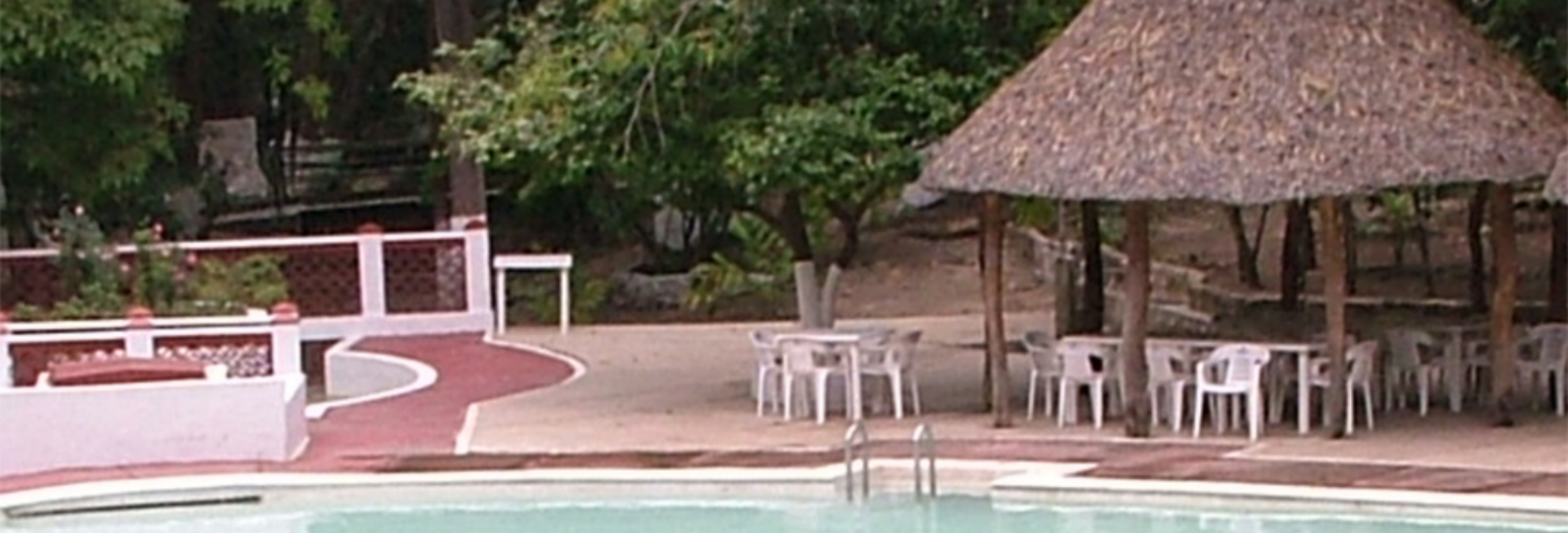


3rd *Astyanax* International Meeting

INTRODUCING THE CAVEFISH GENOME

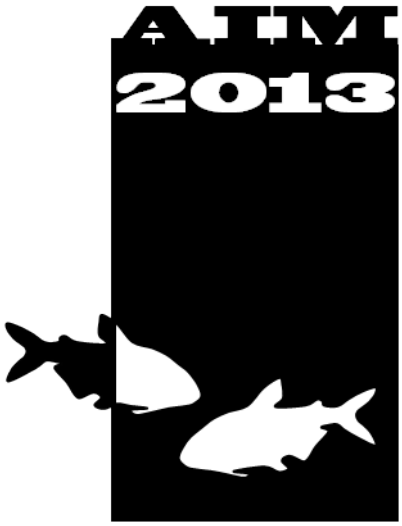
March 17-21, 2013

Ciudad Valles, Mexico

AIM 2013

Š.G., M.P.

Conference Scientific Program



AIM 2013 Scientific Program

Sunday, March 17th

Arrival of participants

5pm – 7pm:	Meeting Registration in the Hotel Taninul Lobby.
7pm:	Welcome Reception in the Hotel Taninul Restaurant.

Monday, March 18th

7:00 – 9:00am:	Breakfast

Morning Session

8:45am:	Meeting Welcome and Acknowledgements: Ernesto Maldonado, Luis Espinasa, Josh Gross.

Invited Plenary Lecture

9:00 – 9:40am: Suzanne McGaugh	"The *Astyanax* genome sequencing project"
9:45 – 10:25am: Hélène Hinaux	"Transcriptome analysis in *Astyanax mexicanus* blind cavefish and sighted surface fish"
10:30 – 11:00am:	*Coffee Break*
11:00 – 11:40am: Josh Gross	"An analysis of gene expression level changes across development in surface and cave-dwelling fish"
12:30 – 2:00pm	*Lunch*

Afternoon Session

2:00 – 2:40pm: Richard Borowsky	"Divergence and Speciation in *Astyanax* of the Sierra El Abra"
2:45 – 3:25pm: Cahill/Yurgel	"Hybridization and the colonization of the cave environment by fish."
3:30 – 4:00pm:	*Coffee Break*
4:00 – 4:40pm: Esquivel Bobadilla	"Genetic structure of *Astyanax mexicanus* at Mexican Atlantic slope"
4:45 – 5:25pm: Luis Espinasa	"Paradigm shifts and pendulum swings regarding the origin of *Astyanax* cavefish: What about geology?"

Monday Evening Poster Session I (First authors listed)

5:30 – 7:00pm

Allison Furterer	"An integrated transcriptome-wide analysis of cave and surface dwelling *Astyanax mexicanus*"

Ana Ofelia Santacruz Vázquez	"Compared phylogenies of monogeneans parasites and their host *Astyanax mexicanus* "
Josh Gross	"An analysis of structural mutations in the gene *Mc1r* in surface and Granadas cave-dwelling *Astyanax aeneus* "
Luis Espinasa	"Caballo Moro breaks Dollo's law: Recuperation of vision in a blind cavefish population"
Maryline Blin	"Development of the olfactory system in *Astyanax* cavefish and surface fish"
Rubens Pazza	"Molecular systematics of the genus *Astyanax* (Characiformes: Characidae) - starter edition"
Bermúdez-González	"Characterization of two trophic ecotypes of Lake Catemaco through diet analysis of stable isotopes"
7:00 – 8:30pm:	*Dinner*

Tuesday, March 19th

7:00 – 9:00am:	Breakfast
<u>Morning Session</u>	
9:00 – 9:40am: Li Ma	"Role of *aA-crystallin* in *Astyanax* Cavefish Eye Degeneration"
9:45 – 10:25am: Nicholas Rohner	"HSP90 as a capacitor for the evolution of eye loss in cavefish"
10:30 – 11:00am:	*Coffee Break*
11:00 – 11:40am: William Jeffery	"Development and Genetics of the *Astyanax* Sclera: An Optic Tissue Organized by the Lens"
12:30 – 2:00pm	*Lunch*
<u>Afternoon Session</u>	
2:00 – 2:40pm: Kelly O'Quin:	"Quantitative genetic analysis of retinal degeneration in the blind cavefish *A. mexicanus*"
2:45 – 3:25pm: Bethany Stahl:	"Pigmentation loss in cave animals: A high-resolution study of destructive genetic mutations"
3:30 – 4:10pm: Masato Yoshizawa:	"Adaptive changes in vibration attraction behavior and its sensory receptors promote eye degeneration

	and disparity between the nuclear and mitochondrial genomes in Pachón cavefish"

Tuesday Evening Poster Session II (First authors listed)
5:30 – 7:00pm

Aaron Stahl	"An evaluation of eyelessness in cave-dwelling *Astyanax mexicanus* using RNA-seq technology"
Amanda Krutzler	"Fragmentation, fusion and asymmetry in the craniofacial skeleton of *Astyanax mexicanus*"
Laurent Legendre	"Transgenesis methods in *Astyanax*"
Manuel Stemmer	"Unravelling continuous eye growth in teleosts by studying blind cavefish"
Oscar Manuel García-González	"Isolation and characterization of *V1r* pheromone receptor gene in cave and surface variants of *Astyanax mexicanus*"
Stéphane Père	"Statistics on *Astyanax* husbandry in the Gif facility"
Claudia Patricia Ornelas García	"Parallel evolution within the *Astyanax* genus in Mesoamerica "
7:00 – 8:30pm:	*Dinner*

Wednesday, March 20th

7:00 – 9:00am:	Breakfast
8:00am:	Pick Up Packed Lunch from the Hotel Lobby
9:00am – 6:00pm:	Meet in the Hotel Lobby at 9am for the Day Trip to the Micos Cave
7:00 – 8:30pm:	AIM Meeting Banquet

Thursday, March 21st

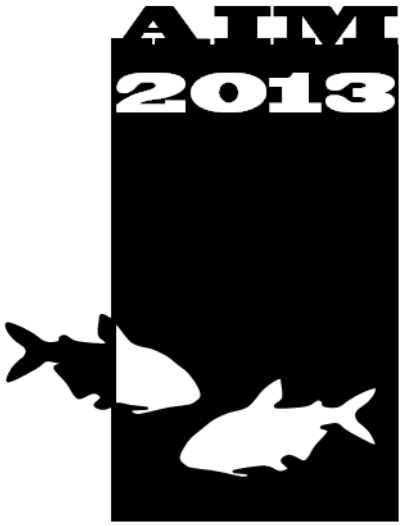
7:00 – 9:00am:	Breakfast

Morning Session

9:00 – 9:40am: Jonathan Bibliowicz	"Olfactory evolution in cave-dwelling *Astyanax mexicanus* "
9:45 – 10:25am: Alex Keene	"Metabolic Regulation of Sleep in *A. Mexicanus*"

10:30 – 11:10am: Sylvie Rétaux	"Feed or fight: developmental origin of a behavioral shift in blind cavefish "
11:15am – 11:45am:	*Coffee Break*
<u>Invited Closing Lecture</u> 11:50am – 12:30pm William Elliott:	"*Astyanax*: Looking Back 45 Years".
1:00pm	*Lunch*
2:30pm:	Afternoon Bus Departure for Tampico

Field Excursion to the Micos cave locality



Micos Locality Cave trip

Wednesday, March 20th

In past years, trips have been organized to the classic caves of Pachón and Chica, which were easy caves that almost anyone can enter, and Tinaja, which involved a higher outdoor challenge. This year we have decided to visit a different cave. While it is exciting to visit a new cave, especially if you have previously explored any of the aforementioned caves, it means that the trip involves a higher outdoor challenge: a short pit half way into the cave and a low ceiling passage where you will need to crawl for a few meters.

