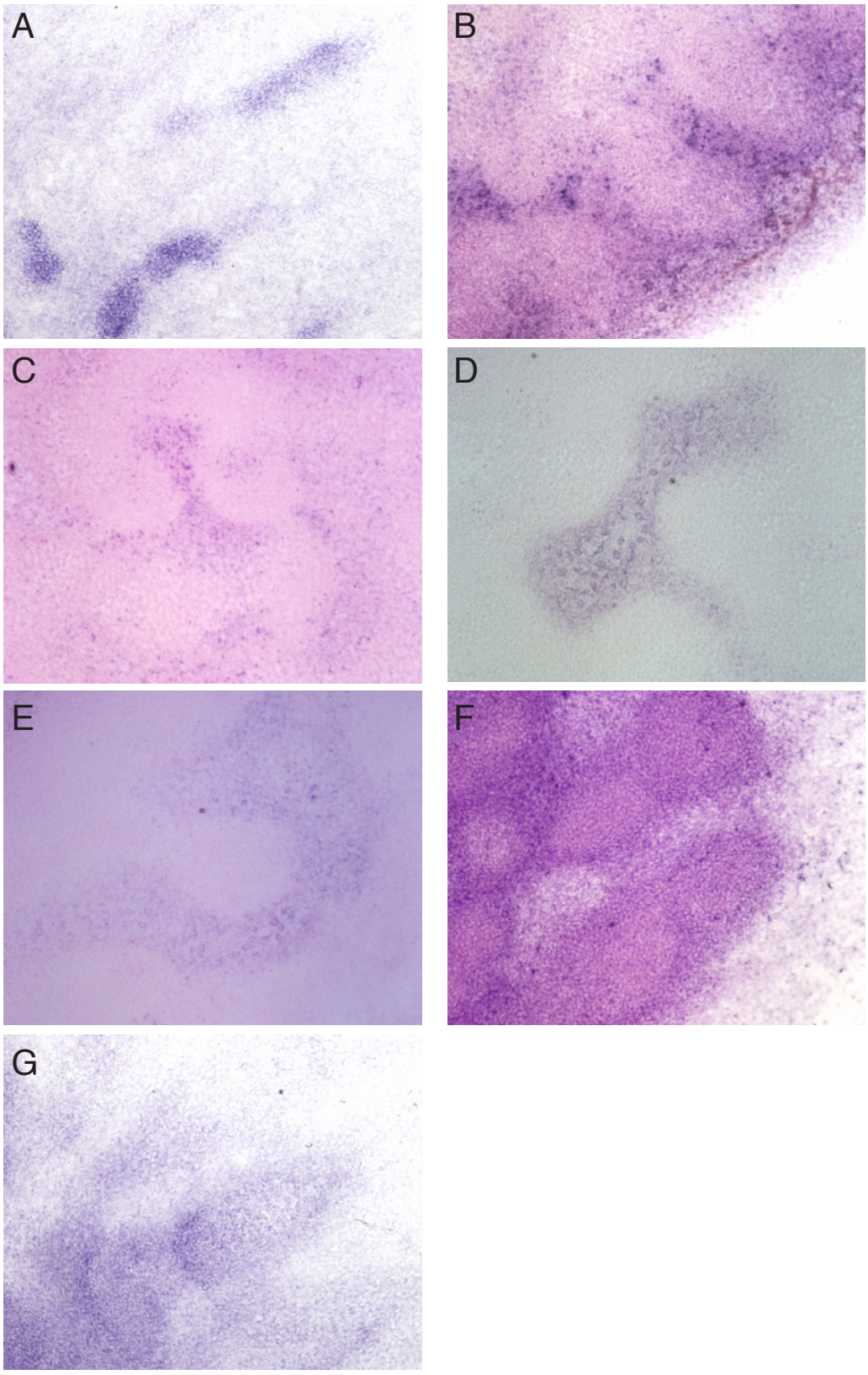

**Figure A4.1. Micromasses display digit-like cartilage projections around their edges.**

A. Micromass stained with alcian blue. B. Close up of the edge of a micromass.

**Figure A4.2. Micromasses have restricted expression of markers of multiple cell types.**

A. In situ for Sox9. B. In situ for Msx2. C. In situ for CRABP1. D. In situ for Tsp2. E. In situ for Bmp2. F. In situ for Bmp4. G. In situ for GDF5.