We are planning on visiting Rio Subterraneo, in the Micos area, although field conditions at the time of the meeting may make us change plans. For Rio Subterraneo you will need to climb down to the cavefish areas with the help of cable ladders and ropes down a 5 meter pit. This is a "wild" cave that requires a short hike in and a 2-4 hr cave time. It will require some non-technical climbing and boulder hopping. Clothing and especially shoes should be adequate for outdoor activities.

PRELIMINARY ITINERARY: The excursion will leave at about 9:00 am from the lobby of Hotel Taninul. In the meantime those not visiting the cave could take an unguided hike to Naciemiento de Rio Choy. We will travel to the entrance of the cave, which is approximately 2 hrs west of Hotel Taninul. There may be a Mexican police checkpoint along the way, so you are REQUIRED to bring your passport on the trip. We should be back at Hotel Taninul by about 7 pm.

WHAT TO EXPECT: The hike to Rio Subterraneo Cave is short and mostly over level ground (about 15 min). The path is slightly strenuous, although brief, and should be manageable for anyone in reasonable health. There will be need for hands and knees crawling in the cave and we will need to use cable ladders and ropes to descend the short pit (about 5 m) to the cave fish level. The passage may be muddy and slippery in spots. We will rest at the underground lake for a while, view the cavefish, and then return to the surface in the opposite direction. The temperature in the cave will be warm (the mean annual temperature of the region, about 80F or 27C), a little cooler than outside but not uncomfortable, although the humidity will be high (your glasses might fog). One can swim in the final pool, so if you want to see cavefish underwater in their natural habitat, you can bring mask and snorkel.

NOTICE: In the caves, especially those harboring bats, there is risk of infection with histoplasmosis (http://www.cdc.gov/niosh/docs/2005-109/default.html). The cave we will visit is not particularly risky in this regard, as it does not contain a large bat population and our visit will be relatively short. However, to be cautious, participants are recommended a respirator and asked to wear it on their face throughout their visit to the cave. The N95 air-purifying respirator (NIOSH 42 CFR 84 standard) offers 95% protection from solid particles larger than 3 microns, including fungal spores

CLOTHING AND EQUIPMENT:
….Your respirator
….Sunglasses and hat with a wide brim (for the hike to the cave)
….Flashlight (torch) with extra batteries and/or a light attached to your helmet (see below)
…. A SMALL backpack, but not heavily packed or otherwise cumbersome, to carry extra batteries, camera, water, and personal items, etc
….Pair of cotton or leather gardening gloves to protect your hands from thorns while hiking and damp mud in the cave
….Helmet
….Jeans or other sturdy trousers
….T shirt
….Hiking shoes or boots with corrugated soles and cotton stockings
….Change of clothes if you don't feel like returning in the same (possibly) muddy trousers

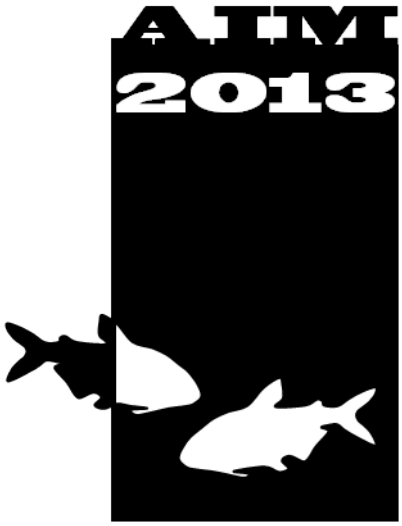
Note 1: There will be several experienced and well equipped cave explorers to guide you through the cave.

Note 2: You will be asked to sign a release of responsibility form to take the trip and enter the cave.

Note 3: There is absolute no collecting of any kind allowed in the cave (unless you have your own permit), although photography is permitted.

Note 4: We want to suggest particular caution if you are considering exploring the cave on the grounds of Hotel Taninul as there have been past reports of histoplasmosis from this locality.

Conference Abstracts



Transcriptome analysis in *Astyanax mexicanus* blind cavefish and sighted surface fish

Hélène Hinaux[1], Julie Poulain[2#], Corinne Da Silva[2#], Céline Noirot[3#], William R Jeffery[4], Didier Casane[5], Sylvie Rétaux[1]

1 DECA group, N&D laboratory, CNRS Gif sur Yvette, France, 2 Génoscope-CEA Sequencing Center, Evry, France, 3 INRA Bioinformatics platform, Toulouse, France, 4 Department of Biology, University of Maryland, College Park, MD,USA, 5 LEGS, CNRS Gif sur Yvette and Université Paris Diderot, Sorbonne Paris Cité, France.

# equal contribution to this work

hinaux@inaf.cnrs-gif.fr

Astyanax mexicanus, a teleost species with surface dwelling (surface fish) and cave adapted (cavefish) morphs, is an important model system in evolutionary developmental biology (evodevo). *Astyanax* cavefish differ from surface fish in numerous traits, including the enhancement of non-visual sensory systems, and the loss of eyes and pigmentation. The genetic bases for these differences are not fully understood as genomic and transcriptomic data are lacking.

We here present *de novo* transcriptome sequencing of embryonic and larval stages of a surface fish population and a cavefish population originating from the Pachón cave. This effort represents the first large scale sequence and clone resource for the *Astyanax* research community. The analysis of these sequences shows low levels of polymorphism in cavefish compared to surface fish, confirming previous studies on a small number of genes. A high proportion of the genes mutated in cavefish are known to be expressed in the zebrafish developing visual system. Such a high number of mutations in cavefish putative eye genes may be explained by relaxed selection for vision-related genes during the evolution in the absence of light. Based on these sequence differences, we provide a list of 11 genes that are potential candidates for having a role in cavefish visual system degeneration.

As the lens plays a major role in triggering eye degeneration in cavefish, we have surveyed the cavefish and surface fish transcriptomes with particular attention for the crystalline gene superfamily. In *Astyanax*, we uncovered 6 beta crystalline sequences (cryba1b, cryba1l, cryba2, crybb1, crybb1d and crybb1c), at least 5 gamma crystalline sequences (crygn2, crygn, crygmxb, crygm5 and genes related to crygm2), 2 sequences of the beta-gamma family of crystallines (aim1a, crybg3), the lambda crystalline cryl1, the mu crystalline crym and the zeta crystalline cryzl1. Among those, we found that crybb1c is strongly down-regulated in cavefish. Thus, at least two cristallines, crybb1c (this work) and cryaa (Behrens et al., 1998) are misexpressed in the cavefish lens, and this may (partly) contribute to lens apoptosis and lens degeneration in cavefish.

Work supported by ANR [ASTYCO] and [BLINDTEST].

An analysis of gene expression level changes across development in surface and cave-dwelling fish.

Joshua B. Gross, Michael Matthews

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The blind Mexican tetra, *Astyanax mexicanus*, is a powerful system for evolutionary and developmental studies owing to our ability to directly compare "ancestral" surface morphs to cave morphs. Utilizing this comparative approach, a wealth of prior research has established that a number of obvious differences in adult phenotype between these two forms arise during embryogenesis. Dramatic differences in gene expression levels likely accompany many of the phenotypic changes occurring during development. To investigate the nature of these expression level differences, we adapted an RNA sequencing (mRNA-seq) approach to assess transcriptome-wide changes occurring during a critical developmental period. We interrogated gene expression changes from pooled RNA derived from groups of whole embryos ranging from 10 hours post-fertilization (hpf) through 3 days post-fertilization (dpf). A prior analysis using normalized next-generation sequencing libraries indicated that many genes in the adult of one morphotype are undetectable in the other morphotype. For instance, cave morphs express a number of genes involved in metabolism that are not evident from surface fish. Conversely, surface fish express a number of genes relevant for the visual system that are not expressed in cavefish. We adopted a similar approach by investigating gene sets from non-normalized libraries, subjected to high-throughput Illumina sequencing, that demonstrated "exclusive" expression in one morphotype but not the other. To understand the function of these groups of genes, we performed enrichment analyses for gene ontology (GO) terms associated with exclusively expressed genes at each developmental stage. As a parallel approach, we also identified and assessed genes associated with a variety of traits categorically defined in past studies as "regressive" or "constructive". Herein, we sought to determine whether genes associated with these traits demonstrate dramatic gene expression differences between morphotypes. Transcriptome-level studies such as this provide an additional tool for identifying essential genes that accompanied evolution of cave-associated characteristics in the subterranean habitat.

Divergence and Speciation in *Astyanax* of the Sierra El Abra

Richard Borowsky, Department of Biology, New York University, New York, NY, USA.
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Extensive studies of microsatellite (mSat) and SNP variation among cave and surface populations in the Sierra de El Abra region clearly establish that the cave populations of the region evolved independently at least five times. They are derived from at least two distinct lineages of surface fish which occupied the area consecutively. The same patterns of genetic relationships among populations emerge from independent analyses of both mSats and SNPs. The population genetic signatures unambiguously establish the lineage identity of all nine studied cave populations. The ancient separation of the two lineages previously established (6.7 mya, Ornelas-Garcia, et al. 2008) suggests that they may represent different species. This hypothesis is strongly supported by studies of inter-population hybrids. Hybrids between lineages have sex ratios strongly skewed towards females. Those that do develop as males exhibit extreme transmission bias of parental alleles, with the biased loci organized into large blocks throughout the genome. In addition, sperm from inter-lineage hybrid males, in comparison to non-hybrid males, are abnormal in several ways, including lower percent motilities, swimming velocities and endurances. Hybrids between populations within lineages do not exhibit these abnormalities. These results argue for the resurrection of the species name, *Astyanax jordani*, for all cave populations of the Sierra de El Abra proper, from Cueva Chica in the south to Cueva de El Pachón in the north.

Hybridization and the colonization of the cave environment by fish.

Amy Cahill, Maria Yurgel and Luis Espinasa

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Populations of cave *Astyanax* have two main types of mitochondrial DNA whose differences are of such magnitude as to be derived from separate species. The commonly accepted view is that an old stock colonized the cave environment and a younger stock is currently found in the surface populations and in some of the cave populations. Hybridization has occurred between both stocks at multiple cave localities. It is often assumed that gene flow from this hybridization reduces the level of troglomorphy in cave population of *Astyanax* but, are there cases in which the hybridization of two surface species actually promoted the evolution of troglomorphy in cave fish?

In the case of two populations of the Pennsylvania Grotto Sculpin, the northern-most cave adapted fish in the world, hybrid populations did show an increase in troglomorphic development. One of these populations, the Nippenose cave fish, has a suite of modifications that readily identify them as cave-adapted: Smaller eyes, elongated pectoral fins, more numerous and enlarged cephalic lateralis pores, and a broader head/mouth. On the contrary, the population of Tytoona cave fish do not have the aforementioned suite, with the exception of slightly enlarged cephalic pores. When looking at mitochondrial markers, the Tytoona cave population has a single haplotype, identical to *C. bairdi* from surrounding surface streams. Interestingly, the more troglomorphic Nippenose cave population shares mitochondrial haplotypes with two species of sculpin —*Cottus cognatus* and *C. bairdi*. Molecular data as well as morphology support that in the Nippenose grotto sculpin, a hybridization event of *C. cognatus* and *C. bairdi* generated an adaptationally distinct sculpin lineage, while in those localities where a single species colonizes the cave, gene flow may have prevented it.

The case of the Pennsylvania Grotto Sculpin raises questions about our understanding of hybridization in *Astyanax.* It has become a paradigm that *Astyanax* cave forms were established by an ancestral surface-dwelling form that is either extinct, or no longer present in this region of Mexico. This may be incorrect since surface populations with mitochondrial haplotypes related to both old and young stocks are current inhabitants of the surrounding areas of Sierra de El Abra: Rascon, and Tamasopo for the old stock and Tampaon and Boquillas for the young stock. While questionable, it may be worth considering that in the evolution of cave *Astyanax*, a hybridization event could have been implicated in the successful colonization of the cave environment in light of the fact that in other cave-adapted fish, hybridization appears to have been important.

Genetic structure of *Astyanax mexicanus* at Mexican Atlantic slope

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The Mexican tetra, *Astyanax mexicanus* is successful species with high capacity to dispersion and adaptation to different habitats, including aquifers. Along its range this species have two morphtypes; a normal epigean and a blind hipogean, this last one without eyes or pigmentation and habiting aquifers in Northeastern of Mexico. The morphological monotony a long its range had raised confusion on specie's taxonomic status. The most studies of population genetic in *Astyanax mexicanus* have focused primarily on populations of cave and near of these, and the incomplete sampling along the Atlantic slope of Mexico, can lead to wrong interpretations.

Using a battery of 10 microsatellite loci and increased sampling coverage along of hydrological basins the Atlantic slope, we study the genetic diversity, structure and genetic relationships between different localities of *Astyanax*, including samples of *Bromocharax caballeroi* from Catemaco Lake.
In the north the individuals were taxonomically identified as *Astyanax mexicanus*, while those from south were *Astyanax aeneus*, however the genetic distances between them do not support such separation. The individuals from Catemaco Lake showed morphological characteristics and genetic distances similar of *Astyanax*, so it appears to be a variant in *Astyanax*. Various analyses (AFC, F*ST*, AMOVA and STRUCTRE) detected 10 genetically homogeneous populations, where the caves populations showed higher levels of differentiation and genetic distances than the surface populations. The surface populations near to the caves showed lower degree of divergence from the southern populations than with the caves populations, these could indicate two waves of invasion from south to north as was proposed previously by others. The dendrogram constructed with genetic distances (DCE) confirm these results. For the first time in genetic studies, we add locations north of the caves, with this data we observed that the caves populations are an intermediated group between northern and southern populations, besides the fact find the isolation by distance among northern populations and the correlation of these with each one the southern populations, we propose a third wave invasive south-north direction in recently time, so the Tans-Mexican Volcanic Belt no appears to be a geographical barrier for *Astyanax* dispersion as it has been for other species. Finally the individuals of Catemaco showed low values of genetic differentiation with regard to northern populations of Garza Valdez/Troncones, probably due to an ancestral genetic signal or translocation by human.

Key word: *Astyanax mexicanus*, microsatelites, population genetic.

Paradigm shifts and pendulum swings regarding the origin of *Astyanax* cavefish: What about geology?

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A scientific paradigm is a framework containing all of the commonly accepted views about a subject. As new knowledge is acquired, paradigms shift. In many cases, these shifts tend to follow pendulum swings. For example, depending on if the analysis was on their morphology, mitochondrial DNA or genomes, perception of Neanderthal man has swung between them being of the same species as humans, to different species, and back to the same species multiple times. A similar pendulum swing can be seen regarding single or multiple origin models for *Astyanax* cavefish. A plethora of bibliography has accumulated term such as phylogenetically old/new populations, lineages A/B, phylogenetically old/new clusters, and old/new epigean stocks, with individual cave fish populations having been assigned contradictorily to one or the other. Much of the confusion has developed from failing to incorporate data obtained by previous authors, the reliance of single gene phylogenies, or conversely, on averaging phylogenetic data when gene flow is a confounding factor.

Geology and an understanding of erosional processes are also underused. Within the single/multiple paradigms, a mistaken assumption is that the *Astyanax* populations that we currently see in caves are the result of original colonization events of those particular caves, without taking in consideration that those particular caves may be of more recent geological age than the cavefish themselves. It will be reviewed how the Sierra de El Abra has been "emerging" as limestone is exposed by erosion, following the progressive lowering of the base level to the current elevation of the present coastal plain. Throughout this process, the rivers Tampaon and Boquillas, which currently limit the southern and northern portions of the Sierra de El Abra, had vastly different courses over the Sierra de Guatemala and the Sierra de El Abra. It will be highlighted that, at the time of cave colonization by *Astyanax,* few of the caves we currently see with populations of fish had yet formed. The cave systems have varied greatly and under this scenario, a karstic and therefore biologic differentiation between the Sierra de El Abra and the Sierra de Guatemala may be of recent origin.

When data from mitochondrial DNA guided the Neanderthal/*Homo* and the Guatemala/El Abra single or multiple origin paradigms, the accepted view was for separate entities. With the advent of genome analyses, will the pendulum swing back to a single unit?

An integrated transcriptome-wide analysis of cave and surface dwelling *Astyanax mexicanus*

Allison Furterer, Brian M. Carlson, Bethany A. Stahl, Joshua B. Gross

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Astyanax mexicanus, a freshwater fish species native to the northeastern region of Mexico, is an emerging model for the study of regressive evolution due to its support of multiple cave-adapted populations in addition to an ancestral surface population. The geographically isolated cave populations are phenotypically distinct from their surface-dwelling counterparts, having converged on a striking suite of traits including reduced pigmentation and a highly regressed visual system within the confines of a nutrient-poor subterranean environment, though interbreeding remains possible. To address the limited amount of genetic information available for this system, we utilized Roche/454 pyrosequencing for an integrated *de novo* assembly of the adult *A. mexicanus* transcriptome, using whole-fish RNA collected from two surface-form fish and two fish from the Pachón cave. 22,596 high-quality contiguous sequences were generated using this approach, then identified using the NCBI's BlastX database, with the integrated nature of the assembly allowing for rapid identification of sequence polymorphisms between morphotypes. Sequences were assigned a wide variety of Gene Ontology (GO) terms using Blast2Go, indicating that this transcriptome effectively represents the genetic makeup of *A. mexicanus*. We adapted our dataset for an RNA-seq study to reveal that certain genes showed expression that was exclusive to just one of the morphotypes. We detected the expression of several genes involved in visual system maintenance in surface-form fish that were not expressed in the cave-form fish. Additionally, many genes related to metabolism that showed expression in cavefish, which previous studies have shown to possess more efficient metabolisms, could not be detected in surface fish. Our results serve as a resource that will enable powerful genetic and genomic analyses in the future that will better clarify the heritable genetic changes governing adaptation to the cave environment.