Figure A4.2, cont.



To determine if these patterns were set up based on the original cell's location within the limb bud, we labeled different regions within the original limb bud and generated micromasses. Sonic hedgehog (Shh) is expressed in the posterior portion of the developing limb bud. To test whether anterior-posterior patterning of the limb bud affects micromass patterning, we made micromasses from Shh-cre heterozygous animals crossed to a Tdtomato reporter line, so that limb bud cells derived from the posterior portion of the limb bud were labeled red. Micromasses derived from these crosses had red cells in both cartilage and non-cartilage spaces, as determined by cell shape (Figure A4.3A). Therefore, anterior-posterior position in the limb bud is not responsible for only cartilage or non-cartilage fate in the micromass culture.

It is also possible that the fate of the micromass cells is determined by the original proximal-distal position of the limb bud cells. To test this hypothesis, we crossed an ubiquitously expressed Cre, Human beta actin-cre, heterozygous mice with a Tdtomato reporter line. This resulted in either red (Cre-positive) or wildtype (Cre-negative) embryos. Limb buds harvested from these embryos were divided into proximal and distal portions, and red-proximal cells were mixed with non red-distal cells, and vice versa. The resulting micromasses had red cells in both the cartilage and the non-cartilage areas (Figure A4.3B). Thus, patterning seen in the micromasses did not result from original proximal-distal location in the limb bud.

Last, we tested the effect of factors expressed in the limb on micromass pattern formation. We treated micromasses with a range of concentrations of

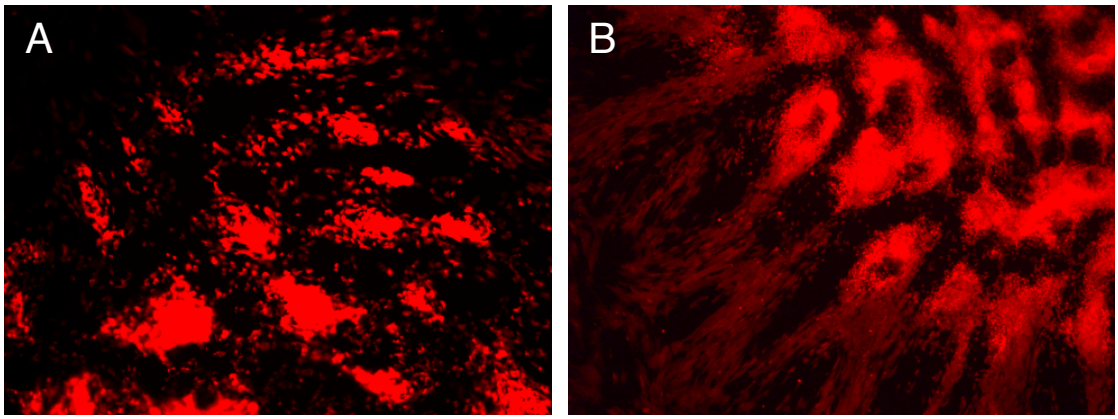



**Figure A4.3. Position in the limb bud does not dictate cartilage versus non-cartilage fate for micromass cells.**

A. Shh-cre Tdtomato micromasses after plating for 2 days. B. Micromasses after 4 days with the distal cells labeled with Tdtomato and the proximal cells unlabeled.