Compared phylogenies of monogeneans parasites and their host *Astyanax mexicanus*

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Monogeneans are fish parasites with high host specificity and a direct life-cycle, which enforce a tight relationship with their host (Huyse & Volckaert, 2005). These features, as well as their capacity for colonization makes them an excellent model for studying speciation. The host populations of *Astyanax mexicanus* cave dwelling cave and surface variants present important differences in terms of their morphology, physiology and behavior. *A. mexicanus* is an excellent model system in evolutionaty biology. Here, we propose the association between monogeneans parasites and their host *A. mexicanus* as a model to study co-evolutionary hypotheses. For this, we identified the parasites morphologically and sequenced the cytochrome oxidase subunit 1 (COX 1) of both parasites and cave and surface fishes collected in the Abra region. We have found *Gyrodactylus* species of monogeneans located in the gills and anus of Astyanax hosts. We are constructing the phylogenies of both parasites and host to test evolutionary hyphotheses like cospeciation, hots-switching and parallel divergence.

References:

HUYSE T & VOLCKAERT FA. 2005. Comparing host and parasite phylogenies: Gyrodactylus flatworms jumping from goby to goby. Syst Biol, 54(5): 710-718. Doi: 10.1080/10635150500221036.

An analysis of structural mutations in the gene *Mc1r* in surface and Granadas cave-dwelling *Astyanax aeneus*

Joshua B. Gross[1], Amanda J. Krutzler[1], Luis Espinasa[2]

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Pigmentation reduction and loss is remarkably common among animals that colonize the cave microenvironment. Genetic analyses in the model system *Astyanax mexicanus* have revealed the identity of causative genes leading to two Mendelian traits (albinism and *brown*) that evolved in cave-adapted fish within this species. Both genes harbor coding sequence mutations in multiple, independently-derived cave populations. It remains unclear why these particular genes (*Oca2* and *Mc1r*) are repeatedly implicated in the convergent evolution of reduced pigmentation. In 2001, a new population of cave-adapted *Astyanax aeneus* was reported from the Granadas cave locality in southern México. These cave-adapted fish represent fish of the same genus, which have independently colonized the subterranean environment. These fish harbor classic troglomorphic phenotypes such as reduced eye size and pigmentation, albeit in a variable fashion at the population level. To determine if *Mc1r* is widely vulnerable to the accumulation of coding sequence mutations, we investigated the coding sequence structure of *Mc1r* in both depigmented cave forms (n=11) and surface-dwelling forms (n=12). We found that full-length sequence primers that successfully amplified *Mc1r* in *mexicanus* failed to amplify the entire open reading frame in *aeneus*. This implies sequence divergence between the two species in the 5' or 3' regions of the Mc1r open reading frame (ORF). A second approach using degenerate PCR primers successfully amplified a significant region of the ORF of *Mc1r* in all individuals. We observed a low level of spurious amplification, which often resulted in amplification of a fragment of the *Mc4r* gene. However, we also observed significant sequence variation, including a variety of SNPs, which were shared broadly among cave and surface-dwelling morphs. One individual harbored a deletion that is predicted to cause loss-of-function of the *Mc1r* gene product. Interestingly, this deletion is different from the 2-bp deletion that was originally identified from the northern *Astyanax mexicanus* Pachón cave population. We did not observe a correlation between *Mc1r* genotypes and reduced pigmentation phenotypes, implying that this gene may not mediate reduced melanic phenotypes in *aeneus* cavefish. Moreover, the unexpectedly high level of sequence and allelic variation may indicate copy number variation of the *Mc1r* locus in the southern *Astyanax aeneus* population.

Caballo Moro breaks Dollo's law: Recuperation of vision in a blind cavefish population

Luis Espinasa and William Jeffery

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Using tissue transplantation, Yamamoto and Jeffery (2000) accomplished the restoration of eyes lost in the evolution of cavefish. In essence, despite a cavernicole genetic background, fish with eyes were created. A similar case appears to also be present in nature, but brought about instead by the effect of natural evolutionary forces.

Caballo Moro Cave (CMC) is a karst window in which the cave system was exposed to light by ceiling collapse. The cave is 4 km away from the nearest surface fish locality. The entrance pit leads to a large pool. Light only reaches the upstream half of the pool, while the downstream half remains in darkness. The pool contains mostly blind depigmented fish in the dark area and eyed pigmented fish in the illuminated area. Results with RAPD fingerprint markers (Espinasa and Borowsky, 2000) indicate that the eyed fish may be distinct from the nearby surface population. Eyed fish of CMC appeared to be genetically closer to blind cave fish from the region, than to the surface fish. These results were confirmed with microsatellite data (Strecker et al 2012).

Through experimental manipulation, Cave fish that have regained eyes undergo many bone modifications, making their skulls resemble surface fish. Nonetheless, they have a signature difference in bone distribution. While in all surface, F1 and F2 fish studied the suborbital bones extend to almost the level of the preopercular and opercular bones, in experimentally eyed cave fish the suborbital bones retain the small size of cavefish, creating a gap between the preopercular and the outer border of the suborbital. When the skulls of eyed CMC fish are examined, they had a distinctive gap and were indistinguishable from laboratory treated cavefish that had regained the eyes.

Histologically the eyes of the eyes fish of CMC are similar to surface fish except for a detail: the retina has a vascularization not previously recorded either for surface fish or surface and cave hybrids. Blood vessels are seen deep within the retina, in between the ganglionic layer and the fibers of the optic nerve.

In agreement with previous genetic information, the skull and eye morphology of the eyed CMC fish does not support an origin via surface fish. Instead, it would appear that blind cavefish had an evolutionary history with some sort of independent "reconstruction" that led to an eye and skull not quite identical to surface fish.

Development of the olfactory system in *Astyanax* cavefish and surface fish

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BACKGROUND

The anatomical organization and the size of the prosencephalon are set up during embryogenesis by morphogen signals which are secreted by the organizer centers of the neural plate and neural tube. These molecules activate signaling pathways and developmental gene networks which specify the fate of neural progenitors by controlling proliferation, neurogenesis, migration and differentiation events. Two of these essential signaling centers are the ventral midline center secreting Shh which is expanded in space in cavefish (heterotopy), and the rostral midline center producing Fgf8 which is shifted in time in cavefish (heterochrony).

In another hand, the formation of the olfactory system results from association of the olfactory epithelium of placodal origin with the olfactory bulbs which are part of the pallial telencephalon. The olfactory bulbs in vertebrates contain two main types of neurons: the mitral cells (neurons projecting to other forebrain regions) and the inhibitory GABAergic interneurons (born in the ventral telencephalon) and migrate through the RMS (the rostral migratory stream) to integrate the olfactory bulbs and contribute to olfactory learning and discrimination.

Our preliminary observations have shown that the olfactory bulbs are bigger (larvae stages examined) in cavefish and that this difference may stem in part from a differential neurogenesis of the interneurons population, as suggested by the increased expression of the transcription factor Lhx6. Indeed, expression analysis using gene markers of the basal telencephalon, coupled to pharmacological treatments of cavefish embryos using cyclopamine (an inhibitor of the Shh signaling pathway) have shown increased migrations of GABAergic interneurons from the ventral telencephalon to the olfactory bulbs in cavefish embryos (Menuet et al, 2007).

AIM

We would like to test the possibility that the olfactory system is coordinately modulated in cavefish, due to modifications of organizer centers (see also Bibliowicz‘s talk).

We quantified proliferation and neurogenesis of interneurons in cavefish and surface fish olfactory system using EdU incorporation methods. In parallel, we compared mRNA expression of different factors such as *pcna, ngn1, neurod, ascl1* to analyze proliferation and neurogenesis events in the olfactory system. In a near future, we will test the Shh- or Fgf-dependence of these events and we will test olfactory capabilities of fishes in these developmentally-modified contexts.

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Molecular systematics of the genus *Astyanax* (Characiformes: Characidae) - starter edition

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The genus *Astyanax* has more than 100 species distributed throughout the Neotropical region. Certain denominations comprise groups of species that gradually are undergoing taxonomic revisions. The main evidence for these species complexes are due to cytogenetic studies, morphological and, more recently, molecular markers and sequencing of mitochondrial DNA. The diploid numbers in *Astyanax* ranging from 36 to 50 chromosomes, with great numerical and structural variation. Aiming to understand the natural history of this group, the ATPase 6/8 region of the mitochondrial DNA of 18 nominal species were sequenced. Due to the morphological and chromosomal diversity of the group, different cytotypes/populations of some of these nominal species or species complexes were sequenced, totaling approximately 180 individuals. The sequences with 650pb were aligned and a Maximum Likelihood tree based on 500 bootstrap replicates according to the test of evolutionary models (TN93 + G) was constructed. The obtained tree rebuilt three major clades, encompassing 28 evolutionary units. The first one is formed by species belonging to the coastal rivers of Brazil, whose morphological characters includes elongated body and hyaline fins. Among the four nominal species, it is suggested there are seven independent evolutionary units. The chromosomal plesiomorphies that support the group are 2n = 50, with a prevalence of acrocentrics, absence of As51 satellite DNA sites; six to 10 5S rDNA sites distributed in acrocentric chromosomes. The second clade is formed by *A. mexicanus* and the species possessing oval humeral spot. All of them have 2n = 50 and predominance of submetacentric chromosomes; presence of As51 satellite DNA in few sites; *A. mexicanus* has six 5S rDNA sites, including a pericentromeric site at a submetacentric pair, that is a marker, meanwhile the other group have a pair of 5S rDNA, just this marker. The third clade is formed by complexes of species *A. scabripinnis* and *A. fasciatus*, among others. It is characterized by high chromosome variability with Robertsonian rearrangements, causing several decreases in chromosome number; loss and gain of As51 satellite DNA sites; maintenance of chromosome submetacentric marker with the 5S rDNA site, usually with four sites; in general, few acrocentric chromosomes. Previous work from our group has demonstrated the occurrence of three major clades, which have been strengthened with the addition of further species to the analysis. Despite representing only about 15% of the nominal species, and only one region of mitogenome, the simplesiomorphies observed in morphological and cytogenetic level reinforce the reconstruction obtained by the present analysis and demonstrate its phylogenetic signal in the genus *Astyanax*.

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"CHARACTERIZATION OF TWO TROPHIC ECOTYPES OF LAKE CATEMACO THROUGH DIET ANALYSIS OF STABLE ISOTOPES"

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The study of the mechanisms that promote speciation is one of the main questions in evolutionary biology. In this sense, environmental and biological interactions could influence populations' evolution, particularly in the phenotypic and genotypic variation. Divergent natural selection between and within populations exploiting different resources, is still one of the primary causes of trophic polymorphism. Within the lake fauna, fish communities are characterized, among other things, by a high degree of ecological specialization, mostly associated with trophic specialization. The trophic morphology (shape and position of the mouth, the tooth shape etc.) and body patterns, as height and width of the body, can respond plastically to differential habitat use. Our study system comprises a complex of sister species of the genus *Astyanax* that were originally described as different genera (*Astyanax* and *Bramocharax*), and previous molecular and morphological analysis, showed that both correspond to lacustrine ecotypes tentatively associated to trophic specializations. In our present study we access the trophic habits of two ecotypes present in Lake Catemaco (*A. aeneus* and *B. caballeroi*). A total of 5 sampling sites were studied, during both dry and rainy seasons, using gill nets at two different depths in the Lake. Dissected stomachs were fixed in 70% ethanol and posteriorly the content was analyzed under a stereoscopic microscope. The material was classified into 5 categories of food, and within each category we identify the items to the lowest taxonomic level. A total of 82 stomachs were analyzed. The analysis of frequency and comparisons among the sampling sites showed some differences among the different ecotypes, as well between some regions within the Lake Catemaco. In general terms the algae *Alaucoseira granulata* and *Fragilaria construensis* were more often in *Astyanax aeneus*, while *Cilindroespermosis catemaco*, fish muscle, fish scales and insects, were more frequent in *Bramocharax caballeroi* ecotype. Based on the results, we concluded that there is evidence of trophic segregation among lacustrine ecotypes in the characid system of Lake Catemaco.

Role of αA-crystallin in *Astyanax* Cavefish Eye Degeneration

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Lens apoptosis plays a critical role in eye degeneration in *Astyanax* cavefish but the underlying molecular mechanisms are poorly understood. Developmental and genetic studies suggest that multiple genes are involved in cavefish eye degeneration. One of these genes is *αA-crystallin (αA-crys*), which is downregulated during cavefish lens development and maps near an eye QTL on relevant *Astyanax* genetic maps. Here we investigate the role of *αA-crys* in cavefish lens degeneration. Knockdown of *αA-crys* expression by injection of translation blocking and pre-mRNA splice-blocking morpholinos (MO) into surface fish eggs resulted in the inhibition of lens development and induction of apoptosis. Thus far, overexpression of *αA-crys* by injection of synthetic capped mRNA into cavefish embryos has not rescued lens apoptosis or eye development. Together, these results suggest that *αA-crys* is required but insufficient for normal lens development. Experiments were conducted to determine whether *αA-crys* downregulation is caused by *cis*- or *trans*-acting regulatory mutations in cavefish. First, the levels of *αA-crys* expression in F1 hybrids, containing both the surface fish and cavefish *αA-crys* alleles in a *trans*-acting background, were compared to the levels in surface fish by *in situ* hybridization and semi-quantitative PCR, and were found to be the same. Second, about 10 kb of DNA sequence including the *αA-crys* locus (through the upstream adjacent *cystathionine β-synthase* gene) was sequenced in surface fish and cavefish. There are two conserved non-coding regions with minor sequence differences in cavefish: one is in the 5'upstream deleting a "CA" repeat and another is in the second intron, deleting a "CT". These two sequence differences could be part of enhancers responsible for *cis*-acting regulation. In addition to *cis*-acting regulation, *aA-crys* downregulation in cavefish may also be caused by changes in *trans*-acting factors. The *pax6* and *sox2* genes encode transcription factors responsible for the activation of *crystallin* gene expression. Of the two candidate genes, *in situ* hybridization showed that *sox2* is downregulated during cavefish lens development. Furthermore, most surface fish embryos lost *αA-crys* expression in the lens after MO-based knockdown of the *sox2* gene, while *αA-crys* expression was not affected after MO knockdown of the *pax6* gene. The results suggest that a genetic pathway leading from *sox2* to *αA-crys* is involved in normal lens development in *Astyanax* surface fish and that defects in this pathway result in lens apoptosis and eye degeneration in cavefish.

HSP90 as a capacitor for the evolution of eye loss in cavefish

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More than seventy years after Conrad H. Waddington first introduced the concept of canalization in evolutionary biology, and fifteen years after its uncovered nature as the heat shock protein 90 (HSP90), the actual role of HSP90 and its canalizing effect in evolution and speciation remain controversy. The theory of HSP90 keeping standing genetic variation poised for activation by an environmental stressor until subsequent selection of adaptive alleles in new environments can occur, however, remains a gripping and compelling concept for evolution. The cavefish model system *Astyanax mexicanus* has many characteristics which makes it a good candidate for studying a potential role of HSP90 in ecological evolution. The prime reason being, that the river forms had suddenly to live in and adapt to a new and rather extreme environment, the cave. Here, we test the role of HSP90 in eye and orbit size of surface and cave *Astyanax mexicanus*, two important and most likely adaptive traits in cave evolution. We show that HSP90 influences eye and orbit size variation in natural populations of surface *Astyanax*, suggesting corresponding underlying standing genetic variation in these river populations. Additionally and most importantly we provide evidence for selection of particular alleles affecting orbit size which are regulated by HSP90 during transition from surface to cave forms, making a scenario likely in which HSP90 has contributed to cavefish evolution. Finally we discuss potential environmental stressor scenarios in the cave environment and analyze their impact upon HSP90 regulation in surface fish development.

Development and Genetics of the *Astyanax S*clera: An Optic Tissue Organized by the Lens

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The vertebrate sclera is an elastic tissue consisting of fibrous and cartilaginous or ossified layers. Despite its importance in visual acuity, little is known about the molecular basis of sclera development. *Astyanax mexicanus* can help resolve this issue because the surface fish (SF) and cavefish (CF) morphs exhibit different scleral phenotypes. A large sclera with ossified elements is present in SF adults, whereas a smaller and entirely cartilaginous sclera is present in CF (Pachón) adults. Sections of adult eyes show that the SF and CF sclera also differ in thickness, with the CF type almost three times thicker than the SF variety. The differences in *Astyanax* scleral morphology first appear near the end of the larval stage. The larval SF sclera initially develops a cartilaginous layer that gradually becomes ossified in the adult, whereas the CF larval sclera remains cartilaginous throughout life. Several experiments show that scleral morphology is controlled within the developing eye by the lens. First, removal of the CF eye and its replacement with a SF eye during embryogenesis results in the development of a SF-type ossified sclera in the CF host. Second, removal of the embryonic lens in SF results in the formation of a thick sclera resembling that of CF. Third, transplantation of a SF embryonic lens into a CF optic cup causes the development of a SF-type ossified sclera in the CF host. To understand the genetic basis of scleral diversity, the thickness of the fibrous and cartilaginous/ossified scleral layers was determined in 41 members of a SF x CF F2 hybrid family. The overall thickness of the two scleral layers was found to be continuously and normally distributed in these F2 hybrids, suggesting that multiple genes control the cavefish scleral phenotype. A preliminary QTL scan revealed three regions in the genome (with LOD scores approaching 3): one, on chromosome 2, responsible for fibrous layer thickness, and two, on chromosomes 17 and 24, responsible for cartilaginous/ossified layer thickness. Interestingly, the preliminary QTL on chromosome 24 covers the same genomic region as previously described *Astyanax* QTL for pupil (lens) area and retinal outer nuclear layer thickness, suggesting that the same or closely linked genes may control the CF lens, retinal, and scleral traits. We conclude that the lens controls phenotypic differences between the SF and CF sclera and that genetic approaches may be useful in revealing the molecular basis of this optic interaction.