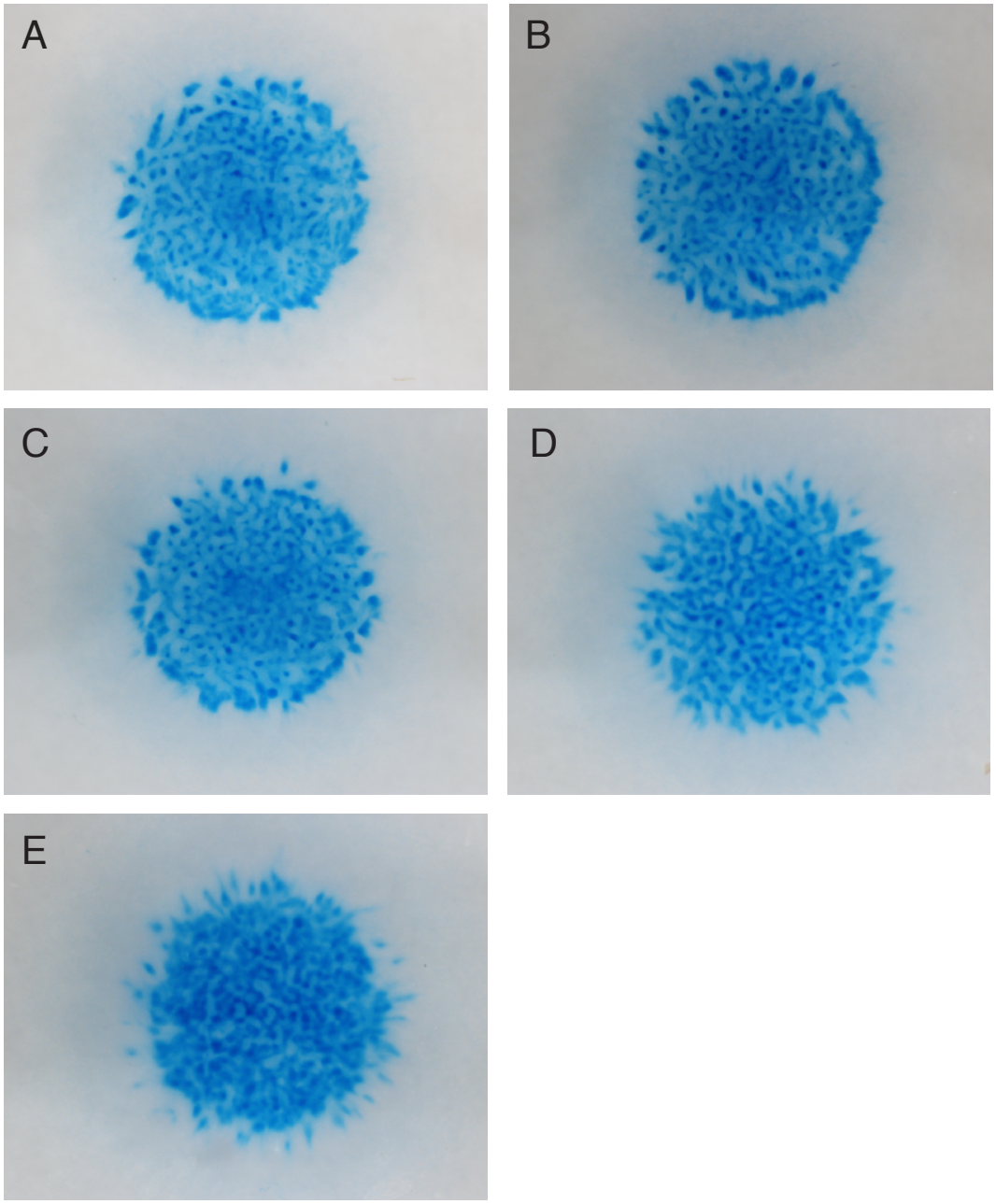



**Figure A4.4. FGF-8 treated micromasses.**

Micromasses after 5 days treated with A. 0 ng/uL B. 2.5 ng/uL C. 10 ng/uL D. 50 ng/uL and E. 150 ng/uL of FGF-8.

Fibroblast growth factor 8 (FGF-8) protein. FGF-8 treatment resulted in an increase in the number and length of digit-like structures around the edge of the micromass (Figure A4.4). Increasing FGF signaling in the limb can induce extra phalanges in the developing chicken limb (Sanz-Ezquerro and Tickle, 2003). Therefore, micromasses may be useful for determining in vivo effects of signaling molecules on digit patterning.

## Materials and Methods

Micromasses were generated from mouse embryonic limb buds stages E10-E11.5, using a protocol modified from (Stadler et al., 2001). In situs were performed using a modified version of the Cepko/Tabin lab section in situ protocol. Micromasses were FGF-8 treated during flooding.

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