Quantitative genetic analysis of retinal degeneration in the blind cavefish *A. mexicanus*

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The retina is the light-sensitive tissue of the eye that facilitates vision. Mutations within genes affecting eye development and retinal function cause a host of degenerative visual diseases, including retinitis pigmentosa and anophthalmia/microphthalmia. The characin fish *Astyanax mexicanus* includes both eyed (surface fish) and eyeless (cavefish) morphs that initially develop eyes with normal retina; however, early in development, the eyes of cavefish degenerate. Since both surface and cave morphs are members of the same species, they serve as excellent evolutionary mutant models to study retinal degeneration. In this study, we crossed the eyed and eyeless forms of *A. mexicanus* and quantified the thickness of individual retinal layers among 115 F_2 hybrid progeny. We used next generation sequencing (RAD-seq) and microsatellite mapping to construct a dense genetic map of the *Astyanax* genome, scan for quantitative trait loci (QTL) affecting retinal thickness, and identify candidate genes within these QTL regions. The map we constructed for *Astyanax* includes nearly 700 markers assembled into 25 linkage groups. Based on our scans with this map, we identified four QTL, one each associated with the thickness of the ganglion, inner nuclear, outer plexiform, and outer nuclear layers of the retina. For all but one QTL, cavefish alleles resulted in a clear reduction in the thickness of the affected layer. Comparative mapping of genetic markers within each QTL revealed that each QTL corresponds to an approximately 35 Mb region of the zebrafish genome. Within each region, we identified several candidate genes associated with the function of each specific retinal layer. Our study is the first to examine *Astyanax* retinal degeneration in the context of QTL mapping. The regions we identify serve as a starting point for future studies on the genetics of retinal degeneration and eye disease using the evolutionary mutant model *Astyanax*.

Pigmentation loss in cave animals: A high-resolution study of destructive genetic mutations

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The precise genetic mechanisms accompanying the regression of phenotypic characters, and whether one mechanism explains all regressive phenotypes, remain largely unknown. Cave animals represent salient examples of regressive evolution, owing to the recurrent loss of pigmentation and eye reduction in organisms living amidst the dark subterranean environment. Among these cave-dwelling examples, the blind Mexican cavefish *Astyanax mexicanus* has converged on depigmented phenotypes in at least 7 known cave populations. Although the mechanism through which these traits regress is poorly understood, one possibility is that the genes responsible for maintaining pigmentation become non-functional through loss-of-function mutations. An alternative scenario is that genes acquire regulatory mutations leading to altered expression levels that may account for degenerative phenotypes in caves. We set out to determine if a pattern exists between the accumulation of coding sequence mutations in genes of exclusive function, supported by prior QTL studies identifying the genes *Oca2* and *Mc1r* in the evolution of albinism and *brown*, respectively. We hypothesized that genes with pleiotropic functions may be constrained from the accumulation of loss-of-function coding alterations because of antagonistic effects on other phenotypes. We utilized an integrated *de novo Astyanax* transcriptome to perform large-scale genetic comparisons specifically evaluating pigmentation gene expression differences between surface and Pachón cave-dwelling fish. From this transcriptome, we identified ~440 genes with pigmentation-related functions. We then evaluated these for potential loss-of-function coding mutations in Pachón cavefish. This gene set was then assessed for robust expression level differences between morphotypes using RNA-seq. Our preliminary data indicate that by 3 dpf, more than 50 pigmentation-related genes are down-regulated in cave-dwelling forms. Additionally, we found evidence of both coding and regulatory alterations affecting pigmentation genes having accumulated in cavefish lineages. Future studies will clarify whether genes acting exclusively on pigmentation are more likely to harbor destructive loss-of-function coding sequence mutations, while regulatory alterations are more frequently associated with pleiotropic genes.

Adaptive changes in vibration attraction behavior and its sensory receptors promote eye degeneration and disparity between the nuclear and mitochondrial genomes in Pachón cavefish.

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The surface-dwelling ancestors of cavefish adapted to dark cave environments by shifting multiple morphological and behavioral traits. Vibration attraction behavior (VAB), which is the ability of fish to swim toward the source of a water disturbance in darkness, is common in cavefish but rarely observed in surface fish. VAB is heritable, has an advantage for feeding in the dark, and is mediated by superficial neuromasts (SN), which are increased in size and number in cavefish. Mating experiments between surface fish and Pachón cavefish indicated that the enhancement of VAB and SN is paternally inherited and that SN specifically located within the cavefish eye orbit (EO) are genetically correlated with eye reduction. Ablation of EO SN in cavefish demonstrated a major role for these sensory receptors in VAB expression. The quantitative trait loci (QTL) for VAB, EO SN, and reduced eye size form two congruent or overlapping clusters on *Astyanax* linkage groups (LG) 2 and 17. However, despite the known involvement of the Shh signaling pathway in eye degeneration, no QTL responsible for any of these traits were found at the *shh* locus on LG 13. Furthermore, experimental induction of eye regression in surface fish via *shh* overexpression indicated that the absence of eyes is insufficient to promote the appearance of VAB or EO SN. We conclude that natural selection for VAB and EO SN enhancement may indirectly promote eye regression in Pachón cavefish through genetic linkage or antagonistic pleiotropy among the genetic factors underlying these traits. We also report the narrowed genomic intervals for VAB and EO SN genes through sequenced restriction site associated DNA tags (RAD-tags), a next generation sequencing (Illumina)-based genotyping method. We are currently identifying the genes responsible for the evolution of VAB and EO SN with the help of this method. We further show by mathematical simulation that paternal inheritance of VAB and SN can lead to the evolutionary disparity between the nuclear and mitochondrial genomes previously observed in Pachón cavefish. The results show how studies of an adaptive behavior and its sensory receptors can provide insights into cavefish evolution as well as how interdisciplinary approaches can lead to new insights into the mechanisms of the constructive evolution.

An evaluation of eyelessness in cave-dwelling *Astyanax mexicanus* using RNA-seq technology

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Across animal evolution the visual system plays an essential role in the ability to find food perceive the surrounding environment and escape predation. However, when an organism colonizes an environment of total darkness, vision is no longer necessary for survival. As a consequence of millions of years of evolution in the subterranean habitat, troglobitic animals such as *Astyanax mexicanus* have "lost" their eyes and associated visual system structures. The identity of the genes and genetic mechanisms governing eye loss in cave-dwelling morphs remain unclear, though several genes have been hypothesized. To investigate the genetic changes that accompany eye loss in *Astyanax*, we performed a large-scale analysis of gene expression level changes across critical developmental stages (10 hpf, 24 hpf, 36 hpf, 72 hpf, and juvenile). We subjected an integrated *Astyanax* transcriptome to gene ontology (GO) term searches to identify 802 genes in *Astyanax* with previously known eye-related functions. This gene set was then subjected to direct mRNA Illumina sequencing technology (RNA-seq) to characterize expression level alterations with robust fold-change differences between surface and cave-dwelling forms. Preliminary analyses indicated the most dramatic variation began roughly 24 hours post-fertilization, when the eye normally begins to degenerate in Pachón cavefish morphs. Additionally, several genes including *crybb2, crygm3*, *prph2*, and *arr3a* exhibited differences of 4-fold or greater in cave v. surface fish. These genes will be optimal candidates for forthcoming functional analysis and validation. Utilizing this powerful approach will lend additional insight to the complex mechanisms underlying to the regressive evolution of the eye in *Astyanax mexicanus*.

Fragmentation, fusion and asymmetry in the craniofacial skeleton of *Astyanax mexicanus*

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When an animal colonizes an extreme environment, numerous regressive and constructive morphological traits undergo rapid evolution. Blind Mexican cavefish, which reside in total darkness, have evolved a spectrum of defects in the craniofacial skeleton including fragmentation, reduced bony area, and dermal bone fusion. These traits remain difficult to categorize as obviously regressive or constructive. Modifications to the cavefish cranial structure, which deviate from the normal morphology of surface-dwelling fish, were first reported in the 1940s. The genetic and developmental bases of these cranial bone alterations, however, remain only partially understood. In this study, we investigated a number of osteological traits in the context of a large F_2 mapping pedigree of surface x cave hybrids (n=227). We subjected each individual to an exhaustive set of craniofacial measurements, including area, count and fusion assessment within the circumorbital bone series and nasal region. In addition, we also assessed a variety of other well-characterized traits that segregated in this cross (e.g., eye size, albinism). We discovered a previously unappreciated asymmetry in suborbital bone fragmentation, as well as frequent fusion of bones. Consistent with prior studies, our preliminary results do not indicate these craniofacial abnormalities are strictly predicted by visual system regression. Here, we present results of additional correlation studies testing the participation of other morphological traits (e.g., nasal region area) on the incidence of fragmentation. Further, we investigated the presence of QTL mediating both left and right sides of the craniofacial skeleton to determine if identical genetic loci actuate the asymmetric presence of fragmentation and fusion. Significant QTL were discovered exclusively on the right side for both fragmentation and fusion, suggesting that there may be a genetic basis for asymmetrical development. For example, pleiotropic interactions between genes selected in the cave environment for constructive traits, such as cranial neuromast expansion, may interfere with normal patterns of bone ossification.

Transgenesis methods in *Astyanax*

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Transgenesis techniques in *Astyanax mexicanus* have been poorly used in the past years although this fish has become an increasingly popular microevolution model (cf Yamamoto et al. Dev Biol. 2009). It is however a fruitful way to analyze gene functions or to visualize expression patterns in live animals. Here, we compared the two main methods of transgenesis usually used in zebrafish. We used two vectors provided by the AMAGEN platform (aquatic animals transgenesis platform). One is a βcrystallin promoter driving CFP, the other is a cardiac actin promoter driving RFP.

First we used Involin, an I-SceI meganuclease-mediated method. Meganucleases are restriction enzymes which cut 18bp recognition sites, typically absent of most genomes. The vector carrying I-SceI site was co-injected together with the meganuclase protein in one cell stage surface fish and cavefish embryos.

Second we used the Tol2 system. The Tol2 transposable element was originally found in the genome of the Japanese medaka fish, and contains a gene encoding an active transposase that catalyzes DNA transposition in vertebrate cells. Vectors carrying the transgene sided by the transposon LTR (Long Terrminal Repeat) were co-injected together with Tol2 transposase RNA in one cell stage embryos.

Forty-one batches of surface and cave fish embryos (total 3095) were successfully injected with these two methods. Between 8.70 to 57.70 % of the surviving injected larvae expressed the reporter. A good overall survival rate after injection was achieved showing no statistical difference between injected eggs and controls. As expected, various types of mosaic expression were observed in F0 animals, from monolateral to bilateral and from strong to low expression.
We established the first *Astyanax* surface fish trangenic lines expressing β-crystallin in the lens with the Involin method. Two FO (founders) males transmitted transgene to 14.60% and 23.60% of their progeny. Some transgene extinction was observed in a family but not in the other, for which F3 individuals still express strongly CFP in the lens.
A new method started to be used for fish transgenesis, named TALEN (Transcription Activator-Like Effector Nucleases). TALEN-mediated gene knock-out and knock-in should be feasible. We will start investigating this new technique in *Astyanax*.
All observations made so far let us think that surface and cave *Astyanax* will be very amenable to transgenesis.

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Unravelling continuous eye growth in teleosts by studying blind cavefish

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The eyes of many vertebrates are already determined in size at birth and won't change much afterwards. By contrast, the teleost eye grows life-long, adapting its relative size to the rest of the body. To achieve this, all necessary cell types have to be produced continuously and in a highly organized fashion. A special tissue around the lens called CMZ (ciliary marginal zone) accomplishes this. The CMZ comprises stem cells, which divide, go through a transit amplifying zone and finally differentiate into the exact number of cells of each type. This is particularly necessary to maintain the growing retina and RPE functional at all time during growth. The mechanisms, which conduct this orchestra of constant eye growth in teleosts are not understood so far. Which genes are involved? Which cells express these? How is the CMZ organized, structured and how does it contribute to constant growth? To address these questions, cave and surface fish models will be used for comparisons. In particular *Astyanax mexicanus*, which is a powerful model to investigate constant eye growth. During development of the cavefish the eye initially starts developing, but degenerates eventually in comparison to its surface dwelling form. Differentiation of later retinal cell types seems to be missing at a certain developmental stage and apoptosis finally leads to the degenerated eye in the adult cavefish. *Phreatichthys andruzzii*, another cave dwelling teleost with a similar phenotype of degenerated eyes, shows that only the early born retinal ganglion cells develop, while the other six cell types are not detectable. Hence, layering of the retina does not proceed. We propose a similar scenario for *A. mexicanus*. Detailed in situ analyses for *P. andruzzii* will be presented and discussed.

Isolation and characterization of V1r pheromone receptor gene in cave and surface variants of *Astyanax mexicanus*

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The V1 pheromone receptor belongs to the V1r receptor super family with more than 150 members in various mammals. Some studies indicate the existence of a single V1r-like pheromone receptor gene in multiple fish species, (Pfister and Rodriguez 2005). We sought to investigate the V1r.like gene in *Astyanax mexicanus*.

Whether there exist differences in the sequences of this gene among different *A. mexicanus* morphotypes as well as the evolution of this receptor in this species is still unknown. The objective of our work is to characterize the pheromone receptor gene V1r-like in *A. mexicanus* variants and investigate whether there are differences between the two populations, as well as to establish the relationship that exists between V1r-like receptor of *A. mexicanus*. With the sequences of other teleosts. In order to obtain the complete sequence of *Astyanax* V1r-like gene, we are using inverse PCR. We are currently fitting a phylogenetic tree of the V1r-like gene among different fish species including *A. mexicanus.*

References:

Pfister P, Rodriguez I: Olfactory expression of a single and highly variable V1r pheromone receptor-like gene in fish species. *Proc Natl Acad Sci U S A* 2005, 102:5489-5494.

Statistics on *Astyanax* husbandry in the Gif facility

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Labs working with non-conventional animal models should develop a very specific strategy for their facility. Regular and intensive eggs production is essential for the team. We will report data collected in our Gif *Astyanax* facility, focusing on the photoperiod, the temperature, the water quality, number of spawning, number of eggs, and growth of animals.

In our facility, we have about 500 animals, half surface fish, half Pachon cavefish, all originating from W. Jeffery's lab-raised individuals (plus 2 wild-caught Molino cavefish). They are all maintained on a 12/12 photoperiod (8 a.m. to 8 p.m), at a temperature of 20-22°C for cavefish, and 26°C for surface fish. There are 7 main re-circulating racks, each with 4 levels of 120 liter tanks. Breeding tanks host groups of 20-25 fish. To induce breeding, the temperature of the water is changed: increased from 20°C to 26°C for the cavefish, and decreased from 26°C to 20°C for the surface fish. They lay on "MOPS" artificial plastic substrates, usually during the night. Eggs are collected and counted in the morning and we determine the fertilization hour with the developmental staging table.

I have collected data since August 2010 until now. First, to optimize eggs production we wanted to determine if there is an "egg-laying season". Although they give eggs each week, the egg laying is variable. The surface population lays more with a maximum at 6000 eggs per month against 1400 eggs maximum for cavefish. There may be a periodicity, either a natural one or one due to human activities along the year, but none correlated to the lunar cycle. Spawning occurs almost exclusively during the dark period for both morphs, with a peak between midnight and 2 a.m.

We will also report some statistics on the growth of the fish, based on dissection data from other experiments. There is a good correlation between weight and size, between size and age and between weight and age in the two populations. Females are always heavier. A 100% sure method to discriminate sex is the presence of denticles which are only found on the anal fin of males (see also Borowsky, 2009). In our colony, the sex ratio is about 50/50, except for hybrids with a Pachon mother, for which only females have been observed.

Work supported by ANR [ASTYCO]

Parallel evolution within the *Astyanax* genus in Mesoamerica

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The fish genus *Astyanax* is characterized by a high phenotypic plasticity, which is more conspicuous in extreme habitats, as caves. In recent studies we have proposed that trophic adaptations could drive the major morphological evolution within the genus *Astyanax,* similarly to other characids. In the present study we evaluate the morphological evolution in a pairs of species in two different systems of Mesoamerica, Lake Catemaco in México and the Nicaragua great Lakes (Managua and Nicaragua Lakes), Nicaragua. The main goal of the present study is to evaluate parallel evolution of the morphological traits in both systems, having its fundament in two evolutionary processes: phylogenetic inertia and selection. We examined body size and shape variation in 210 individuals from Nicaragua populations and Catemaco Lake, in order to evaluate the main morphological patterns as well as evaluate the effect of the phylogenetic inertia. In both regions parallel evolutionary patterns were found mainly in the body shape (spatial configuration of the head and body depth), sharing similar morphological adaptations among ecomorphs. Based on our results we support the lack of phylogenetic signal in our study system.

Olfactory evolution in cave-dwelling *Astyanax mexicanus*

Jonathan Bibliowicz, Yannick Elipot, Maryline Blin, and Sylvie Rétaux

Development and Evolution of the Forebrain

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While the general features of the olfactory system are well-conserved across vertebrates, olfactory capabilities have been known to evolve rapidly in response to changing environmental conditions. In cave-dwelling populations of *Astyanax mexicanus*, several morphological and behavioral shifts occurred in adaptation to cave life characterized by total and permanent darkness. Previous studies have shown that sensory systems such as the lateral line and taste buds are modified in cavefish. Increases in the size and/or number of these sensory structures have been shown to accompany the loss of vision in cave animals, and are thought to provide a sensory compensation for life in dark cave environments. Our comparison of the olfactory system in *Astyanax* surface fish and Pachón cavefish showed an increase in the overall size of the olfactory organ (nose) in cave-dwelling morphs at both embryonic and post-embryonic stages. Molecular and cellular analyses further revealed a higher number of olfactory sensory neurons (OSNs), as well as increased neurogenesis within the post-embryonic olfactory epithelium (OE) of cave-fish, when compared to surface-fish.

Current research efforts are focused on investigating the functional/behavioral consequences of increased size and modified neurogenesis in the cavefish OE. Recent work in mice suggests that increased turnover of neurons in the OE might affect olfactory capabilities by providing a dynamic mechanism to adapt the olfactory neuronal repertoire to the environment. Could the observed increase in olfactory epithelial neurogenesis provide such mechanism for adaptation to cave environments? What effects would this modification have on the ability of an animal to smell in the dark? To address these questions, we have developed an assay protocol to test olfactory function in response to food-related odors (amino-acids) in the dark. By combining behavioral and molecular assays, we are able to not only test the behavioral responses of both populations to various odorants, but also to quantify neuronal activation in response to odorant exposure. These studies should provide new insights into the mechanisms underlying olfactory adaptations in cave animals specifically, as well as the functional role of olfactory neurogenesis in general.

Work supported by ANR grant [ASTYCO]

Metabolic Regulation of Sleep in *A. Mexicanus*

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While sleep is a near-universal behavior throughout animal kingdom, its function and evolutionary basis remain unclear. Sleep quantity and quality are regulated by metabolic state. Neural regulation of sleep, appetite and energy homeostasis is critical for an animal's survival and under stringent evolutionary pressure. Across phyla, starvation results in reduced or disrupted sleep suggesting animals suppress sleep in order to forage for food. Understanding the evolutionary basis for sleep loss in nutrient poor environments will provide critical insight into how animals adapt to an altered habitat. We are currently investigating the biological basis of sleep in the Mexican cavefish *Astyanax mexicanus,* a model system for investigating evolutionarily derived sleep loss. It is possible that cavefish sleep less than surface fish because they are more sensitive to small perturbations in food availability. Alternatively, these sleep changes could be evolutionary hard-wired, as an adaptation constant nutrient shortage in caves. To differentiate between these two possibilities we are currently rearing fish on high and low calorie diets to determine whether high-metabolic stores can rescue the reduced sleep in cavefish. We are collaborating with Erik Duboue and Richard Borowsky (NYU) to pharmacologically interrogate the molecular basis for reduced sleep in cave populations. Blocking ß-adrenergic signaling with propranolol increases sleep in cavefish while having no effect on surface fish raising the possibility that increased catecholamine signaling is responsible for reduced sleep in *A. mexicanus* cave populations. We are currently using RNA-seq in starved or sleep-deprived cave and surface populations to determine additional molecular pathways that may underlie reduced sleep in cave populations. Additionally, we are examining cortisol levels, a conserved indicator of stress and sleep deprivation, to determine whether cave populations are chronically sleep deprived or have evolved reduced sleep need.

Feed or fight: developmental origin of a behavioral shift in blind cavefish

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Astyanax mexicanus, with its surface dwelling (surface fish) and cave adapted (cavefish) morphs, is an important model system in evolutionary developmental biology. *Astyanax* cavefish differ from surface fish in numerous traits, including morphological, physiological, and behavioral traits. We will report on the developmental and neural bases for the evolution of social and feeding behavior in cavefish.

We first focused on the loss of aggressive behavior in cavefish. We used an intruder-resident behavioral assay to compare aggressiveness quantitatively (attack counts) and qualitatively (pattern and nature of attacks) between the surface and Pachón cave morphs of *Astyanax*. Using this paradigm, we characterized aggressive behavior in surface fish, bring support for the genetic component of this trait, show that it is controlled by hindbrain raphe serotonergic neurons and that it corresponds to the establishment of dominance between fish. Cavefish have lost such aggressive/dominance behavior. The few attacks performed by cavefish during the behavioral test instead correspond to food searching behavior, driven by the developmental evolution of their hypothalamic serotonergic neurons, itself due to increased Hedgehog signaling during early forebrain embryogenesis.

The latter result led us to focus on the developmental evolution of the hypothalamus, a forebrain region known to be involved in the control of homeostasis, including the control of feeding behavior and food intake. Previous analyses in our group on early embryos have shown that hypothalamic development and size is modified in cavefish. We therefore mapped and compared in surface fish and cavefish hypothalamus the neuropeptidergic (NPY, POMC, VP, OT, GnRH, SS) and dopaminergic neurons. We found significant differences in the sizes of some of these neuronal groups that may account, together with the difference in serotonin neurons reported above, in the differences of feeding behavior in the two *Astyanax* morphs.

We propose that during evolution and adaptation to their cave habitat, cavefish have undergone a behavioral shift, due to modifications of their hypothalamic neuronal networks. They have lost the typical aggressive behavior of surface fish and evolved a food searching behavior that is probably more advantageous to survive in the dark. We have therefore demonstrated a link between the development of a neuronal network and the likely adaptive behaviors it controls.

Work supported by ANR [ASTYCO] and [BLINDTEST].

Astyanax: Looking Back 45 Years

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In this presentation I look back 45 years to the way we did field work in the 20th Century, culminating in the big *Astyanax* paper by Mitchell, Russell, and Elliott, 1977. This ecological/ hydrological/biological paper has been an important source of information to many cavefish researchers. If only GPS had existed back then! We did not discover Mexican topographic maps until the late 1970s!

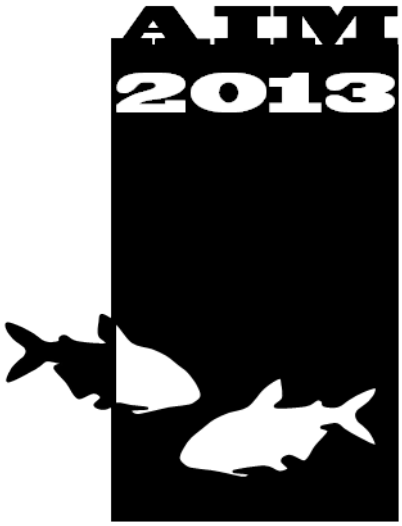
I started caving as a University of Texas biology student in 1967. Soon I was collecting invertebrates and mapping caves in Texas and Mexico. Bill Russell taught me how to map caves, and James Reddell amazed me when he reported that some of my inverts were new species. I was recruited by Dr. Robert W. Mitchell to attend grad school at Texas Tech University, and join his research on *Astyanax* cavefishes. I started this work for Bob in the summer of 1969. Don Broussard, Jim McIntire and I spent six weeks working in many caves around Ciudad Valles in the Sierra de El Abra. A number of cavers and biologists joined us. We also descended into Bee Cave near the northern end of the cavefish range, Chamal area, Sierra de Guatemala, near Ciudad Mante. Our task was to map cavefish caves and collect fish. During this work I discovered a new species of troglomorphic cave scorpion while mapping Sótano de Yerbaniz.

I earned my MS and PhD under Bob Mitchell. I have studied many aspects of cave biology. My career also took me to caves in Belize, many western states, Alaska, and finally Missouri.

I have not been in the El Abra since 1974, or the northern range of *Astyanax* cavefish since 1981. I will share my ideas on what ought to be done to further research on these cavefishes. I also will show you some of my work on biogeography, biodiversity, conservation, and bats. We have three cavefish species in Missouri, including the amblyopsids *Ambyopsis rosae, Typhlichthys subterraneus,* and an interesting "cave sculpin," *Cottus* sp., that is evolving sympatrically, some think, from a surface form.

I recently posted a pdf of Mitchell, Russell, and Elliott on my new website, http://mocavelife.com/ for you to download. I hope it will help you in your work!

Conference Attendees



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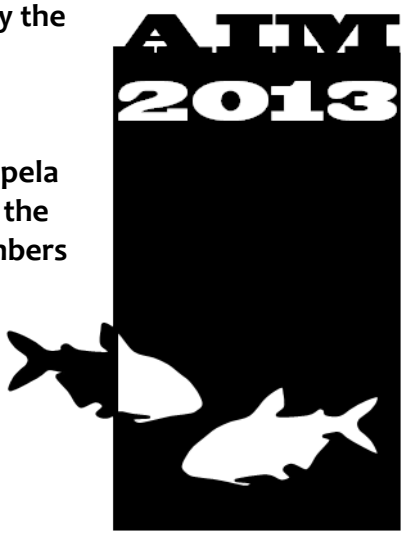
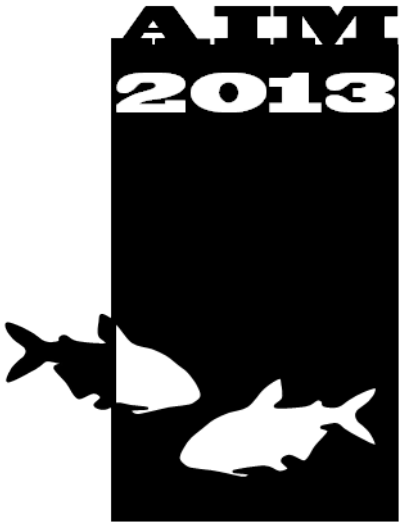
We are also very grateful to past meeting founders and organizers, specifically William Jeffery, Sylvie Rétaux, Spela Goricki and Luis Espinasa for invaluable assistance with the organization of this meeting. Finally, we thank the members of the global *Astyanax* research community without whom this stimulating and inspiring meeting would not be possible.



AIM2013 Meeting Organizers

Ernesto Maldonado
Luis Espinasa
Joshua Gross

Meeting Notes



2013 *Astyanax* International Meeting March 17th - 21st

Sunday March 17th	Monday March 18th	Tuesday March 19th	Wednesday March 20th	Thursday March 21th
	Breakfast served 7am to 8.45am	Breakfast served 7am to 9am	Breakfast served 7am to 9am	Breakfast served 7am to 9am
Arrival of Participants	Morning session 8:45am to 12:00pm	Morning session 9am to 12:00pm	*Collect bag lunch at 8am and meet in the Hotel lobby at 9am for excursion to the Micos cave locality*	Morning session 9am to 12:30pm
	Lunch served 12:30 to 2pm	Lunch served 12:30 to 2pm		Lunch served at 1pm
	Afternoon session 2pm to 5:30pm	Afternoon session 2pm to 4:30pm		*Close of the meeting*
Meeting Registration 5pm to 7pm Hotel lobby	Evening poster session 5:30pm to 7:00pm	Evening poster session 5:30pm to 7:00pm	*Return from Micos locality*	
Welcome Reception 7pm	Dinner served 7pm to 8:30pm	Dinner served 7pm to 8:30pm	AIM Meeting Banquet served at 7pm	